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Interaction between Iron Metabolism and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Mice with Variants of the *Ahr* Gene: A Hepatic Oxidative Mechanism

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ABSTRACT

The binding of 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) with the aryl hydrocarbon (AH) receptor and subsequent changes in gene expression have been studied intensively, but the mechanisms by which these lead to toxicity are unclear. We investigated the influence of iron, previously implicated in TCDD-induced hepatic porphyria, in mice with alleles of *Ahr* that encode receptors with varied affinity for TCDD. The administration of iron to Ahr^{b-1} C57BL/6J (AH-responsive) mice before a single dose of TCDD (75 μg/kg) markedly potentiated not only the hepatic porphyria but also general hepatocellular damage and elevation of plasma hepatic enzymes. The formation of hydroxylated and peroxylated derivatives of uroporphyrins formed from uroporphyrinogen and the induction of a μ-glutathione transferase (GST) were consistent with the operation of an oxidative mechanism. In a comparison of C57BL/6J mice with Ahr^{b-2} BALB/c (AH-responsive) and Ahr^{d} SWR and DBA/2 (AH-nonresponsive) mice, iron overcame the weak hepatic porphyria and toxicity responses in BALB/c and SWR strains but not in DBA/2. CYP1A isoforms are strongly implicated in the mechanism of porphyria, but activities were lowered by 20–30% with iron treatment, and a comparison of levels between strains did not fully account for the resistance of DBA/2 mice. Studies with the use of gel shift assays and cytosolic aconitase of the capacity of the iron regulatory protein controlling the translation of some iron metabolism proteins showed a significant difference between C57BL/6J and DBA/2 mice after the administration of TCDD. We conclude that iron potentiates both the hepatic porphyria and toxicity of TCDD in susceptible mice in an oxidative process with disturbance of iron regulatory protein capacity. Iron even overcomes the AH-nonresponsive Ahr^{d} allele in the SWR strain but not in DBA/2 mice, which remain resistant.

TCDD is the prototype and most potent member of a large class of polyhalogenated aromatic and polycyclic aromatic chemicals that have a wide range of toxic effects *in vivo*, including "wasting," immune suppression, teratogenicity, hepatotoxicity, and carcinogenicity (Pohjanvirta and Tuomisto, 1994). The mechanisms by which TCDD exerts its effects have been the subject of intense investigation. Most actions probably are ultimately the consequences of TCDD acting as a ligand for the AHR (Poland and Glover, 1979), a transcription factor that functions as a heterodimer with another protein, aryl hydrocarbon nuclear translocator. Both proteins are members of a subclass of basic helix-loop-helix transcription factors. The TCDD/AHR/aryl hydrocarbon nuclear translocator complex binds to a DNA dioxin-responsive promoter element and leads to increases in transcription of a variety of genes, of which the most understood is *cyp1a-1* (Swanson and Bradfield, 1993). A number of variants of the *Ahr* gene have been identified in mice, leading to protein products with markedly different affinities toward TCDD (Poland *et al.*, 1994; Poland and Glover, 1990). The strains, such as C57BL/6J, possessing the Ahr^{b-1} allele encode a receptor (~95 kDa) with a much higher affinity for TCDD than that produced by other strains, such as DBA/2 (~104 kDa), with the Ah^{d} allele. A third type, with the Ahr^{b-2} allele, which is present in BALB/c mice, gives rise to a receptor (~104 kDa) with an affinity for TCDD nearly as great as that from C57BL mice. Inductions of the hepatic enzymic activity

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GST, μ-glutathione transferase; AH, aryl hydrocarbon; AHR, aryl hydrocarbon receptor; ALT, alanine aminotransferase; IRP, iron regulatory protein; IRE, iron responsive element; HPLC, high performance liquid chromatography; UROD, uroporphyrinogen decarboxylase; HCB, hexachlorobenzene; PCNA, proliferative cell nuclear antigen; PCT, porphyria cutanea tarda; PCB, polychlorinated biphenyl; EROD, ethoxyresorufin dealkylation; MROD, methoxyresorufin dealkylation; BROD, benzyloxyresorufin dealkylation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

by AHR agonists in C57BL/6J, BALB/c, and DBA/2 mice reflect these receptors with differing affinities, as apparently do some aspects of the toxicities (Pohjanvirta and Tuomisto, 1994, Poland and Glover, 1979, Swanson and Bradfield, 1993).

Despite huge advances that have occurred as a result of molecular studies of the action of the *Ahr* gene, the mechanisms by which the gene is related to toxicity remain unclear. In one approach to understanding its intracellular role, mice deficient in the expression of AHR were found to be very resistant to the toxicity of TCDD (Fernandez-Salguero *et al.*, 1996). However, because the action of TCDD varies markedly among organs of mice and among species, interactions with other genes must govern ultimate toxic responses. For instance, the *hr* locus modulates both the promoting influence of TCDD on skin carcinogenesis in mice and the hepatic toxicity (Greig *et al.*, 1987; Knutson and Poland, 1982).

One aspect of the hepatic toxicity of TCDD still unexplained is the development of a porphyria caused by a block at the UROD step of heme synthesis (Pohjanvirta and Tuomisto, 1994; Smith *et al.*, 1981). At first, this seemed to be associated totally with the *Ahr* genotype (Jones and Sweeney, 1980), but importantly, depletion of hepatic iron was found to protect C57BL/6J mice from the heme synthesis block, whereas iron overload potentiated the phenomenon (Greig *et al.*, 1984; Jones *et al.*, 1981; Sweeney *et al.*, 1979). For agonists of the AHR much less potent than TCDD, such as HCB, elevated iron status altered the response to the extent that porphyria developed significantly only after combination with iron overload (Smith and Francis, 1983). In fact, iron alone eventually causes uroporphyria in mice, but this does not depend on possession of the Ahr^b allele (Smith and Francis, 1993).

Although original studies suggested that iron potentiated general hepatic toxicity as well as porphyria, this has never been explored fully (Greig *et al.*, 1984; Jones *et al.*, 1981). In the present study, we show that the influence of iron impinges on more general aspects of hepatic toxicity, not just porphyria, and that a susceptible "iron metabolism gene" appears to overcome partially the resistance of the Ahr^d allele. The formation of oxidized products from uroporphyrinogen and the induction of GST support the hypothesis that an oxidative sequence is involved. Gel shift assays showed a significant difference between Ahr^b C57BL/6J and Ahr^d DBA/2 mice in the influence of TCDD on the operation of the IRP, which acts by binding to mRNA iron-responsive elements controlling translation of some iron metabolism proteins such as ferritin and transferrin receptor.

Materials and Methods

Chemicals. TCDD was purchased from Greyhound Chemicals (Birkenhead, UK) and was defined as 99% pure by gas-chromatography spectrometry. To make up dosing solutions, 1 mg was dissolved in 15 ml of acetone over 2 days at room temperature. Aliquots were then mixed with corn oil (Sigma Chemical, Poole, Dorset, UK). MROD, EROD, BROD, cumene hydroperoxide, hydrogen peroxide, and 1,2 dichloro-4-nitro- and 1-chloro-2,4-dinitrobenzenes were purchased from Sigma. Iron-dextran solution (100 mg Fe^{2+} and 100 mg dextran/ml) was also purchased from Sigma. Dextran was a gift from Fisons Chemical (Loughborough, UK). The cDNA probe for CYP1A1 was purchased from American Type Culture Collection (Rockville, Md).

