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Altered Hepatic Iron Distribution and Release in Rats after Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent toxin and tumor promoter in rodents, and is prototypical of many polycyclic halogenated hydrocarbons that occur as environmental contaminants. Most pathologic lesions produced by these compounds may be mediated by an intracellular receptor protein called the TCDD (Ah) receptor which functions as a trans-acting effector of gene expression (Poland and Knutson 1982; Whitlock 1987). However, the post-translational pathways and mechanisms involved in the expression of the toxic manifestations of TCDD and its bioisosteres have received little attention and remain largely unknown.

Various studies have shown that TCDD induces hepatic lipid peroxidation (Stohs et al. 1983; Al-Bayati et al. 1987a; Mohammadpour et al. 1988), alters the distribution of divalent cations (Wahba et al. 1988), and produces DNA single strand breaks (Wahba et al. 1989) which occur in conjunction with lipid peroxidation (Ames et al. 1982).

The role of iron in the initiation of lipid peroxidation is well known (Bus and Gibson 1979; Alleman et al. 1985). Evidence suggests that chelated iron acts as a catalyst of the Haber-Weiss reaction, facilitating the conversion of superoxide anion and hydrogen peroxide to hydroxyl radical, a species frequently proposed to initiate lipid peroxidation (Imlay et al. 1988; Halliwell and Gutteridge 1986). Although hydroxyl radical is highly reactive, its in

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vivo formation is dependent upon the availability of physiological iron (Thomas and Aust 1986, 1988).

A role for iron in the toxic manifestations of TCDD may exist. TCDD induces porphyria in rats and mice, and depletion of body iron stores effectively prevents TCDD-induced porphyria (Rowley and Sweeney 1984; Yao and Safe 1989). A decrease in TCDD-induced hepatic lipid peroxidation is observed in rats fed an iron deficient diet (Al-Turk et al. 1988), and iron is involved in the enhanced lipid peroxidation associated with hepatic microsomes from TCDD-treated rats (Al-Bayati and Stohs 1987). Previous studies have indicated that TCDD administration to female rats alters iron distribution in hepatic mitochondria and microsomes (Al-Bayati et al. 1987b). Therefore, we have examined the effect of TCDD administration to male and female rats on the hepatic subcellular distribution of iron as well as on the availability of free or releasable iron. Rowley and Sweeney (1984) have shown that the release of iron from ferritin by microsomes from TCDD-treated mice correlated with NADPH-dependent c cytochrome reductase activity, and we have also examined the effect of TCDD on microsomal and mitochondrial cytochrome c reductase activity.

MATERIALS AND METHODS

TCDD was obtained from the National Cancer Institute Chemical Carcinogen Repository (Bethesda, MD). Ferric chloride standard was purchased from Fisher Scientific Co. (St. Louis, MO). All other chemicals utilized in these studies were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO). Male and female Sprague-Dawley rats, weighing 140 to 160 g, were obtained from Sasco Inc. (Omaha, NE). The animals were maintained on standard Purina laboratory chow and water ad libitum, with a 12 hr light/dark cycle 7:00 A.M. to 7:00 P.M. at 21° C. TCDD-treated animals received 40 μg TCDD/kg/day for 3 days orally in a 10% acetone in corn oil vehicle. Control rats received the vehicle. The animals were killed by decapitation 3, 7 or 10 days post-treatment between 6:00 a.m. and 7:00 a.m. to eliminate possible effects due to diurnal variation.

The iron content in whole liver and subcellular fractions was measured by atomic absorption spectrophotometry as previously described (Wahba et al. 1988). Iron released from microsomes and mitochondria was measured using the bleomycin chelated iron-dependent degradation of DNA procedure of Gutteridge et al. (1981). This assay method measures only free iron and not iron bound to transport protein or to enzymes. Hepatic cytochrome c reductase activity was determined

according to the procedure of Dallner (1963). The amount of cytochrome c reductase/min/mg microsomal or mitochondrial protein was calculated using the molar extinction coefficient of $18.5 \times 10^{-3} mM^{-1} cm^{-1}$. Protein concentrations of whole liver homogenate, microsomes, mitochondria, and cytosol were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Data for each group were subjected to analysis of variance (ANOVA) and Student's t test. The data are expressed as the mean $\pm$ standard deviation (SD). Each value is derived from 8-12 animals. The level of statistical significance employed in all cases was $P<0.05$.

