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TRANSPLACENTAL INDUCTION OF MIXED-FUNCTION OXYGENASES IN EXTRA-HEPATIC TISSUES BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

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Abstract—Treatment of pregnant rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in a dose-dependent induction of a mixed-function oxidase system in fetal and maternal extra-hepatic tissues. At doses of 6 μg/kg, aryl hydrocarbon hydroxylase (AHH) activity was increased 24-, 22- and 4-fold in fetal lung, kidney and skin, respectively, while maternal lung, kidney and adrenal AHH activity was increased 4-, 2- and 2-fold respectively. High-pressure liquid chromatographic (H.P.L.C.) analysis of benzo(a)pyrene (BP) metabolism after TCDD induction indicated that fetal lung, kidney and skin produced significant quantities of benzo(a)pyrene-7,8-dihydrodiol (BP-7,8-diol), benzo(a)pyrene-4,5-dihydrodiol (BP-4,5-diol) and 9- and 3-phenols of BP. The fetal liver produced benzo(a)pyrene-9,10-dihydrodiol (BP-9,10-diol), BP-4,5-diol, BP-7,8-diol and 9- and 3-phenols of BP. Maternal lung also produced BP-9,10-diol, while maternal adrenal gland yielded primarily the 9-phenol of BP. Epoxide hydratase activity was increased 2- to 3-fold in maternal lung, fetal lung and skin after TCDD pretreatment, but was not affected significantly in liver, kidney or placenta. Treatment of pregnant rats with TCDD increased the covalent binding of BP to DNA in preparations containing maternal liver, lung and placenta as well as fetal liver, lung and skin. Pretreatment with TCDD resulted in increased epoxide hydratase and AHH activities in extra-hepatic tissues but only AHH was increased in hepatic tissues, indicating that the inducing capabilities of TCDD differ from, but share some similarities with, both phenobarbital (PB) and 3-methylcholanthrene (MC). Thus, TCDD appears to provide an exceptionally potent and broad-spectrum transplacental induction of carcinogen-transforming enzymes in extra-hepatic tissues.

Chlorinated dibenzodioxins and chlorinated dibenzofurans occur as contaminants in many technical and/or industrial products related to the polychlorinated phenols. The most toxic of the dibenzodioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was the teratogenic contaminant found in the heavily used defoliant "Agent Orange", a phenoxyacetic acid herbicide [1]. This highly toxic compound possesses a percutaneous LD_{50} for guinea pigs of less than 1 μg/kg [2], is toxic to primates [3], chickens, mice and horses [4], and has an oral LD_{50} in rats of 100 μg/kg [3]. It has also proven to be an extremely potent teratogen in laboratory animals [5]. Subacute or chronic exposure to low levels of TCDD produces chloracne in man [6] and causes acute and chronic histopathologic changes in liver, kidney and gastric mucosa [3].

The potency of TCDD as an inducer of the mixed-function oxygenase system in rats was evidenced by a 30-fold induction of aryl hydrocarbon hydroxylase (AHH) with as little as 10 μg/kg [7]. Exposure to TCDD increased AHH activity by as much as 3000 times [8], also increased δ-amino-levulinic acid synthetase activity, and has appeared to be similar to 3-methylcholanthrene (MC) as an enzyme inducer [1]. High levels of induction have been reported in rats [1], human lymphocytes [9], mice [10] and 'non-responsive' mice [11]. Previous studies from our laboratory [12] and other laboratories [13, 14] indicate that TCDD is capable of inducing the hepatic mixed-function oxygenases transplacentally, while less potent inducers such as MC [15] and polychlorinated biphenyls [16] are much less effective transplacental inducers.

This report represents an extension of our earlier studies on the effects of TCDD on pregnant rats [12]. Here we report a study of the dose-related effects of TCDD on extra-hepatic maternal and fetal tissues with respect to AHH activity, BP metabolism in the same tissues as revealed by high-pressure liquid chromatographic (H.P.L.C.) analyses, and catalysis of covalent binding of BP to DNA *in vitro*. We also determined the effects of TCDD on hepatic and extra-hepatic epoxide hydratase activity in maternal and fetal tissues.