Mice and treatment. Male mice of the C57BL/6J/Ola, BALB/c/Ola, SWR/Ola, and DBA/2/Ola inbred strains were purchased from Harlan Olac (Bicester, UK) and acclimatized for 2 weeks before use at 7–10 weeks of age. Animals were housed in plastic cages on corn bedding at 21°, with a 12-hr light/dark cycle, and fed RM3 diet *ad libitum*. Cages were kept in plastic isolators under negative pressure with eight changes of air/hr. Mice were dosed with an iron dextran solution (0.2 ml/25 g, i.e., 800 mg of Fe^{2+}/kg body weight) or dextran by subcutaneous injection in the flank. After 1 week, the animals received 75 μg of TCDD in oil/kg of body weight or oil alone by gavage and then left for periods of 1–5 weeks. This dose level of TCDD and the time for maximum effect were determined previously (Smith *et al.*, 1981). Mice were killed under terminal anesthesia using CO_2, and blood was removed by cardiac puncture for plasma or serum analyses. All animal procedures were conducted under Home Office regulations. Organs were weighed, and representative samples were fixed in buffered formalin or frozen in liquid N_2 and stored at −70° until analysis. For PCNA analysis, livers were fixed in Carnoy's fluid.

For enzymic and other biochemical analyses, livers were homogenized in 0.25 M sucrose at 4°, and microsomes and cytosol were prepared as described previously (Madra *et al.*, 1996).

Histological examination. Liver sections were stained with hematoxylin and eosin. PCNA expression was detected by immunohistochemistry, and the labeling index was determined as a percentage of hepatocytes (Madra *et al.*, 1995).

Plasma and serum analyses. Estimations of ALT, alkaline phosphatase, and bilirubin were performed with kits obtained from Sigma.

Microsomal dealkylations. EROD, MROD, and BROD, as catalyzed by microsomal cytochrome P450 isoforms, were performed as reported previously (Madra *et al.*, 1996) but at 21° with substrates of 2, 5, and 5 μM concentrations, respectively, and 100 μM NADPH. Product formation was estimated by fluorimetry with excitation at 540 nm and emission at 585 nm. Rates were calculated by fluorimeter software giving least-squares fit. With induced mouse hepatic microsomes, EROD, MROD, and BROD are good indicators of CYP1A1, CYP1A2, and CYP2B, respectively (Nerurkar *et al.*, 1993).

GST and peroxidase assays. GST activity with 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene and glutathione peroxidase activity of GST toward cumene hydroperoxide and selenium-dependent glutathione peroxidase with H_2O_2 were assayed by standard methods referred to previously (Madra *et al.*, 1996).

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting were performed as described previously (Madra *et al.*, 1996) using a polyclonal antibody against mouse GST Yb_1 subunit provided by Prof. J. Hayes (University of Dundee, Dundee, Scotland).

Northern blots. Northern blot analysis of CYP1A1 and CYP1A2 mRNA was performed with a cDNA for CYP1A1 that detected both CYP1A1 and CYP1A2 mRNA species using total mRNA (Chaloupka *et al.*, 1995). Because of their homology, this probe can detect both CYP1A1 and CYP1A2 transcripts (3.3 and 1.9 kb, respectively). Bands were quantified by densitometry and normalized against glyceraldehyde phosphate dehydrogenase gene expression for standardization.

Porphyrin analysis. Porphyrin accumulation in liver homogenate was determined by spectrofluorimetry according to the method of Grandchamp *et al.* (1980) assuming that three basic porphyrin types, uroporphyrin, coproporphyrin, and protoporphyrin, might be present. Results were standardized with reference porphyrins, and concentrations were estimated by matrix analysis. As expected, treatment with TCDD gave uroporphyrin isomers as the major porphyrins present, and results are expressed in these terms.

HPLC of porphyrins. Detailed analysis of liver porphyrins was conducted by HPLC as described previously (Guo *et al.*, 1996) using a Hypersil ODS column (250 × 5 mm; Shandon Scientific, Runcorn, UK), and gradients were constructed from 9% acetonitrile in 1 M

ammonium acetate buffer, pH 5.16, and 10% acetonitrile in methanol. Identification of peaks was made by comparison with previously prepared standards after detection by emission fluorescence at 618 nm following excitation at 400 nm.

IRP assays. The IRP assays were largely based on previously published methods [Leibold and Munro, 1988; Haile *et al.*, 1989 (and references cited therein)]. Liver samples of 100–150 mg were homogenized in 10 volumes (w/v) of extraction buffer (10 mM HEPES, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM dithiothreitol supplemented with 0.5% Igepal, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin) and centrifuged at 10,000 $\times$ *g* for 5 min. Supernatants were removed and stored at $-70°$ until use. Two DNA oligonucleotides were synthesized (Applied Biosystems, Norwalk, CT). The first contained the IRE sequence in the 5′-to-3′ direction followed by the T7 promoter sequence in the 3′-to-5′ direction (52 bp), and the second contained the T7 promoter sequence in the 5′-to-3′ direction (19 bp): IRE sequence, 5′-GGGTTCCGTCCAAACACTGTTGAAGCAGGAAAC CCTATAGTGAGTCGTATTA-3′ ; and T7 sequence, 5′-TAATACGACTCACTATAGG-3′ (the underlined sequence is the T7 promoter). The IRE oligonucleotide was purified on a 12% denaturing acrylamide gel using a variation on UV shadowing. Localization of the 52-bp oligonucleotide on the gel was made with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The DNA was excised from the gel and incubated in elution buffer (0.1% sodium dodecyl sulfate, 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0) at room temperature overnight and subjected to phenol-chloroform extractions and ethanol precipitation. The 19-bp T7 oligonucleotide was purified using a double ethanol precipitation and again resuspended in H_2O. Both oligonucleotides were diluted to a concentration of 50 ng/μl. The IRE and T7 oligonucleotides (950 ng of each) were annealed to form a partially single-stranded DNA molecule with a double-stranded region containing the T7 promoter. The transcription reaction was carried out using the annealed oligonucleotides; 1$\times$ Transcription Optimized Buffer; 10 mM dithiothreitol; 20 units of RNasin; 0.5 mM concentration each of UTP, GTP, and ATP; 12 μM CTP; 50 μCi of [α-^{32}P]CTP; and 20 units of T7 RNA polymerase in 20 μl of reaction. Reactions were incubated at 37° for 1 hr; 1 unit of DNase (RNase free) was added, and incubation was continued for an additional 30 min (T7 Riboprobe System; Promega, Madison, WI).

The transcription product was purified through a microspin column (S200; Pharmacia, Piscataway, NJ), and specific radioactivity was assessed. For the RNA binding assay (Park *et al.*, 1996), 0.1 μg of cytosol was diluted with extraction buffer to a final volume of 18 μl; 40 units of RNAsin was added with 10,000 cpm of ^{32}P-labeled RNA. (Before addition of the labeled RNA, it was denatured at 65° for 5 min and allowed to cool slowly to room temperature for ~30 min to ensure the stem-loop structure was favored.) The reaction was incubated at room temperature for 30 min. Heparin was added to a final concentration of 5 mg/ml, and the incubation was continued for an additional 10 min. Two microliters of glycerol loading buffer was added, and the reaction was analyzed on a nondenaturing acrylamide gel. Visualization was performed with a PhosphorImager. To assess total IRP capacity, reactions first were incubated with 2-mercaptoethanol to deplete the IRP of all iron. As a check of the specificity of binding illustrating IRP, no binding was observed with a randomly generated oligonucleotide or with the IRE oligonucleotide differing at a critical position by a single base-pair deletion as described by Haile *et al.* (1989).