RESULTS AND DISCUSSION

The effect of TCDD on the hepatic distribution of iron in whole liver and its subcellular fractions from female rats 7 days after treatment is presented in Table 1. TCDD had no effect on the iron content of whole liver or cytosol. However, after treatment a 46% increase in the iron content of mitochondria was observed relative to the control group, while a 40% decrease in microsomal iron content occurred when the iron distribution was expressed per milligram of protein. When the data were expressed relative to wet weight, after TCDD administration the iron content of mitochondria increased by 41%, while a 16% decrease in microsomal iron was observed.

The effect of TCDD on the distribution of iron in the liver of male rats was also examined 7 days post-treatment (Table 1). The iron content of whole liver of untreated male rats was approximately one half the iron content of the liver of female rats (Table 1). Administration of TCDD had no effect on the iron content of whole liver, microsomes or cytosol when the data were expressed relative to milligrams of protein or grams of liver. When the data were expressed per milligram of protein, TCDD resulted in a 46% increase in mitochondrial iron 7 days after treatment, and a 89% increase when the data were expressed per gram liver.

The ability of iron to catalyze formation of reactive oxygen species may depend upon the availability of free or loosely bound iron. Therefore, the amount of free or chelatable iron associated with hepatic microsomes and mitochondria following TCDD administration was determined (Table 2). In female rats, TCDD produced a significant increase (33%) in releasable (chelatable) iron from microsomes, and a significant decrease (58%) in the amount of chelatable iron associated with mitochondria. Administration of TCDD to male rats also resulted in a significant decrease in the amount of

Table 1. Effect of TCDD on the hepatic distribution of iron in female and male rats

Tissue Fraction	Iron Content			
	nmol/mg protein		nmol/g liver	
	Control	TCDD-treated	Control	TCDD-treated
Female Rats				
Whole Homogenate	12.11 ± 1.10	10.88 ± 0.35	2996.6 ± 43.2	3090.6 ± 95.2
Mitochondria	9.91 ± 1.07	14.51 ± 0.43*	155.4 ± 26.8	218.6 ± 22.2*
Microsomes	23.52 ± 1.70	14.01 ± 1.55*	1086.0 ± 61.4	914.6 ± 29.0*
Cytosol	16.95 ± 1.15	16.69 ± 1.74	1288.0 ± 76.1	1139.7 ± 126.8
Male Rats				
Whole Homogenate	6.03 ± 0.93	6.51 ± 0.66	1707.8 ± 122.84	1520.8 ± 149.34
Mitochondria	6.37 ± 1.45	9.27 ± 1.01*	70.47 ± 12.4	133.0 ± 14.2*
Microsomes	7.79 ± 0.51	8.30 ± 1.44*	542.0 ± 36.53	572.1 ± 81.5*
Cytosol	8.07 ± 1.32	10.03 ± 1.10	1024.4 ± 92.2	1083.5 ± 155.6

Rats were treated with 40 μg TCDD/kg/day for 3 days p.o. and killed 7 days post-treatment. Control animals received the vehicle. Each value is the mean ± S.D. of 8-12 animals.
*$P<0.05$ with respect to the control group.

iron released (30%) from mitochondria, with no change in the amount of iron released from microsomes.