MATERIALS AND METHODS

Female Sprague-Dawley rats (Tyler's Laboratories, Bellevue, WA) were received on day 14 of gestation, housed in individual cages and placed in separate rooms to prevent any possible contamination of control animals with TCDD. The animals were dosed on day 17 of gestation with a single intraperitoneal injection of TCDD (0.2 to 6.0 μg/kg) in corn oil or corn oil alone. Animals were sacrificed on day 20 of gestation, and maternal livers, lungs, kidneys,

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adrenals and placentas, and fetal livers, lungs, kidneys and skins were removed and washed with ice-cold 1.15% KCl. Tissues were pooled within groups and homogenized with a Potter–Elvehjem homogenizer (livers) or Polytron PT-10, with 2 parts of 0.1 M phosphate buffer (pH 7.35) at 0–4°. The homogenates were centrifuged at 9000 *g* for 20 min to remove cell debris, and aliquots of the supernatant were utilized for all subsequent assays of BP metabolism. Microsomal preparations were utilized for assays of epoxide hydratase activities.

Benzo(a)pyrene (BP) was obtained from Eastman Chemical Co., Rochester, NY, and recrystallized from warm benzene and methanol. BP-7,10-[^{14}C] (sp. act. 18.6 μCi/μmole) and BP-G[^{3}H] (sp. act. 6.3 Ci/mmole) were obtained from Amersham/Searle Corp., Arlington Heights, IL. TCDD [98.6% pure by gas–liquid chromatography (G.L.C.), lot No. 851-144-II] was a gift from the Dow Chemical Co., Midland, MD. 1,1,1-Trichloro-2,3-propene oxide (TCPO) was purchased from Aldrich Chemical Co., Milwaukee, WI. Standards of the 3-hydroxy, 9-hydroxy, 3,6-quinone, 1,6-quinone, 6,12-quinone, 4,5-diol, 7,8-diol, 9,10-diol and 4,5-epoxide metabolites of BP were provided by Drs. H. V. Gelboin and J. K. Selkirk, NCI. The 4,5-diol, 4,5-quinone and 4,5-epoxide were synthesized in our laboratory according to the methods described by Goh and Harvey [17] and Cho and Harvey [18].

Aryl hydrocarbon hydroxylase was assayed by the fluorometric method of Wattenburg *et al.* [19] with modifications (Juchau *et al.* [20]). Metabolism of BP was assessed by H.P.L.C. analysis as described by Selkirk *et al.* [21] and modified by Berry *et al.* [12].

Epoxide hydratase was assayed using BP-4,5- epoxide as substrate. The reaction mixture contained 0.5 to 1.0 mg protein, 10.0 μg BP-4,5-oxide (in dimethylsulfoxide) and 120 μmoles Tris buffer, pH 8.5, in a total volume of 1.0 ml. The reaction was incubated at 37° for 5 min and was terminated by the addition of 3 ml of cold dichloromethane. The reaction was then extracted twice with dichloromethane and the residue was dried and redissolved in 1.0 ml methanol. Eight μl was injected into a Micromeritics model 7000-0111 H.P.L.C. fitted with a 2.1 mm × 0.5 m Vydac RP ODS (The Separations Corp., Gardena, CA) column operated at ambient temperature, with an isocratic solvent consisting of 65% methanol and 35% buffered water (pH 7.8). BP-4,5-diol was measured at 254 nm and determined from the peak area utilizing a standard curve.

The covalent binding of BP[^{3}H] to DNA *in vitro* was measured by a procedure based on the system originally described by Gelboin [22] and Grover and Sims [23]. The reaction mixture contained the following components in a final volume of 3 ml: 50 μmoles sodium phosphate, pH 7.4; 100 μmoles EDTA; 0.5 mg NADPH; 2 mg of calf thymus DNA; 0.2 ml of tissue homogenate (2–6 mg protein) and 143 nmoles BP[^{3}H] in 25 μl ethanol (72 μCi). The reaction was started by adding the hydrocarbon substrate, and the mixture was incubated for 15 min at 37° in total darkness. The reaction was terminated by the addition of 3 ml of a solution containing 2% sodium dodecyl sulfate; 0.03 M NaCl; and 0.003 M Na citrate, pH 7.0. The mixture was extracted and DNA was isolated as described by Buty *et al.* [24]. Covalent binding of BP[^{3}H] to DNA was expressed as pmoles hydrocarbon bound/μg of DNA/mg of protein/15 min of incubation. Values from unincubated flasks were subtracted as 'zero time' controls and each experiment was performed in triplicate.