Aconitase activity. Aconitase was determined by following the rate of disappearance of *cis*-aconitate at 240 nm using 100 μg of cytosol in 1 ml of 0.1 M Tris·HCl buffer, pH 7.4, containing 200 μM *cis*-aconitate at 25° (Gosiewska *et al.*, 1996). To check for complete activation of aconitase, cytosol was preincubated for 5 min in the presence of 1 mM $FeSO_4$/5 mM cysteine.

Nonheme iron content. Hepatic iron levels were determined according to the methods of Constantin *et al.* (1996) and Madra *et al.* (1996).

Statistics. Results are given as mean $\pm$ standard deviation, and significance was assessed by analysis of variance, with a value of $p < 0.05$ taken as significant.

Results

Synergism of iron and TCDD in C57BL/6J mice. Previously, a single dose of 75 μg of TCDD/kg to male mice of the C57BL/10ScSn strain caused a progressive depression of hepatic UROD activity (significant within 1 week) and subsequent maximum porphyria development (measured as uroporphyrin) after 5 weeks (Smith *et al.*, 1981). At this dose, depression of enzyme activity and uroporphyria were potentiated by prior loading of mice with iron dextran (Greig *et al.*, 1984). As a first step in investigating further this phenomenon, mice of the closely related C57BL/6J strain received the same dose of TCDD, with and without prior iron loading, and were examined 5 weeks later. Iron pretreatment greatly potentiated the levels of porphyrin accumulating in the liver (Table 1). This was not just an indication of an effect on heme synthesis but an aspect of general hepatic toxicity because elevated levels of plasma ALT and alkaline phosphatase activities were observed, which is indicative of greater hepatocyte injury. Bilirubin levels were affected only slightly by the iron treatment before TCDD. Histological examination of the livers showed mild to moderate toxicity and inflammation after TCDD (Shen *et al.*, 1991), which was markedly potentiated in the dual-treatment group. Iron greatly enhances the carcinogenicity of PCBs in mice (Smith *et al.*, 1990). To determine whether iron potentiated TCDD-induced proliferation, the expression of PCNA in the liver was quantified by immunocytochemistry. Despite a greater degree of damage in the iron/TCDD group, no increase in proliferation, as estimated by PCNA expression, was detectable with this dose of TCDD, although a greater number of fields had to be exam-

TABLE 1

Interaction of TCDD and iron in C57BL/6J mice

Mice (4 or 5/group) were treated with dextran or iron-dextran (800 mg of Fe^{2+}/kg/g) and then after 1 week received oil or TCDD in oil (75 μg/kg). Five weeks later, mice were killed, and plasma enzymes and hepatic porphyrins were estimated. Results are mean $\pm$ standard deviation.

TCDD group	Iron	Body weight	Liver weight	Thymus weight	Plasma			Hepatic
					Bilirubin	Alkaline phosphatase	ALT	Uroporphyrin
		g	*g*	*mg*	*mg/dl*	*units/liter*		*nmol/g*
Control	−	27 ± 3	1.33 ± 0.23	54 ± 7	0.26 ± 0.21	42 ± 4	31 ± 3	0.25 ± 0.01
Control	+	28 ± 3	1.85 ± 0.23	64 ± 17	0.15 ± 0.13	51 ± 6	43 ± 17	0.17 ± 0.01
TCDD	−	27 ± 1	1.65 ± 0.10	21 ± 9[a]	0.14 ± 0.08	56 ± 19	70 ± 54	50 ± 90[a]
TCDD	+	24 ± 1[a]	2.04 ± 0.09	23 ± 7[a]	0.41 ± 0.03	108 ± 15[b]	379 ± 118[b]	650 ± 318[b]

[a] Significantly different from control.
[b] Significantly different from group not given iron.

ined to accumulate the 4000 hepatocyte nuclei/sample. This again was consistent with increased liver damage after interaction with iron (Fig. 1). In contrast to the effects on the liver, thymus atrophy, another characteristic feature of TCDD-induced toxicity in C57BL/6J mice, was unaffected by prior iron treatment (Table 1).

Formation of oxidized derivatives of porphyrinogens. The hepatic porphyrins that accumulated in this study were analyzed by HPLC. The major porphyrins identified were uroporphyrin I and III isomers and heptacarboxylic porphyrin IIId, which are expected as a consequence of inhibition of UROD (Guo *et al.*, 1996; Madra *et al.*, 1996). However, a variety of other minor porphyrins were also detected, some of which were identified as peroxyacetic acid, mesohydroxypropionic acid, and β-hydroxypropionic acid derivatives of uroporphyrins (Fig. 2). These products have been identified previously in *in vitro* reactions of uroporphyrinogens, not uroporphyrins, with systems generating oxygen free radical species, especially in the presence of iron (Guo *et al.*, 1996).

Induction of GST and CYP1A1. GST activities were estimated with 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene as substrates at 1 and 5 weeks. At 5 weeks, a marked induction of transferase activity toward DCNB was observed (3–4-fold) in the iron/TCDD group indicative of a μ class of GST (Fernandes *et al.*, 1996). Confirmation that this was due to induction of the Yb_1 GST subunit of a μ-class isoform was obtained by Western blotting with an anti-Yb_1 polyclonal antibody (Fig. 3). Concomitant with this induction was a 60% decrease in glutathione peroxidase activity toward cumene hydroperoxide, although the decrease was less with H_2O_2 as a substrate.

To determine the influence of iron overload on cytochrome P450 induced by TCDD, the activities of CYP1A1, CYP1A2, and CYP2B, measured as microsomal EROD, MROD, and BROD, respectively, were also measured 1 and 5 weeks after TCDD (Table 2). CYP1A1 and CYP1A2 activities were markedly induced by TCDD at both time points, but in contrast to GST, activities in control and induced samples were depressed by prior iron treatment despite the enhancing action of iron on porphyria and toxicity development. Western blotting with antibodies against CYP1A1/2 protein confirmed these findings (results not shown).

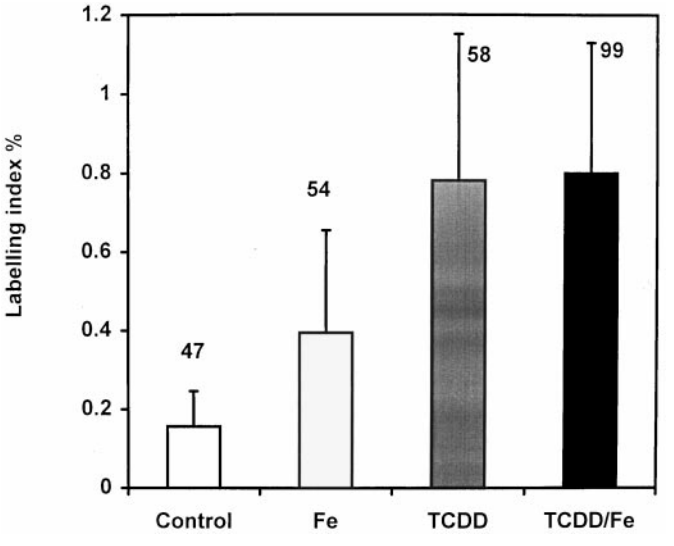

Fig. 1. PCNA labeling index for hepatocytes from C57BL/6J livers (4 or 5 mice/group) exposed to iron and TCDD. Labeling indices were estimated by examination of 4000 nuclei/mouse liver and are expressed as a percentage labeling index. Results are given as mean ± standard deviation. The TCDD and TCDD/iron groups were significantly different from each other. However, a greater number of fields (*numbers above each bar*) had to be examined to accumulate 4000 nuclei in the TCDD/iron samples.