The activity of NADPH dependent cytochrome c reductase was determined in hepatic microsomes and mitochondria of TCDD-treated female rats, and expressed in nmoles cytochrome c reduced/min/mg protein (Table 3). The activity of the enzyme in the microsomes significantly increased (23%) 3 days after TCDD administration, with no change in the enzyme activity at either 7 or 10 days post-treatment. Activity of the enzyme in mitochondria decreased 15%, 18% and 18% after 3, 7, and 10 days post-treatment with TCDD, respectively. Therefore, no correlation appears to exist with respect to the effect of TCDD on the amount of iron that can be released and the activity of NADPH-dependent cytochrome c reductase in microsomes and mitochondria.

A sex difference is evident in the iron content of whole liver and its subcellular fractions from Sprague Dawley rats. The hepatic iron content of female rats is approximately twice as great as the iron content of male rats (Table 1). These results are similar to the observations of Vidnes and Helgeland (1973) who observed a 2-fold higher concentration of hemosiderin iron in livers of female Wistar rats as compared to male rats, while Linder et al. (1973) observed that Charles River female rats exhibited 4- to 5-fold greater concentrations of nonheme iron and ferritin iron in the liver.

The role of iron in the formation of reactive oxygen species, lipid peroxidation and tissue damage is well known. Greater levels and rates of lipid peroxidation occur in female rats as compared to male rats (Al-Bayati et al. 1987a) which may be explained by the higher hepatic iron content in female rats. However, lipid peroxidation is dependent upon the form in which the iron exists and not upon the total amount of the iron present.

The administration of TCDD to female rats may result in the release of greater amounts of free or catalytically active iron than in male rats. This hypothesis is supported by the observations in Table 2. Hepatic microsomes from TCDD-treated female rats exhibited significantly higher amounts of releasable iron than the corresponding control group.

Table 2. TCDD induced iron release from mitochondria and microsomes of livers of female and male rats

Treatment	Iron Released (in 2 hours)	
	Microsomes	Mitochondria
	nmol/mg protein	nmol/mg protein
Female Rats		
Control	1.85 ± 0.07	1.84 ± 0.32
TCDD-Treated	2.46 ± 0.35*	0.78 ± 0.21*
Male Rats		
Control	1.84 ± 0.51	5.14 ± 1.01
TCDD-Treated	1.54 ± 0.22	3.62 ± 0.22*

Rats were treated with 40 μg TCDD/kg/day for 3 days p.o. and were killed 7 days post-treatment. Control animals received the vehicle. The amount of released free ferrous iron was determined by using the bleomycin technique. Each value is the mean ± S.D. of 8-12 rats. *$P < 0.05$ with respect to the control group.

No significant difference was observed in the amount of releasable iron from microsomes of control and TCDD-treated male animals. Thus, an increase in the amount of releasable iron from microsomes of TCDD-treated female rats occurred in spite of a decrease in the total iron content (Table 1). In male rats, no change occurred in either the amount of releasable iron (Table 2) or iron content (Table 1) in the microsomes of male rats 7 days after treatment with TCDD. These observations provide an explanation for the greater lipid peroxidation in female rats as compared to male rats in response to TCDD. However, since TCDD also elevates hepatic malondialdehyde levels in male rats, other mechanisms must therefore operate as well in TCDD-induced lipid peroxidation. The amount of releasable iron from hepatic mitochondria of both male and female rats decreased 7 days after treatment with TCDD in conjunction with an increase in the total iron content (Table 1).

Table 3. Effect of TCDD on the Activity of Hepatic Microsomal and Mitochondrial NADPH-Dependent Cytochrome c Reductase

Days Post-treatment	nmol cytochrome c reduced/min/mg protein	
	Microsomes	Mitochondria
—	230.1 ± 16.5[a] (100%)	304.8 ± 22.7[a] (100%)
3	283.5 ± 23.1[b] (123%)	258.6 ± 19.8[b] (85%)
7	239.5 ± 23.5[a] (104%)	249.2 ± 7.1[b] (82%)
10	228.4 ± 8.9[a] (99%)	284.8 ± 20.8[b] (82%)

Female rats were treated with 40 μg TCDD/kg/day for 3 days orally and killed 3, 7 or 10 days post-treatment. The control group received the vehicle (corn oil containing 10% acetone). Each value is the mean ± S.D. of 8-12 animals. Values with non-identical superscripts are significantly different ($P<0.05$).