RESULTS

Initial investigations were undertaken to ascertain the magnitude of the transplacental induction of AHH using varying doses of TCDD. Four doses (0.2, 0.5, 2.5 and 6.0 μg/kg) were given to groups of pregnant rats on day 17 of gestation and the results of the treatments on fetal lung, kidney and skin, and maternal lung, kidney, adrenal and placental AHH activities are shown in Table 1. Fetal extra-hepatic tissues were found to be responsive in a dose-dependent fashion, with the kidney exhibiting the largest

Table 1. Effects of varying doses of TCDD on AHH activity in fetal and maternal rat tissues*

	Dose (μg/kg body wt)				
Tissue	Control	0.2	0.5	2.5	6.0
Fetal					
Lung	17 ± 8	54 ± 19	161 ± 48	358 ± 121	408 ± 142
Kidney	11 ± 7	15 ± 9	23 ± 7	99 ± 24	242 ± 71
Skin	7 ± 3	9 ± 5	15 ± 8	23 ± 10	28 ± 8
Liver	15 ± 4	320 ± 62	356 ± 85	898 ± 213	917 ± 204
Maternal					
Lung	15 ± 3	37 ± 9	34 ± 6	46 ± 9	56 ± 12
Kidney	65 ± 10	67 ± 15	74 ± 13	123 ± 21	131 ± 20
Adrenal	98 ± 19	109 ± 27	115 ± 36	137 ± 32	170 ± 46
Liver	221 ± 27	362 ± 59	583 ± 102	2077 ± 341	6981 ± 516
Placenta	12 ± 10	18 ± 13	23 ± 14	31 ± 14	36 ± 17

* Doses are presented as intraperitoneal injections of TCDD.

AHH activity is expressed as pmoles 3-hydroxy-BP formed/mg of protein/min. Assays were performed in triplicate on three pools of 9000 *g* supernatant fractions with a minimum of four organs/pooled sample. Final protein concentrations in incubation flasks were adjusted to 0.7 mg/ml in each case. Experiments were repeated twice with closely similar results. Standard deviations are indicated.

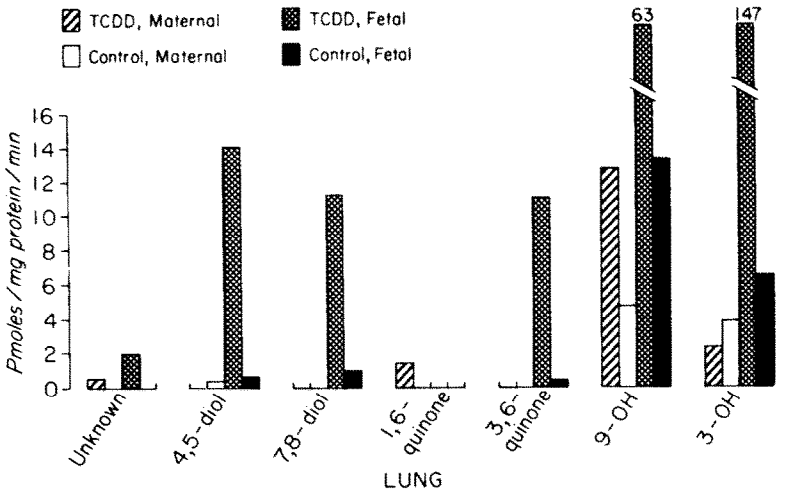

Fig. 1. Specific activity and metabolites formed by maternal and fetal lung homogenates of control and TCDD-treated tissues.

percentage of increase, followed by the lung and skin. Unlike the liver, which appeared to reach a maximum induction at the 2.5 μg/kg dose, lung, kidney and skin exhibited non-maximal induction. As evidenced by the data presented in Table 1, maternal lungs, kidneys, placentas and adrenals demonstrated dose-dependent increases in AHH activity. The low placental AHH response to TCDD may relate to toxicity [3] and/or to degeneration of placental tissues at term [12]. The lung and kidney were less responsive to TCDD than the liver [8], and a small but significant induction in the adrenal gland also was observed. Induction in this organ has not been previously reported.