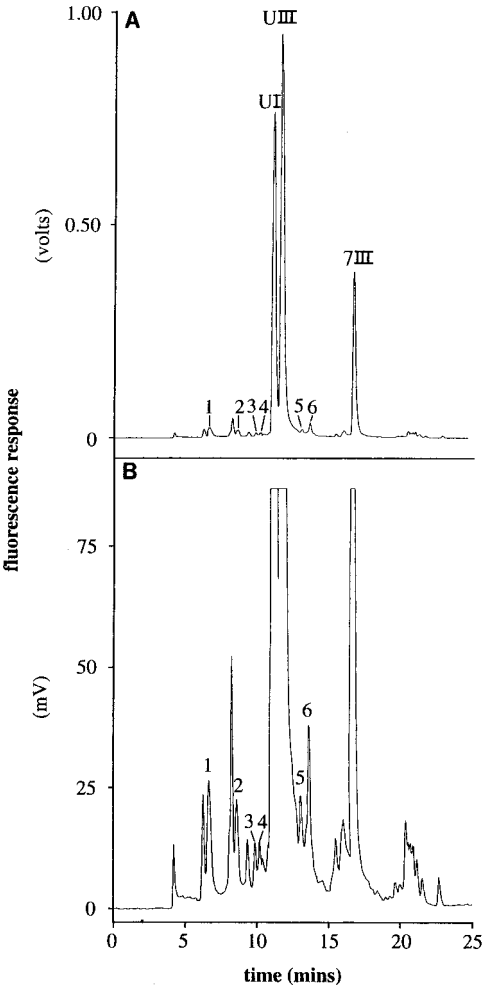

Fig. 2. HPLC of porphyrin sample from a C57BL/6J mouse exposed to TCDD after iron pretreatment. A, Low sensitivity; at this sensitivity, no peaks were observed in control samples. B, Expanded sensitivity. *UI*, uroporphyrin I; *UIII*, uroporphyrin III; *7III*, heptacarboxylic porphyrin III; *1*, mesohydroxy uroporphyrin III; *2*, β-hydroxypropionic acid uroporphyrin III; *3*, hydroxyacetic acid uroporphyrin III; *4*, peroxyacetic acid uroporphyrin III; *5*, hydroxyacetic heptacarboxylic porphyrin III; *6*, β-hydroxypropionic acid heptacarboxylic porphyrin III.

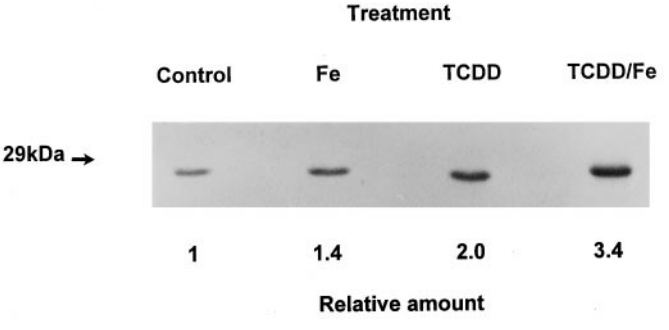

Fig. 3. Immunoblot of liver cytosol from C57BL/6J mice exposed to TCDD with and without prior iron treatment and probed for Yb_1 subunit of mouse GST. Values are the relative amounts of protein estimated by densitometry.

Comparison of *Ahr* alleles. After the clear influence of iron on both porphyria and hepatic toxicity caused by TCDD in C57BL/6J mice was demonstrated, a comparison was made among the three common variants of the *Ahr* gene in inbred mice ($Ahr^{b\text{-}1}$, $Ahr^{b\text{-}2}$, and Ahr^{d} alleles), which confer different sensitivities to maximum induction of CYP1A1 and toxicity (Table 3). Although iron again potentiated hepatic porphyria development and serum ALT levels in C57BL/6J mice with the $Ahr^{b\text{-}1}$ allele, no porphyrin increases and only slight increases in ALT were observed with DBA/2 mice, which is consistent with their possessing the Ahr^{d} allele coding for a receptor with reduced affinity for TCDD. An important observation, however, was that SWR mice, which also possess the Ahr^{d} allele (Constantin *et al.*, 1996; Poland and Glover, 1990), developed a small but significantly greater response to TCDD than DBA/2, and this response was potentiated markedly by iron. These porphyric and ALT responses in SWR mice after the interaction of iron and TCDD approximated the levels observed in the C57BL/6J strain without iron and were marginally greater than that observed for $Ahr^{b\text{-}2}$ BALB/c mice after dual treatment (Table 3). Histopathological examination of livers from these four strains of mice with three variants of the *Ahr* allele confirmed ALT data showing that hepatic damage in those mice exposed to iron and TCDD was of the order C57BL/6J > SWR > BALB/c > DBA/2 (Table 3 and Fig. 4). At this dose and time, DBA/2 mice showed only slight effects of TCDD and even with iron had only diffuse fat accumulation. Thus, iron significantly potentiated both the hepatic toxicity and porphyria in Ahr^{d} SWR mice but had little enhancing effect in another Ahr^{d} strain (DBA/2). This demonstrates that the low hepatic toxicity of TCDD associated with the Ahr^{d} *geno-*

TABLE 2

Influence of iron on hepatic activities of glutathione transferases and peroxidases and CYP1A1, CYP1A2, CYP2B, and after TCDD in C57BL/6J mice

Cytosolic and microsomal enzymes were assayed 1 or 5 weeks after TCDD treatment of 4 or 5 mice/group. 3,4-Dichloronitrobenzene probably reflects activity of Yb_1 subunit of a μ-class glutathione transferase, whereas 1-chloro-2,4-dinitrobenzene represents overall transferase activity. EROD, MROD, and BROD are believed to represent CYP1A1, CYP1A2, and CYP2B activities, respectively. Values are mean ± standard deviation.

TCDD group	Time	Iron	Cytosolic				Microsomal		
			1-Chloro-2,4-dinitrobenzene	3,4-Dichloronitrobenzene	CuOOH	H_2O_2	EROD	MROD	BROD
	weeks		*μmol/min/mg of protein*		*nmol/min/mg of protein*			*pmol/min/mg of protein*	
Control	1	−	14.8 ± 1.5	62 ± 23	150 ± 25	116 ± 18	21 ± 4	24 ± 6	6 ± 2
Control	1	+	10.1 ± 2.0	56 ± 17	153 ± 30	125 ± 31	11 ± 5	10 ± 3	17 ± 8
TCDD	1	−	15.5 ± 0.5	65 ± 3	205 ± 13	183 ± 19	742 ± 142	511 ± 101	123 ± 9
TCDD	1	+	13.7 ± 3.5	97 ± 4[a]	202 ± 36	150 ± 32	563 ± 105	425 ± 66	108 ± 22
Control	5	−	7.4 ± 1.2	54 ± 6	121 ± 7	142 ± 6	114 ± 68	144 ± 66	30 ± 12
Control	5	+	7.2 ± 1.8	70 ± 13	133 ± 13	152 ± 16	20 ± 13	51 ± 21	13 ± 6
TCDD	5	−	6.6 ± 1.2	78 ± 18	132 ± 35	192 ± 15	1211 ± 593	739 ± 375	146 ± 60
TCDD	5	+	8.1 ± 0.8	233 ± 25[a]	54 ± 15[b]	133 ± 28[b]	470 ± 257	402 ± 179	64 ± 34

[a] Significantly greater than TCDD group.
[b] Significantly less than TCDD group.
There was a significant tendency for EROD and MROD activities to be lower after iron treatment than corresponding noniron groups.

