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2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-MEDIATED DEPRESSION OF RAT TESTICULAR HEME SYNTHESIS AND MICROSOMAL CYTOCHROME P-450*

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Abstract—Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) produces hirsutism, alopecia, and chloracne, symptoms that suggest a possible alteration of endocrine function. Therefore, the effects of TCDD on rat testicular cytochrome P-450 content were investigated. Forty-eight hours after a single, oral dose of TCDD (25 μg/kg) testicular microsomal cytochrome P-450 levels were depressed by approximately 24%. Microsomal cytochrome P-450 continued to decrease to 62% of control levels at 4 days and remained at approximately the same levels 7 days following treatment. Testicular microsomal heme content exhibited a similar pattern after administration of TCDD. No alterations in testicular δ-aminolevulinic acid (ALA) synthase were detected. The incorporation of [^{14}C]ALA into microsomal heme was decreased to approximately 36% of control values at 24 hr after TCDD administration. Testicular weights were not altered during the 7-day experimental period. These data suggest that TCDD depresses cytochrome P-450 levels in the rat testis through an inhibition of the synthesis of testicular heme.

As an undesired by-product in the manufacturing of phenoxyherbicides, TCDD has been released into the environment through industrial accidents and the spraying of contaminated herbicides [1, 2]. Human exposure to TCDD has been associated with chloracne, skin hyperpigmentation, hirsutism, alopecia, neurological dysfunction and porphyria [3–5].

Laboratory studies have shown TCDD to be very toxic to various animal species. The LD_{50} of TCDD for animal species investigated, while varying greatly, is at the level of μg/kg [6]. Death is typically delayed, occurring from 1 to 6 weeks following the administration of a single, oral dose of TCDD [6]. A wide variety of toxic effects are produced by TCDD; however, the precise mechanism by which death occurs is not known. It appears that animals treated with TCDD waste away in a starvation-like manner [7, 8].

TCDD has been reported to produce testicular hypoplasia and an impairment of spermatogenesis in animal models [5, 9, 10]. These observations, along with a possible endocrine basis for other reported TCDD toxic effects (hirsutism and alopecia), prompted the examination of the effects of TCDD on rat testicular microsomal cytochrome P-450, a hemoprotein essential in the synthesis of testicular androgens.

MATERIALS AND METHODS

Materials. Pyridoxal-5′-phosphate, disodium EDTA, and porcine heart succinyl-CoA synthetase (EC 6.2.1.4) were obtained from the Sigma Chemical Co., St. Louis, MO. Guanosine triphosphate, δ-aminolevulinic acid (ALA), and coenzyme A were obtained from Calbiochem, La Jolla, CA. δ-[4-^{14}C]Aminolevulinic acid (42 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and [2,3-^{14}C]succinic acid (15 mCi/mmole) from ICN Pharmaceuticals, Irvine, CA. TCDD was a gift from the Dow Chemical Co., Midland, MI. Other chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats (250–300 g; 60–75 days of age) were obtained from the Holtzman Co., Madison, WI. The rats were permitted food (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL) and water *ad lib.* except for a 12-hr period prior to TCDD administration, when food was withheld. TCDD was dissolved in acetone, and aliquots were added to corn oil to provide an acetone/corn oil ratio of 1:2 (v/v). TCDD was administered orally (25 μg/kg; 2.5 ml/kg) with a 16-gauge, curved animal feeding needle (Popper & Sons, Inc., New York, NY). Control animals received 2.5 ml/kg of acetone/corn oil (1:2), which did not alter any variables measured in these experiments. Animals were killed at the same time of day (7:00 to 8:00 a.m.) to avoid any potential differences in effects caused by circadian rhythms.

Preparation of tissues. Animals were decapitated, and the testes were removed, decapsulated and weighed. Testes from each animal were pooled, and homogenates (10%, w/v) were prepared in 0.25 M sucrose (5°) using a motor-driven glass Potter–Elvehjem homogenizer and a Teflon pestle (0.15 mm clearance). Subcellular fractions of mitochondria and microsomes were prepared by the methods of Cammer and Estabrook [11] and Simpson and Boyd [12].

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Microsomal preparations were ascertained to be free of hemoglobin by spectrophotometric assay. Protein was measured by the method of Bradford [13], with bovine serum albumin as the standard.

Microsomal cytochrome P-450 determination. Concentrations of cytochrome P-450 were determined from the carbon monoxide difference spectrum (450–490 nm) of dithionite-reduced microsomes with a millimolar extinction coefficient of 91 $mM^{-1} cm^{-1}$ [14].

Microsomal heme determination. The levels of microsomal heme were determined from the difference spectrum of the oxidized/reduced pyridine hemochromogen between 541 and 557 nm, with a millimolar extinction coefficient of 20.7 $mM^{-1} cm^{-1}$ [15].