Most iron is bound to heme and non-heme proteins. Ferritin, a ubiquitous iron storage protein, is a good source of iron for catalysis of lipid peroxidation (Samokyszyn et al. 1989). However, the iron must be in a free or easily releasable form in order to catalyze the formation of reactive oxygen species. Xenobiotics which undergo redox cycling facilitate reduction of ferric iron in ferritin with the resultant release of catalytically active ferrous iron, and a role for NADPH-dependent cytochrome c reductase in the reduction of ferritin associated iron has been demonstrated (Rowley and Sweeney 1984; Vile and Winterbourn 1988). Therefore, we examined the effect of TCDD on NADPH-dependent cytochrome c reductase activity associated with microsomes and mitochondria of female rats (Table 3). The amount of releasable iron (Table 2) does not correlate well with the activity of NADPH-dependent cytochrome c reductase in either microsomes or mitochondria (Table 3). Thus, although the reductive release of iron from ferritin may depend on NADPH, other factors may be involved.

Superoxide anion may also be involved in the reductive release of iron from ferritin (Reif et al. 1988), and several possible sources for the production of superoxide anion exist. Mitochondria produce superoxide anion and can mobilize iron from ferritin (Ulvik and Romslo 1979), while cytochrome P-450 may

constitute another source (Morehouse and Aust 1988). Activated macrophage, which produce reactive oxygen species, can also release iron from ferritin (Koster et al. 1986). We have recently observed that TCDD administration to rats results in the activation of macrophage with a resulting increase in the production of superoxide anion (unpublished observations). Thus, macrophage activated by TCDD may facilitate the release of iron which then participates in the subsequent production of additional reactive oxygen species, contributing to the development of an oxidative stress and tissue damage.

In summary, the results presented in this study indicate that TCDD produces alterations in the hepatic subcellular distribution of iron. A sex difference exists in the iron content of whole liver and hepatic cellular fractions. A sex difference also exists in the releasibility of iron from microsomes. These disparities may explain the differences in lipid peroxidation in male and female rats. In addition, the results suggest that TCDD-induced modulations in NADPH-dependent cytochrome c reductase do not play a major role in the release of iron from ferritin or the subsequent increase in the formation of reactive oxygen species.

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REFERENCES

Al-Bayati ZAF, Murray WJ, Stohs SJ (1987a) 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced lipid peroxidation in hepatic and extrahepatic tissues of male and female rats. Arch Environ Contam Toxicol 16:159-166

Al-Bayati ZAF, Stohs SJ, Al-Turk WA (1987b) TCDD dietary iron and hepatic iron distribution in female rats. Bull Environ Contam Toxicol 38:300-307

Al-Bayati ZAF, Stohs SJ (1987) The role of iron in TCDD-induced lipid peroxidation in rats. Toxicol Lett 38:115-121

Alleman MA, Koster JF, Wilson JHP, Edixhoven-Bosdijk A, Slee RG, Kroos MJ and Eijk HGV (1985) The involvement of iron and lipid peroxidation in the pathogenesis of HCB induced porphyria. Biochem. Pharmacol., 34, 161-166

Al-Turk WA, Shara MA, Mohammadpour H, Stohs SJ (1988) Dietary iron and 2,3,7,8-tetrachlorodibenzo-p-dioxin induced alterations in hepatic lipid peroxidation, glutathione content and body weight. Drug Chem Toxicol 11:55-70

Ames B, Hollstein MC, Cathcat R (1982) Lipid peroxidation and oxidative damage to DNA. In: Lipid Peroxidation in Biology and Medicine, (Yagi, K., Ed.), Academic Press, NY, pp. 339-351

Bus JS, Gibson JE (1979) Lipid peroxidation and its role in toxicology. In: Reviews in Biochemical Toxicology, (E. Hodgson et al. Eds.), Elsevier, North Holland, pp. 125-149