TABLE 3

Comparison of porphyria and hepatic toxicity in mice with differing *Ahr* alleles

Mice (four or five per group) received a single dose of iron dextran (800 mg Fe/kg) (+) or the dextran equivalent (800 mg/kg) (−) by subcutaneous injection. After one week mice received TCDD (75 μg/kg) (+) in oil or the oil equivalent (10 ml/kg) (−). The experiment was terminated 5 weeks after TCDD. Values are mean ± standard deviation.

Strain	*Ahr* allele	Iron	TCDD	Body weight	Liver weight	ALT[a]	Uroporphyrin	Estimate of relative liver toxicity[b]
				g		*units/liter*	*nmol/g*	
C57BL/6J	b-1	−	−	29 ± 3	1.56 ± 0.16	64 ± 35	0.13 ± 0.11	−
		+	−	26 ± 3	1.93 ± 0.21	83 ± 17	0.11 ± 0.07	−
		−	+	26 ± 2	1.93 ± 0.19	417 ± 283	271 ± 129[c]	+++
		+	+	19 ± 1[c]	2.01 ± 0.37	791 ± 368	1964 ± 414[d]	++++
BALB/c	b-2	−	−	22 ± 1	1.36 ± 0.05	90 ± 30	0.18 ± 0.27	−
		+	−	28 ± 2	1.68 ± 0.19	82 ± 10	0.15 ± 0.16	−
		−	+	26 ± 1	1.75 ± 0.12	115 ± 58	1.30 ± 1.06	+
		+	+	26 ± 1	2.43 ± 0.14[d]	336 ± 110[d]	110 ± 128[d]	++
SWR	d	−	−	24 ± 1	1.18 ± 0.13	70 ± 25	0.18 ± 0.23	−
		+	−	25 ± 1	1.52 ± 0.18	66 ± 24	0.25 ± 0.26	−
		−	+	25 ± 3	1.53 ± 0.16	99 ± 31	8.2 ± 11.9	++
		+	+	25 ± 1	2.27 ± 0.18[d]	446 ± 153[d]	246 ± 140[d]	+++
DBA/2	d	−	−	27 ± 1	1.46 ± 0.09	49 ± 13	0.16 ± 0.17	−
		+	−	29 ± 1	2.11 ± 0.22	94 ± 65	0.13 ± 0.10	−
		−	+	28 ± 2	2.62 ± 0.16	65 ± 38	0.28 ± 0.20	−
		+	+	26 ± 2	2.43 ± 0.18	138 ± 79	0.21 ± 0.18	+

[a] Values are per liter of serum.
[b] Toxicity was determined by examination of hematoxylin and eosin-stained liver sections.
+, Mild fat accumulation and hypertrophy of hepatocytes; ++, mild to severe grade hydropic changes in central vein region and hypertrophy; +++, mild to moderate grade toxicity, single-cell necrosis/apoptosis in central vein region, numerous mitoses; ++++, moderate to severe grade inflammation, loss of hepatocytes, proliferation of biliary and endothelial cells.
[c] Significantly different from control.
[d] Significantly greater than group not given iron.

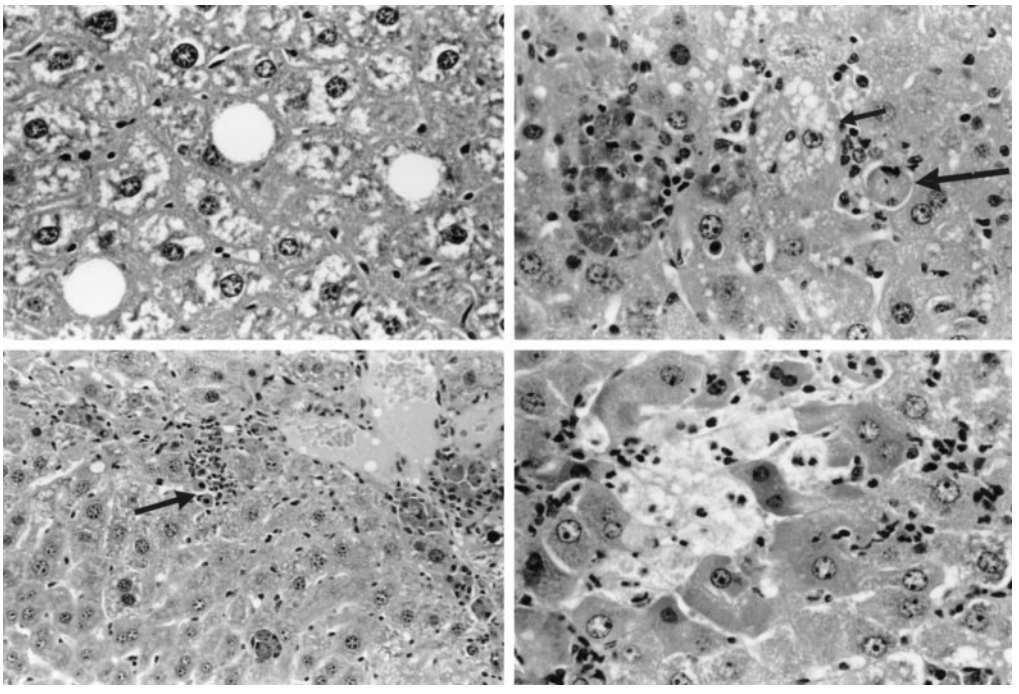

Fig. 4. Comparison of hepatic damage in iron loaded mice 5 weeks after being given TCDD given in order of increasing severity. *Top left*, DBA/2 mouse liver showing accumulation of fat in hepatocytes (hematoxylin and eosin, ×300). *Top right*, BALB/C mouse liver with hepatocyte in the center of the field showing hydropic degeneration (*short arrow*) adjacent to a hepatocyte undergoing cell death with a fragmented nucleus (*long arrow*) (hematoxylin and eosin, ×300). *Bottom left*, SWR mouse liver showing several inflammatory foci adjacent to a central vein with two hepatocytes undergoing cell death with pyknotic nuclei (*arrow*) (hematoxylin and eosin, ×150). *Bottom right*, C57BL/6J mouse liver with focal necrosis with inflammatory cell infiltration (hematoxylin and eosin, ×300).

type in SWR mice can be partially overcome by the interaction with another predisposing gene or genes, which probably are associated with iron metabolism.

Analysis of CYP1A1 activity in the four strains as a measure of relative TCDD-induced gene expression showed that at this dose of TCDD, all levels were still significantly elevated at the end of the experiment and there did not appear to be sufficient difference between the strains to explain the observed marked variations in toxicity and porphyria. Again, iron caused a significant depression of CYP1A1 activity (to ~70%) in both the susceptible strain and the resistant DBA/2 mice (Fig. 5). Northern analysis of mRNAs for CYP1A1 and CYP1A2 in livers from C57BL/6J and DBA/2 mice showed a marked induction after TCDD, as expected (Fig. 6); however, contrary to activity or protein levels, mRNA levels were not decreased with iron overload. There was a tendency for increased levels (≤2.3-fold higher), but this was not statistically significant (see the legend to Fig. 6). Levels of CYP1A1 and CYP1A2 mRNAs in TCDD-treated DBA/2 mice were 40–70% of the levels in C57BL/6J mice, as observed for relative CYP1A1 activities.