Transplacental induction of the AHH system by TCDD was further evaluated by studying rates of formation of specific metabolites of BP with H.P.L.C. analysis. The ability to form phenols, quinones and dihydrodiols of BP by lung tissue preparations is shown in Fig. 1. Not only were rates of phenol formation more rapid in fetal lungs, but dihydrodiols (especially the 7,8-diol) were formed more rapidly on a 'per mg protein' basis. The maternal lung did not appear as susceptible to induction as the fetal lung but TCDD increased specific activities by a factor of 4 as compared with control levels.

Analysis of BP metabolism in the fetal kidney with high-pressure liquid chromatography revealed general agreement with data from the fluorometric assay (Fig. 2). Of particular interest is the predominance of the 9-OH-BP over the 3-OH-BP in fetal and maternal kidney, and the increase by TCDD of rates of formation of 9-OH-BP in the maternal but not the fetal kidney. TCDD also increased the relative amounts of quinone formed in maternal kidney, as well as amounts of the 4,5- and 7,8-diols. The 9,10-diol was not detected, and in the maternal or fetal kidney an unidentified, highly polar compound appeared near the solvent front in kidney of TCDD-pretreated animals.

Elevation of fetal rat skin mixed-function oxygenases as evidenced by BP metabolism is shown in Fig. 3. The 3-OH-BP was induced to the greatest degree, while the level of 9-OH-BP production appeared constant. Pretreatment with TCDD increased the amount of 6,12-BP quinone produced and

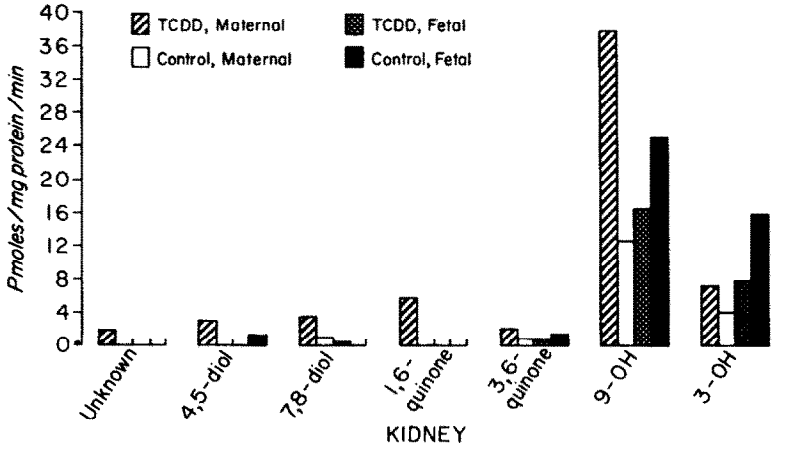

Fig. 2. Specific activity and metabolites formed by maternal and fetal kidney in control and TCDD-induced tissues.

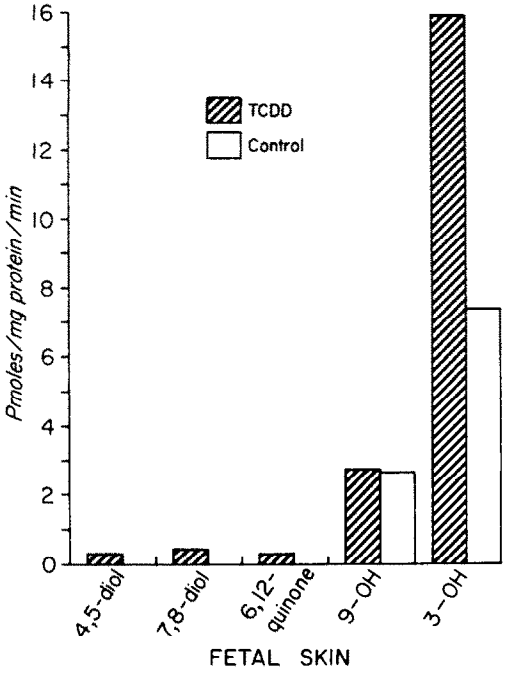

Fig. 3. Specific activity and metabolites formed by fetal control and TCDD-induced skin.