ALA synthase activity. The activity of rat testicular mitochondrial ALA synthase was determined by a radioisotopic method, that had previously been developed in this laboratory [16], which utilizes [2,3-^{14}C]succinate and a succinyl-CoA generating system. The ^{14}C-labeled material was confirmed to be the [^{14}C]ALA-pyrrole by thin-layer chromatography in two different solvent systems with an authentic ALA-pyrrole standard, and recovery of ALA was found to be 91% [16]. The activity of ALA synthase in the rat testis was localized predominantly in the mitochondrial fraction [16].

Incorporation of [^{14}C]ALA into microsomal heme. The rate of testicular heme synthesis was measured in rats 24 hr after a single, oral dose of TCDD (25 μg/kg) and compared to that of rats which had received the vehicle alone. Animals received 6 μCi of [4-^{14}C]ALA [i.p.; 0.75 ml total volume of 50 mM Tris–HCl (pH 7.4)–0.9% NaCl] and were killed 90 min later. Incorporation of [^{14}C]ALA into testicular microsomal heme was linear for 120 min. Testes were pooled from two animals, the microsomes were prepared, and heme was extracted into ethyl acetate/acetic acid (4:1, v/v) followed by washes with water and 1.5 N HCl by the procedure of Bonkowsky *et al.* [17]. For each sample, an aliquot of the heme extract was added to 6 ml of Amersham ACS scintillation fluid and counted for radioactivity in a Beckman LS8000 liquid scintillation counter (93% efficiency), and another aliquot was assayed for heme.

Statistics. Data were analyzed by Student's *t*-test to determine the significances of difference between means.

RESULTS

The effect of a single, oral dose of TCDD (25 μg/kg) on rat testicular microsomal cytochrome P-450 is depicted in Fig. 1. Levels of microsomal cytochrome P-450 were significantly less than control levels at 2 days after administration of TCDD (76% of control). The levels of microsomal cytochrome P-450 were decreased further at 4 days (62% of controls) and remained at approximately the same level 7 days following administration of TCDD. It has been reported previously that the administration of TCDD to guinea pigs results in a depression of testicular microsomal cytochrome P-450 without any appreciable alteration of the levels of microsomal heme [18]. However, as shown in Table 1, rat testicular heme levels were decreased following administration of TCDD. The levels of microsomal heme were significantly lower than control values 2 days after administration of TCDD and remained depressed throughout the 7-day experimental period.

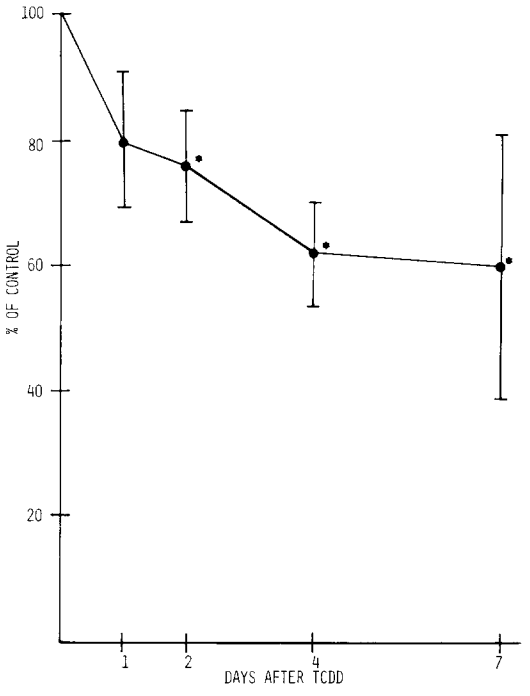

Fig. 1. Effect of TCDD on rat testicular microsomal cytochrome P-450 content. Rats received a single, oral dose of TCDD (25 μg/kg) at time 0 and were killed at intervals indicated; testicular microsomal cytochrome P-450 was determined as described in Materials and Methods. The mean control value for microsomal cytochrome P-450 was 0.162 nmole P-450/mg protein. Each value is the mean ± S.E.M. of at least three determinations. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

Compensatory increases of hepatic ALA synthase activity have been postulated to occur following a

Table 1. Effect of TCDD on rat testicular microsomal heme content*

Days after TCDD treatment	Microsomal heme (% of control)
1	105 ± 2
2	74 ± 5†
4	62 ± 8†
7	71 ± 2†

* Rats received a single, oral dose of TCDD (25 μg/kg) at time 0 and were killed at intervals indicated; testicular microsomal heme was measured as described in Materials and Methods. The mean control value for microsomal heme was 0.258 nmole heme/mg protein. Each value is the mean ± S.E.M. of at least three determinations.