Because changes in GST and glutathione peroxidase seemed to be associated with hepatotoxicity, these activities also were examined and were found to be compatible with an interaction between iron and TCDD leading to hepatocellular damage, especially in the C57BL/6J mice and to a lesser extent in SWR (Fig. 5).

Effect of TCDD on control of iron metabolism. The previous results suggest a role for an aspect of iron metabolism that may be genetically variable in the hepatic toxicity of TCDD. The post-transcriptional regulations of some proteins linked to iron regulatory metabolism, such as ferritin, transferrin receptor, and erythroid aminolevulinate synthase, are thought to be controlled by the interaction of a cytosolic IRP and an IRE in the untranslated regions of the mRNAs, and this is sensitive to iron availability (Hentze and Kuhn, 1996). If saturated with iron, the IRP does not bind to mRNA but possesses cytosolic aconitase activity. Measurements of IRP/IRE binding by a gel shift assay and aconitase activity often are inversely proportional and are used as measurements of intracellular iron availability and traffic. C57BL/6J and DBA/2 mice were treated with iron or dextran and then TCDD as previously described. Cytosolic aconitase activity and IRP/IRE interaction were estimated 2 weeks after TCDD, a time at which it seemed reasonable that any action of the chemical would be detected. A significant decrease in aconitase activity was observed in both C57BL/6J groups administered TCDD regardless of whether they were first dosed with iron (Fig. 7C). Aconitase was not increased in the TCDD groups if cytosols were preincubated with Fe^{2+}/cysteine to maximize iron incorporation (Gosiewska *et al.*, 1996). No compensatory increase in IRP/IRE interaction was detected that was associated with TCDD-induced depletion of aconitase; in fact, a decrease in binding was suggested (Fig. 7A). Treatment of cytosols with mercaptoethanol before the binding assay gives total binding IRP/IRE capacity regardless of endogenous iron concentrations. TCDD significantly decreased such capacity in this strain (Fig. 7B). With DBA/2 mice, which are refractive to the effects of TCDD, no such changes in aconitase activity or total IRP/IRE interactions could be detected. Thus, at this dose and after 2 weeks, even in the presence of iron, TCDD caused a depression of all aspects of IRP function in susceptible C57BL/6J mice, suggesting a down-regulation in IRBP formation or function but less in the resistant DBA/2 strain. We observed little influence of iron overload alone on the aconitase and IRP/IRE parameters.

Discussion

The results demonstrate that iron overload will potentiate the hepatic porphyria induced by TCDD in C57BL/6J mice, consistent with previous results in C57BL strains with TCDD, PCBs, and the weak AHR ligand HCB (Greig *et al.*,

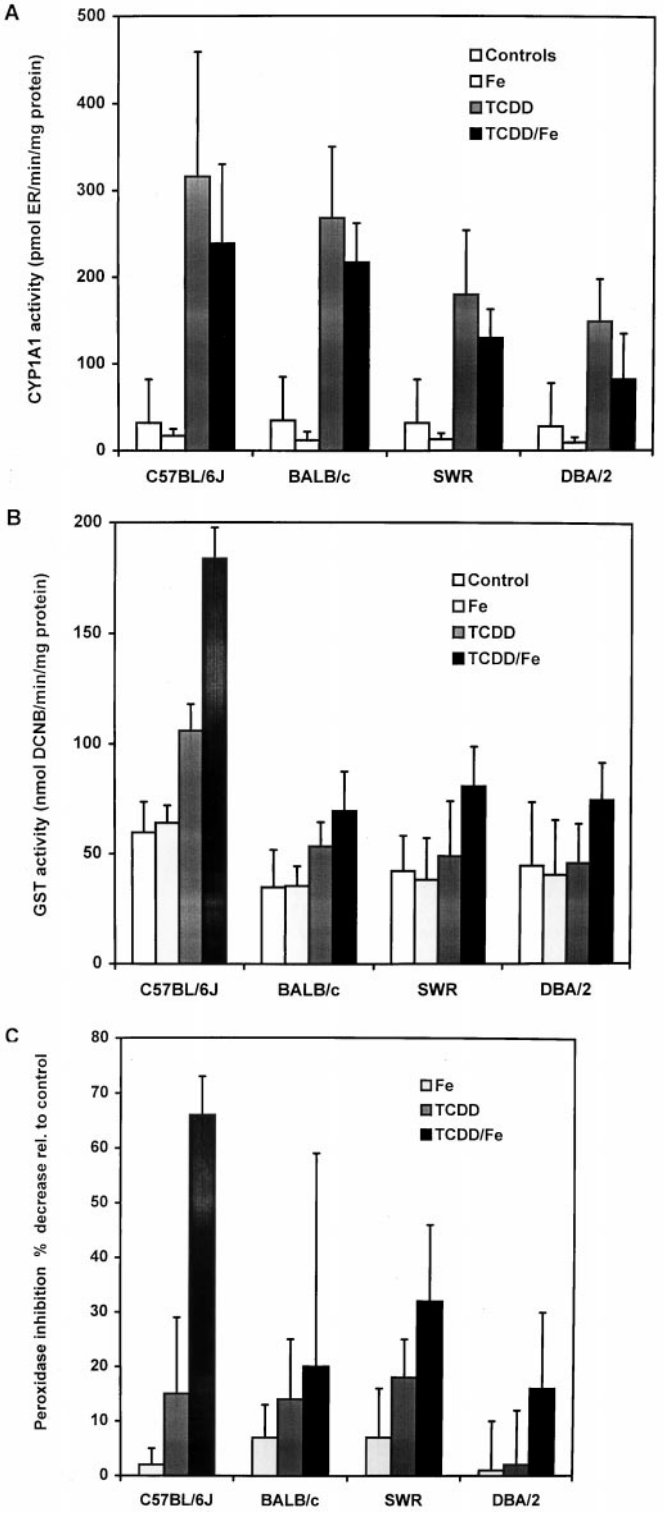

Fig. 5. Comparison of liver microsomal and cytosolic enzyme activities in C57BL/6J, BALB/c, SWR, and DBA/2 mice at 5 weeks after exposure to TCDD and iron. A, CYP1A1 activity determined as EROD (*ER*). B, GST activity with 3,4-dichloronitrobenzene (*DCNB*) as a substrate and indicative of Yb_1 subunit. C, Glutathione hydroperoxidase activity with cumene hydroperoxide expressed as inhibition relative to control samples. Values are mean ± standard deviation for four or five mice/group.

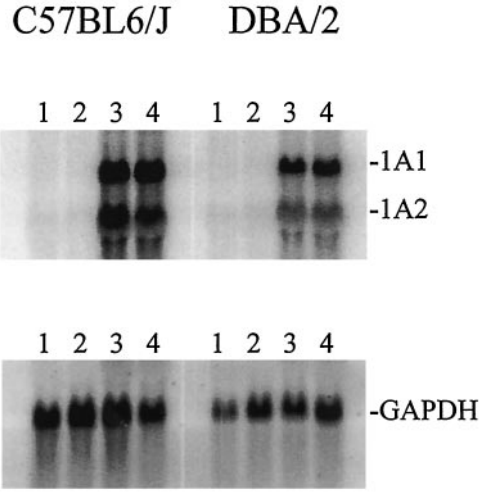

Fig. 6. Example of Northern blot analysis for CYP1A1 and CYP1A2 mRNA from single livers of C57BL/6J mice 2 weeks after TCDD with or without prior iron overload. *Lane 1*, control. *Lane 2*, iron. *Lane 3*, TCDD. *Lane 4*, TCDD/iron. The analyses were repeated twice with mRNA preparations from different mice. Results were quantified by densitometry and normalized relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) intensity. For TCDD-induced C57BL/6J mice, CYP1A1 levels in three different experiments were 1.0-, 2.3-, and 1.9-fold higher with iron than without. Results for CYP1A2 were 1.1, 1.9, and 1.6, respectively. Although there obviously was a tendency for levels to be higher after iron, the changes were not statistically significant.