also increased the production of BP-4,5- and BP-7,8-diols. As with the kidney and lung, TCDD pretreatment did not result in the formation of detectable BP-9,10-diol.

Induction of enzymatic activity in the maternal adrenal by TCDD was demonstrated in both the AHH assay and measurements of metabolism of BP with H.P.L.C. analysis (Fig. 4). As with the kidney, production of the 9-OH-BP was favored over the 3-OH-BP. The adrenal was active in producing the 1,6-, 3,6- and 6,12-quinones of BP. The adrenal also produced detectable quantities of BP-4,5-, 7,8- and 9,10-diol, and two unidentified compounds, one

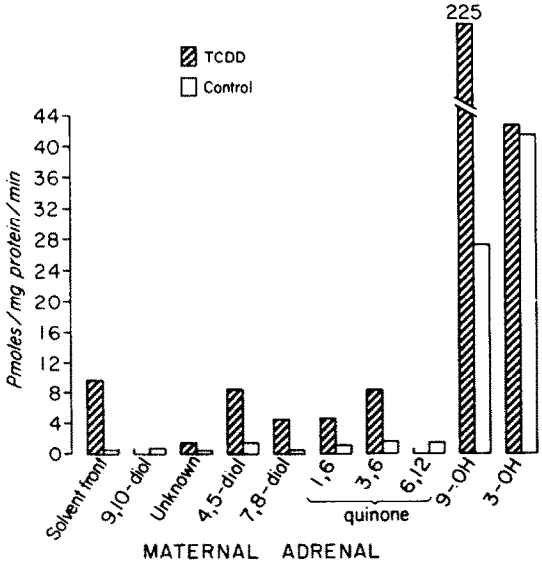

Fig. 4. Specific activity and specific metabolites formed by maternal control and TCDD-treated adrenal gland.

Table 2. Effects of TCDD treatment on epoxide hydratase activity*

Tissue	Control	TCDD†	Ratio (TCDD/control)
Maternal liver	2.18 ± 0.56	2.42 ± 0.64	1.11
Fetal liver	1.40 ± 0.40	1.48 ± 0.58	1.06
Maternal lung	0.30 ± 0.08	0.78 ± 0.19	2.60
Fetal lung	0.47 ± 0.14	1.34 ± 0.35	2.85
Fetal skin	0.76 ± 0.23	1.99 ± 0.39	2.62
Fetal kidney	1.04 ± 0.31	1.10 ± 0.41	1.06
Placenta	0.23 ± 0.09	0.12 ± 0.04	0.52

* Specific activity is expressed as nmoles BP-4,5-dihyrodiol formed/mg of protein/min with standard deviations. Values represent the means of triplicate determinations on three pooled samples with at least four organs in each pool.

† Given at 2.5 μg/kg, i.p., in corn oil on day 17 of gestation; the animals were sacrificed on day 20.

located between the BP-9,10-diol and the BP-4,5-diol, and the other, a highly polar compound, appearing at the solvent front.

The formation of dihydrodiols from BP oxides is a detoxification reaction involving a microsomal epoxide hydratase [25]. TCDD was shown to enhance the formation of BP-4,5-dihydrodiol from BP-4,5-oxide in certain tissue homogenates (Table 2). The least epoxide hydratase induction occurred in hepatic homogenates, while lung and skin homogenates were quite inducible. The placental epoxide hydratase was not induced.