Dallner G (1963) Studies on the structural and enzymatic organization of the membranous elements of liver microsomes. Acta Pathol Microbiol Scand (Suppl) 166:7-41

Gutteridge JMC, Rowley DA, Halliwell B (1981) Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Biochem J 199:263-265

Halliwell B, Gutteridge JMC (1986) Iron and free radical reactions: Two aspects of antioxidant protection. Trends in Biochemical Sciences 11:372-375

Imlay JA, Chin SM, Linn S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction In vivo and In vitro. Science 240:640-242

Koster JF, Biemond P, Swaak AJG, van Eijk HC (1986) Iron mobilization from ferritin. In: Free Radicals and Arthritic Diseases (Swaak AJA and Koster JF, eds.) Eurage, Amsterdam, pp. 51-58

Linder MC, Moore JR, Scott LE, Munro HM (1973) Mechanisms of sex difference in rat tissue iron stores. Biochim Biophys Acta 297:70-80

Lowry OH, Rosebrough NJ, Farr WL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol chem 193:265-275

Mohammadpour H, Murray WJ, Stohs SJ (1988) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced lipid peroxidation in genetically responsive and non-responsive mice. Arch Environ Contam Toxicol 17:645-650

Morehouse LA, Aust SD (1988) Reconstituted microsomal lipid peroxidation: ADP-Fe^{3+}-dependent peroxidation of phospholipid vesicles containing NADPH-cytochrome P-450 reductase and cytochrome P-450. Free Rad Biol Med 4:269-277

Poland A, Knutson JC (1982) 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann Rev Pharmacol Toxicol 22:517-554

Reif DW, Schulast J, Aust SD (1988) Iron release from ferritin and lipid peroxidation by radiolytically generated reducing radicals. Arch Biochem Biophys 264:238-243

Rowley B, Sweeney GD (1984) Release of ferrous iron from ferritin by liver microsomes: A possible role

in the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Can J Biochem Cell Biol 62:1293-1300
Samokyszyn VM, Miller DM, Reif DW, Aust SD (1989) Inhibition of superoxide and ferritin-dependent lipid peroxidation by ceruloplasmin. J Biol Chem 264:21-26
Stohs SJ, Hassan MQ, Murray WJ (1983) Lipid peroxidation as a possible cause of TCDD toxicity. Biochem Biophys Res Commun 111:854-859
Thomas CE, Aust SD (1986) Release of iron from ferritin by cardiotoxic anthracycline antibiotics. Arch Biochem Biophys 248:684-689
Thomas CE, Aust SD (1988) Reproduction release of iron from ferritin by cation free radicals of paraquat and other bipyridyls. J Biol Chem 261:13,064-13,070
Ulvik R, Romslo I (1979) Studies on the mobilization of iron from ferritin by isolated rat liver mitochondria. Biochim Biophys Acta 588:256-271
Vidnes A, Helgeland L (1973) Sex and age differences in the hemosiderin content of rat liver. Biochim Biophys Acta 328:365-372
Vile GF, Winterbourn CC (1988) Microsomal reduction of low molecular weight Fe^{3+} chelates and ferritin: Enhancement by adriamycin, paraquat, menadione, and anthraquinone 2-sulfate and inhibition by oxygen. Arch Biochem Biophys 267:606-613
Wahba ZZ, Al-Bayati ZAF, Stohs SJ (1988) Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic distribution of iron, copper, zinc and magnesium in rats. J Biochem Toxicol 3:121-129
Wahba ZZ, Lawson TA, Murray WJ, Stohs SJ (1989) Factors influencing the induction of DNA single strand breaks in rats by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicology 58:57-69
Whitlock JP (1987) The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Pharmacol Rev 39:147-161
Yao C, Safe S (1989) 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced porphyria in genetically inbred mice: Partial antagonism and mechanistic studies. Toxicol Appl Pharmacol 100:208-216

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