1984; Jones *et al.*, 1981; Madra *et al.*, 1995; Smith and Francis, 1983). Importantly, this potentiation by iron of hepatic porphyria is part of a wider toxic scenario because plasma enzymes and histological evaluations of the liver also were changed significantly. The greatly increased response of Ahr^d SWR mice in the presence of iron suggests that there are other predisposing genes besides *Ahr* that can profoundly influence the hepatic toxicity of TCDD. Previous work has demonstrated that the hepatic toxicity of TCDD is not completely dependent on the AH phenotype (Greig *et al.*, 1984). In addition, Knutson and Poland (1982) showed that the toxic effects of TCDD on the skin of mice were influenced by a battery of genes associated with the *hr* locus and that this locus also may affect hepatic toxicity (Greig *et al.*, 1987). Iron overload markedly influences the carcinogenicity of PCBs in C57BL/10ScSn mice (Smith *et al.*, 1990), but in the current study, we saw no interaction between TCDD and iron on a proliferative index of liver cells. This may be a consequence of the toxic dose of TCDD used in these experiments.

What Are the Molecular Mechanisms Leading Ultimately to These Pathological Processes Arising from TCDD/Iron Interaction?

Evidence for an oxidative process. There is considerable evidence that an oxidative process may play a part in the uroporphyria induced by TCDD and its analogues (Smith and De Matteis, 1990). A number of studies have implicated CYP1A2 regulated via the AHR in the oxidation of uroporphyrinogen to the nonutilized uroporphyrin (Jacobs *et al.*, 1989; Sinclair *et al.*, in press) to explain the uroporphyria that develops on exposure to TCDD and related chemicals, including the nonchlorinated AHR ligands. Although there is strong evidence for the involvement of CYP1A2 in the mechanism of porphyria, the inhibition of UROD that occurs as an early event (Smith *et al.*, 1981) and the more generalized liver damage (Madra *et al.*, 1996; current study) require the operation of processes in addition to the conversion of uroporphyrinogen to its porphyrin analogue. The relatively mild

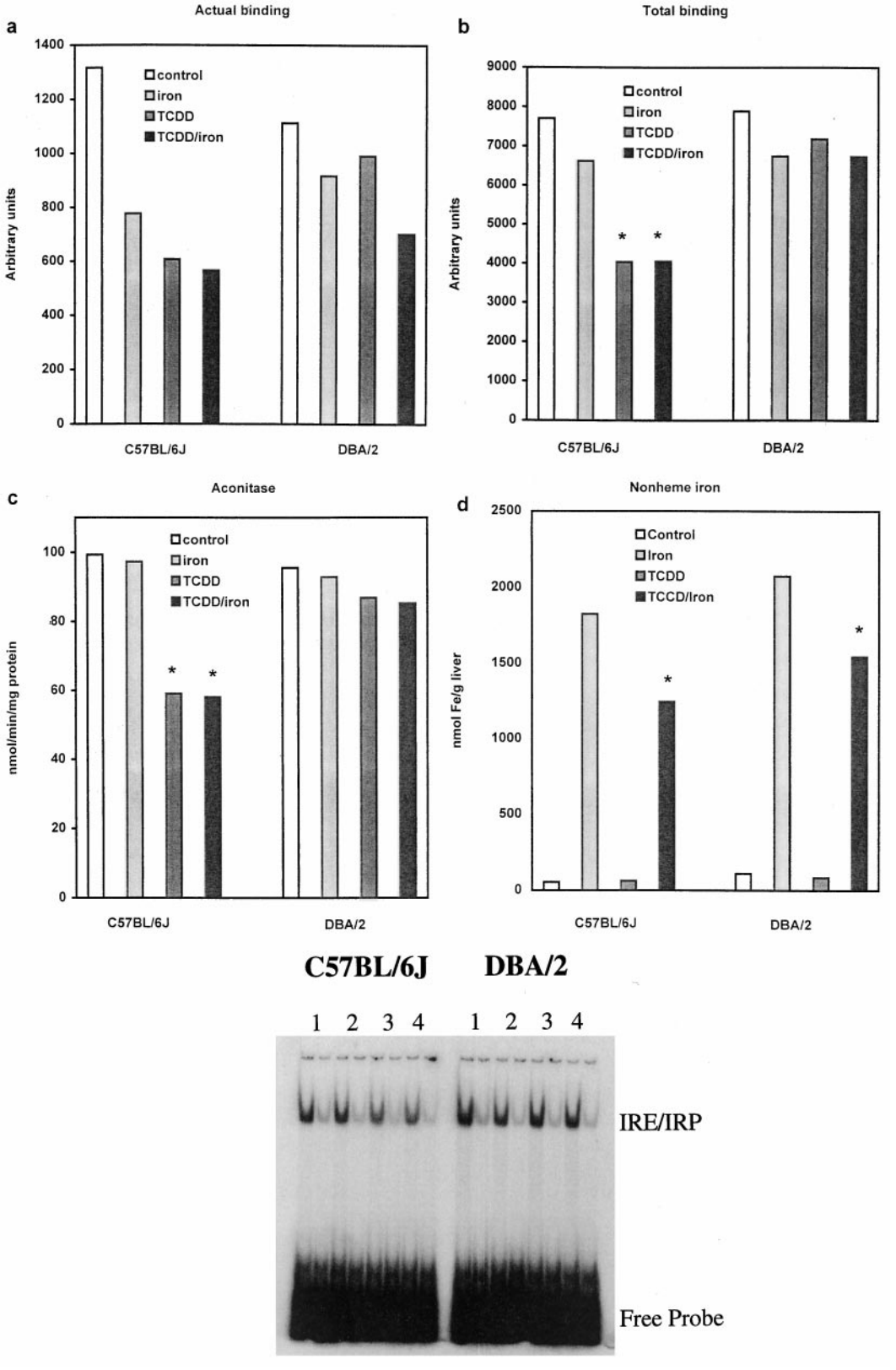

Fig. 7. a–d, IRP/IRE interaction in livers of C57BL/6J and DBA/2 mice 2 weeks after TCDD. a, Actual binding. b, Total capacity after reduction with 2-mercaptoethanol. There were no significant differences detected between the control values of these strains. c, Cytosolic aconitase activity. d, Nonheme iron content of livers. The values are mean for observations from a total of seven to nine mice/group obtained from two separate experiments. For clarity, mean ± standard deviation bars have been omitted. Results were subjected to two-way analysis of variance. Significant effects of TCDD (*) on total IRP capacity and aconitase (b and c) were observed in C57BL/6J but not in DBA/2, and there was no additional interaction with iron. In the nonheme iron analyses (d), values were tested by Student's *t* test after TCDD/iron treatment, and there were no significant differences between the strains. *Gel analysis*, example of assay system for IRP/IRE interaction showing total (*left*) and actual binding (*right*) for control (*lanes 1*), iron (*lanes 2*), TCDD (*lanes 3*), and TCDD/iron (*lanes 4*). *Each lane*, cytosol from a single mouse.