† Indicates that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

Table 2. Effect of TCDD on rat testicular mitochondrial ALA synthase activity*

Days after TCDD treatment	ALA synthase	
	Control	TCDD
	[nmoles·ALA (g protein)$^{-1}$·hr^{-1}]	
1	242 ± 14	234 ± 12
2	245 ± 14	255 ± 10
3	243 ± 6	257 ± 9
4	239 ± 5	253 ± 4
7	242 ± 9	249 ± 6

* Rats received a single, oral dose of TCDD (25 μg/kg) and were killed at the specified time intervals. Mitochondrial ALA synthase activity was determined as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

decrease in hepatic heme levels via feedback repression and/or inhibition [19]. Recently, this phenomenon has also been demonstrated in the rat testis utilizing the inhibitor of rat testicular heme synthesis 1,2-dibromo-3-chloropropane [20]. As shown in Table 2, the activity of testicular mitochondrial ALA synthase activity was not altered by TCDD treatment. The absence of increased ALA synthase activity as an early event following TCDD administration is typical in mammalian species [21, 22]. TCDD has been reported to increase the activity of ALA synthase in the chick embryo 3 hr after administration [23]. However, four weekly oral doses of TCDD (25 μg/kg) were required to cause increased activity of the enzyme in mouse liver [24], which may merely reflect the severe liver damage that was also observed at this time period.

It is possible that TCDD might depress testicular microsomal heme and cytochrome P-450 content through an impairment of the synthesis of heme. To investigate this possibility, the rate of incorporation of [^{14}C]ALA into testicular microsomal heme was measured for both TCDD-treated and control rats. The effect of TCDD on the incorporation of [^{14}C]ALA into microsomal heme is shown in Table 3. The incorporation of [^{14}C]ALA into testicular microsomal heme was decreased to 36% of control values 24 hr after administration of TCDD.

A compound such as TCDD could depress heme and cytochrome P-450 levels through a non-specific inhibition of testicular protein synthesis. If this were the case, the activity of testicular ALA synthase, as well as testicular weight, might be expected to decrease. However, as reported in Table 2, TCDD had no effect on testicular ALA synthase activity. Furthermore, testicular weight was also not changed by TCDD treatment during the 7-day experimental period. These data suggest that TCDD does not act merely by inhibiting testicular protein synthesis.

DISCUSSION

As reported herein, administration of TCDD to mature male rats results in decreased testicular microsomal heme and cytochrome P-450 content and a decreased rate of [^{14}C]ALA incorporation into microsomal heme. Testicular weights and the activity of ALA synthase were not affected by TCDD treatment. These results suggest that one mechanism whereby TCDD lowers testicular heme and cytochrome P-450 is by inhibition of the synthesis of heme. Jones and Sweeney [25] have reported that the administration of TCDD (25 μg per kg per week for 5 weeks) to mice causes an increase in the urinary excretion of uroporphyrin and a decreased activity of hepatic uroporphyrinogen decarboxylase. Thus, it is possible that the activity of this heme biosynthetic enzyme is also decreased in the rat testis after TCDD treatment. The determination of the exact step(s) of the rat testicular heme biosynthetic pathway which is impaired by TCDD requires further investigation.

TCDD has been demonstrated to be a potent inducer of distinct forms of rabbit hepatic, kidney, and lung cytochrome P-450 [26–28] and to decrease the activities of various rat hepatic P-450-dependent enzymes [29, 30], suggesting that the effects of this chemical may be both organ, and species, specific.

It has been reported previously that TCDD depresses guinea pig testicular cytochrome P-450 without decreasing heme levels [18]. This observation suggests that TCDD may also regulate the levels of discrete guinea pig testicular apocytochrome P-450(s). However, higher doses of TCDD might also alter the synthesis of testicular heme in this species.

The data presented herein indicate that TCDD may depress rat testicular cytochrome P-450 levels through an inhibition of the synthesis of testicular heme, the prosthetic group of cytochrome P-450. However, these data do not eliminate the possibility

Table 3. Effect of TCDD on the incorporation of [^{14}C]ALA into rat testicular microsomal heme*

Treatment	[^{14}C]ALA incorporation into microsomal heme (cpm/nmoles heme)
Control	1358 ± 114
TCDD, 24 hr	490 ± 154†

* Rats received 6 μCi of [^{14}C]ALA intraperitoneally 24 hr after administration of TCDD (25 μg/kg; orally). The rats were killed 90 min later, the microsomal heme was extracted and assayed, and the radioactivity was measured as described in Materials and Methods. Each value is the mean ± S.E.M. for six determinations.

† Indicates that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

that TCDD may also alter the synthesis of specific rat testicular apocytochromes.

The decreases in microsomal cytochrome P-450 in both the guinea pig and rat testis do suggest that TCDD may affect testicular function. Cytochrome P-450 is an essential component of the testicular androgen biosynthetic pathway. Therefore, changes in levels of testicular cytochrome P-450(s) by either inhibition of the biosynthesis of heme or regulation of apocytochrome P-450 levels might be expected to produce alterations of plasma androgen concentrations. Future research in this laboratory will involve investigations into the mechanism whereby rat testicular heme synthesis and the subsequent P-450-mediated synthesis of steroids are impaired by TCDD.

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