The ability of the tissue homogenates to catalyze covalent binding of BP to DNA *in vitro* is shown in Table 3. The data show that an NADPH-generating system is necessary to catalyze the covalent binding of BP *in vitro* to DNA, and that the epoxide hydratase inhibitor TCPO does not significantly affect binding of BP to DNA. Stimulation of DNA binding

Table 3. Tissue-mediated covalent binding *in vitro* of [^{3}H]BP to DNA

Tissue	Specific activity*		Ratio (TCDD/control)
	Control	TCDD†	
Maternal Liver	136	8660	63.6
−NADPH	18	53	2.9
+TCPO	158	9100	57.6
Lung	62	87	1.4
−NADPH	20	36	1.8
Fetal Liver	96	1023	10.6
−NADPH	1	10	10
+TCPO	89	1000	11.2
Lung	33	38	1.1
−NADPH	11	8	0.7
Skin	31	164	5.3
−NADPH	14	6.2	0.4
Placenta	11.6	30	2.6
−NADPH	7.5	10.5	1.4

* Specific activity is expressed as fmoles[^{3}H]BP bound/μg of DNA/mg of homogenate protein/15 min of incubation.

† Given at 2.5 μg/kg i.p. in corn oil on day 17; the animals were sacrificed on day 20.

appears maximal in hepatic tissues followed by skin, lung and placenta.

DISCUSSION

Several investigators have reported either no or minimal transplacental induction of mixed-function oxidases from different tissues when polycyclic aromatic hydrocarbons (PAH) were used as the inducing agents. The administration of 80 mg/kg of MC i.p. to rats did not induce hepatic demethylation of 3-methyl-4-monomethylaminoazobenzene (3′-MMAB) [26], and administration of twenty times the amount of BP necessary for maternal induction only slightly raised fetal AHH activity and demethylation of 3′-MMAB [15]. Similar results for rats were reported for transplacental induction of fetal hepatic AHH [27] and for chick and mouse embryos [27, 28]. Transplacental stimulation with polychlorinated biphenyls [16] indicated that only minimal induction occurred in fetal rat livers and that the induction did not differ from that observed with MC. Recent evidence has shown that TCDD given orally [13] increases AHH and glucuronyl transferase activity in fetal rat liver and that TCDD stimulation was dependent on the gestational stage of the rat [14]. TCDD also has been shown to induce hepatic AHH activity in genetically 'non-responsive' mice [11]. Results presented in this paper indicate that fetal AHH activity was elevated transplacentally in lungs, kidneys, skins and liver in a dose-dependent fashion. Elevation of maternal AHH activity in kidney, lung and liver showed dose-dependent relationships and greater activity than the fetal tissues. Recent results indicate that TCDD binds preferentially in the liver [29]. Our data tend to indicate that TCDD is distributed to all tissues with little or no relationship between tissue concentrations and effects on enzymes. The marked decrease in AHH activity of placental tissues perhaps relates to the degenerated state of the tissue at term and the toxicity of TCDD to the tissue [30]. Elevation of AHH activity in the adrenal gland appears unique to TCDD and may relate to increased *de novo* synthesis of the inducible form of AHH, which may be only a minor portion compared to the relatively high levels of constitutive enzyme present in the adrenal. Another possible explanation for the increased transplacental induction by TCDD may relate to a more rapid transport across organ membranes [30] and the lack of metabolism of TCDD *in vivo*, leading to a long half-life. The latter possibility will require further investigation.

Analysis of BP metabolism in preparations of MC-pretreated rat liver indicated that stimulation occurred with respect to all metabolites detected with the largest increase in the BP-9,10-diol [31]. We previously demonstrated that TCDD pretreatment resulted in increases of BP-4,5-diol, BP-7,8-diol and BP-9,10-diol with preparations of maternal and fetal hepatic tissues [12]. Results from this investigation indicate that preparations of fetal lung and skin produced substantial quantities of the 3-phenol and 7,8-diol as major metabolic products. The skin and lung both are primary target tissues for PAH carcinogens and are not nearly as metabolically active as the hepatic system. Additionally, the fetal kidney produced greater quantities of 9-phenol and BP-7,8-diol than skin and lung, but exhibited lower overall activity than the hepatic system. Although previous investigations have demonstrated that adrenal glands exhibit high specific AHH activity [32], the production of 3-phenol was not truly representative of BP metabolism by the adrenal. As was evidenced with H.P.L.C. analysis, the adrenal homogenates produce more 9-phenol than 3-phenol and, to a limited extent, more dihydrodiols. The apparent trend in BP metabolism by extra-hepatic tissues after TCDD pretreatment was toward higher production of the 9-phenol and the 7,8-diol, whereas hepatic tissues produced more 3-phenol and BP-9,10-diol. The significance of this trend is not apparent at present, but it was shown that induction as measured by high-pressure liquid chromatography allowed critical evaluation of subtle metabolic differences between various extra-hepatic tissues as they relate to specific inducers.