six-electron withdrawal from uroporphyrinogen may be a synchronous event along with more powerful oxidizing mechanisms, some of which may lead to other products from uroporphyrinogen (or precursor molecules) as well as to oxidation of other cellular targets. In fact, uroporphyrinogen might be considered a molecule well designed to act as a sensitive indicator of such intracellular oxidative processes. The identification here of peroxylated and hydroxylated uroporphyrins in the liver of mice treated with TCDD, which have been shown previously to be formed in free radical-

generating systems *in vitro* (Guo *et al.*, 1996), is consistent with this proposal. Products of uroporphyrinogen oxidation generated *in vitro* caused UROD inhibition (Francis and Smith, 1988). The operation of such an oxidative process could explain the more generalized hepatic toxicity documented in this work. Previous experiments have shown that TCDD and PCBs induce the formation of 8-hydroxydeoxyguanosine from DNA in mouse liver cells (Faux *et al.*, 1992; Park *et al.*, 1996). With PCBs, this can be potentiated by iron (Faux *et al.*, 1992). Our demonstration in C57BL/6J mice that TCDD eventually caused the induction of GST, especially with iron overload, with a decrease in glutathione peroxidase activity is also consistent with an oxidative sequence of some kind (Fernandes *et al.*, 1996). Similar findings have been reported after the interaction of PCBs and iron in mice (Madra *et al.*, 1996). The elevation of GST has been shown to occur in transgenic mice that overexpress the hepatitis B virus large envelope protein, and it has been proposed that this reflects oxidative damage generating endogenous inducers of ARE-regulated enzymes (Fernandes *et al.*, 1996). Although there is much evidence implicating CYP1A isoforms (especially CYP1A2) in the development of porphyria and DNA oxidation induced by TCDD analogues, perhaps by an uncoupling mechanism (Smith and De Matteis, 1990) that is not yet understood, a number of features of the current study require additional explanation. First, at the dose of 75 μg/kg, induction of CYP1A1 (and probably CYP1A2), even after 5 weeks, differed by only 2-fold among C57BL/6J, BALB/c, SWR, and DBA/2 mice, which is unlikely to be sufficient in itself to explain the marked strain differences in susceptibility. Second, iron overload caused a significant reduction in the CYP1A activities and protein levels induced by TCDD in all strains, although it markedly potentiated the porphyria and toxicity.

Involvement of iron. The experimental uses of iron deficiency and iron overload to modify the porphyrogenic responses to TCDD and HCB (Greig *et al.*, 1984; Jones *et al.*, 1981; Smith and Francis, 1983; Sweeney *et al.*, 1979) have arisen because of the clinical observations that some aspect of iron metabolism is implicated in the related human disease sporadic PCT. This syndrome occurs in a small proportion of patients with mild or moderate liver damage associated with alcohol, estrogenic drugs, hepatitis C, human immunodeficiency virus, and other precipitating factors (Kappas *et al.*, 1995). Hepatic UROD is inhibited, and there is associated high accumulation of uroporphyrins. The majority of cases of sporadic PCT in which biopsy has been undertaken have shown some degree of siderosis, and phlebotomy or desferrioxamine treatment usually brings about remissions. On the other hand, iron treatment has been shown to be one of the precipitating agents. Hence, the original experiment of Sweeney *et al.* (1979) to lower iron status in C57BL/6J mice and protect against TCDD-induced porphyria was extremely relevant as a model of the human disease. The strong influence of iron overload precipitating porphyria and toxicity in SWR mice after TCDD demonstrates that expression of genes associated with iron metabolism plays an important role in determining whether hepatic toxicity is observed with these chemicals. Interestingly, iron overload alone will cause uroporphyria in SWR mice, eventually even more so than in C57BL/6J mice, whereas the DBA/2 strain continues to be refractory (Smith and Francis, 1993). This illustrates that it would be misleading to think of the porphyria in rodents as purely a toxic response to TCDD and HCB. These chemicals potentiate a process that can occur without exogenous factors. By the use of criteria such as sensitivity to induction of CYP1A1 activity, AHR size, and restrictive fragment polymorphism data, SWR mice can be classified as having the Ahr^d allele (Constantin *et al.*, 1996; Poland and Glover, 1990). However, an apparent gene present in SWR mice confers a susceptibility that can partially overcome the resistant Ahr^d allele after TCDD exposure. It seems reasonable to postulate that C57BL/6J and BALB/c strains also possess the susceptible gene or genes and that the resistance of DBA/2 is due to the possession of both the Ahr^d allele and a resistant allele of a gene associated with iron metabolism.

Recent findings suggest that the sporadic nature of nonfamilial PCT might be explained by predisposed individuals being carriers of the hemochromatosis C28Y mutation (Roberts *et al.*, 1997) that has been identified recently, although its function with respect to iron metabolism is not understood (Feder *et al.*, 1996). Probably what might be important is a genetically variable aspect of intracellular iron traffic within liver cells that is linked to the hemochromatosis gene because experimental studies do not depend on differential iron absorption from the intestine. Consistent with this hypothesis is the demonstration that in rats, TCDD causes alterations in the subcellular distribution of hepatic iron (Wahba *et al.*, 1990). The involvement of iron is additional strong evidence for an oxidative mechanism contributing to the hepatic toxicity of TCDD.

In recent years, the concepts concerning iron homeostasis and iron-catalyzed Fenton chemistry leading to oxidative sequences have undergone significant changes. IRPs respond to changes in iron pools and control the expression of mRNAs that have IRP binding sites (e.g., ferritin), thus setting in play mechanisms to restrict the availability of potentially harmful free iron. It has become clear that control of iron metabolism is integrated with regulation of many other cellular pathways, such as energy production and cell proliferation, that have not been envisaged previously (Hentze and Kuhn, 1996). In some cell lines, H_2O_2 leads to IRP activation with aconitase activity loss, although total IRP/IRE capacity is not changed after 2-mercaptoethanol treatment (Gaetano *et al.*, 1996). This process seems to reflect oxidative stress and not purely a change in iron availability. Our current findings demonstrated that TCDD caused a decline of not only aconitase activity but also IRP/IRE interaction as well as total IRP capacity in susceptible C57BL/6J mice but significantly less in the resistant DBA/2 strain. This seems to suggest either a down-regulation of IRP formation or a nonreversible inactivation. Whether this was a consequence of a toxic process or a signaling pathway to cope with oxidative stress cannot be determined from these experiments, although it should be noted that changes occurred to the same extent in the TCDD group as the TCDD/iron mice. The fact that we did not observe much effect of iron on IRP/IRE interaction may suggest that other routes of maintaining iron homeostasis after chronic iron overload are important or that a steady state was reached by 3 weeks after iron treatment.

In summary, iron overload potentiates both the hepatic uroporphyria and generalized toxicity caused by TCDD in

Ahr^{b-1} C57BL/6J mice with associated gene expression and the formation of oxidized porphyrinogens indicative of oxidative stress mechanisms. The influence of the unknown "iron metabolism" gene involved is pronounced, particularly in SWR mice, in response to administered TCDD because it seemed to partially overcome the resistance of the *Ahr*^{d} allele. The hypothesis that iron metabolism changes in the presence of TCDD was supported by findings of iron regulatory mechanisms. Further studies will concentrate on the genetic bases of these interactions.

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