The epoxide hydratase system is primarily responsible for the elimination of electrophilic epoxide intermediates. Previous investigations have demonstrated that epoxide hydratase is not inducible by PAH such as BP [33] and MC [34] in hepatic tissue. Results presented in this paper indicate that TCDD is capable of inducing epoxide hydratase activity to approximately the same degree as phenobarbital in certain extra-hepatic tissues [33]. However, as with the oxidative metabolism, the induction was highly tissue specific. Induction occurred in the fetal skin and lung and in maternal lung but not in maternal liver, kidney or placenta. The stable 'K-region' arene oxide, BP-4,5-epoxide, was used as a substrate for epoxide hydratase because of its direct relationship to the parent BP. Other substrates exhibit higher affinities for the enzyme [35] but the study indicated that TCDD was capable of markedly increasing epoxide hydratase activity, and that the apparent induction was tissue dependent.

Since there is a good correlation between the carcinogenic activity of PAH and their ability to be converted into an electrophilic intermediate(s) and subsequent interaction with cellular nucleophiles such as DNA [24, 36–38], we investigated the effects of TCDD pretreatment on tissue-mediated covalent binding of [^{3}H]BP to DNA. Previous investigations [39, 40] ascertained that covalent binding with hepatic tissue homogenates shows a strong requirement for NADPH, and was inducible by PAH. Investigations with the skin [24] demonstrated a dependence on NADPH when BP was used as a substrate. Our results demonstrate that TCDD is effective in enhancing the binding of BP to DNA in maternal and fetal hepatic tissues in reactions requiring reduced pyridine nucleotides. Maternal and fetal rat lungs exhibited a similar trend, but the requirement for NADPH was not as evident. The fetal rat skin also showed TCDD-inducible covalent binding and a similar requirement for NADPH. The potent epoxide hydratase inhibitor, TCPO, only slightly increased the covalent binding of BP to DNA. The nature of the TCDD enhancement appeared to be tissue-specific, and, moreover, the data from the binding *in vitro* appeared to correlate well with the metabolizing capabilities of the tissue and the metabolites formed.

The extreme toxicity of TCDD [1, 3, 7, 29] prevented the giving of doses above 6 μg/kg to pregnant Sprague–Dawley rats. Doses above 10 μg resulted in fetal death and generally spontaneous termination of pregnancy. However, with fetal liver, plateau levels of AHH were attained at 2.5 μg/kg, and plateau AHH levels were approached in fetal lung and kidney at 6 μg/kg. Plateau AHH levels in maternal tissues were not attained at the dosages used and, in the case of liver, a maximal dose was approximately 10 μg/kg [8] for a nonpregnant rat. A recent report pertaining to a high affinity, low capacity cytosol induction receptor for TCDD in liver [41] furnishes new evidence for the potency of TCDD as an enzyme inducer. In addition, the study demonstrates that PAH compete for the receptor but are much less effective than TCDD.

This study illustrates that administration of TCDD to pregnant rats results in elucidation of metabolic capabilities that are not normally apparent in fetal tissues. The ability of TCDD to induce enzyme activity in a fashion similar to both PB and MC in a tissue-specific manner is of extreme interest when evaluating the overall metabolism of PAH. Moreover, TCDD elevation of AHH and epoxide hydratase activity in fetal tissues provides further information on inducible and constitutive enzyme levels in fetal tissues and their function in continued investigations on the mechanisms of transplacental carcinogenesis.

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