Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on mRNA expression of key enzymes in heme metabolism in rats

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ABSTRACT

We have previously shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes hepatic accumulation of biliverdin in a TCDD-semiresistant rat line B. Here, the effect of TCDD on mRNA expression of key enzymes in heme metabolism [heme oxygenase 1 (HO-1), δ-aminolevulinic acid synthetase 1 (ALAS1), biliverdin IXα reductase (BVRA) and uroporphyrinogen decarboxylase (UROD)] was studied in order to determine, whether the accumulation could be due to their altered expression. We used three rat strains differing in TCDD-sensitivity: line B (LD50 400-800 μg/kg), line A (LD50 >10000 μg/kg) and Long-Evans (*Turku*/AB; LD50 10-20 μg/kg). In Study I, line A and B female rats were given 300 μg/kg TCDD intragastrically and the tissues were harvested 2, 7, 14, 32 or 35 days later. In Study II, which included also a feed-restricted control group, male Long-Evans rats received 100 μg/kg TCDD and were sampled 10 days later. The hepatic and splenic expression levels of the mRNAs were assessed using real-time quantitative RT-PCR and compared with vehicle-treated controls. Most effects of TCDD were similar in all strains. In liver, TCDD unexpectedly did not induce HO-1 and had no effect on UROD, but it strongly repressed ALAS1 and slightly increased BVRA expression. In spleen, TCDD repressed HO-1 in line A and B rats. The primary reason for biliverdin accumulation does not seem to be TCDD-induced disturbance in the expression of the studied mRNAs; most importantly, it is not caused by induction of HO-1. The observations provide new information on mRNA expression of key heme metabolic enzymes after a high dose of TCDD.

Key words: TCDD, heme oxygenase, δ-aminolevulinic acid synthetase, biliverdin reductase, uroporphyrinogen decarboxylase, heme metabolism

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener among the polychlorinated dibenzo-*p*-dioxins (PCDDs), which are widespread and persistent environmental pollutants. TCDD is known to disturb heme metabolism in at least two ways: it causes porphyria and jaundice (Buu-hoi *et al*., 1972; Cantoni *et al*., 1987; Kociba *et al*., 1976; Pohjanvirta and Tuomisto, 1994). In addition, we have shown in our previous work that TCDD causes accumulation of the heme degradation product biliverdin and its derivative biliverdin monoglucuronide in the livers of certain intermediately TCDD-resistant rat lines (Niittynen *et al*., 2003). Accumulation of biliverdin in mammalian tissue is a very rare phenomenon. In undisturbed heme catabolism of most mammals, biliverdin is rapidly and quantitatively reduced to bilirubin by biliverdin reductase [see McDonagh (2001) for a review]. Biliverdin has occasionally been found in human serum in certain pathological conditions such as obstructive jaundice, paracetamol induced hepatic necrosis, and a rare POEMS syndrome (Wardle and Williams, 1981; Yasuda *et al*., 1994), but in experimental mammals, we are not aware of any cases in which marked biliverdin accumulation in liver or serum would have been observed.

The syndrome is genotype-dependent and thus belongs to type II dioxin effects (Niittynen *et al*., 2003; Simanainen *et al*., 2002). Most cases of biliverdin accumulation have been recorded in intermediately TCDD-resistant rats, such as line B (*Kuopio*; see below). These rats are presumably homozygous for the two genes mediating TCDD resistance/sensitivity. They possess the wild-type (sensitive) alleles of the aryl

hydrocarbon receptor (AHR) gene and the resistant alleles of the yet unidentified gene *B*. Gene *B* contributes to TCDD sensitivity by possibly interacting with the AHR (Tuomisto *et al*., 1999). A fully functional transactivation domain in AHR seems to be necessary for the development of the syndrome since no cases have been recorded in rats expressing only the Han-Wistar type AHR (Pohjanvirta *et al*., 1998) with altered transactivation domain structure.

One hypothesis for the accumulation of biliverdin is that the inducible form of the enzyme forming biliverdin, heme oxygenase 1 (HO-1), would be induced due to TCDD exposure (Fig. 1). TCDD is known to cause oxidative stress (Stohs, 1990) and reactive oxygen species (ROS) are one of the many agents that induce the expression of HO-1 (Keyse and Tyrrel, 1990; Stocker, 1990). Despite this connection, we found only one report showing an increase in the amount of HO-1 after TCDD exposure (Nishimura *et al*., 2001). At the molecular level, HO-1 induction is mediated e.g. via the antioxidant response element (ARE) residing in the 5' upstream region of HO-1 gene (Prestera *et al*., 1995). ARE is not a classical target of TCDD but it is responsive to oxidative stress (Rushmore *et al*., 1991) and thus might be influenced by the ROS produced by TCDD. These facts warranted verifying whether TCDD exposure results in induction of HO-1 in rats with biliverdin accumulation.

In addition to HO-1, two other enzymes in heme metabolic pathway were regarded as possible players in the etiology of this syndrome. These are the nonspecific form of δ-aminolevulinic acid synthetase (ALAS1) and biliverdin IXα reductase (BVRA). ALAS1

is the first and the rate-limiting step in heme biosynthesis in nonerythroid cells (May *et al*., 1995; Fig. 1). TCDD and many other cytochrome P450 inducers have been shown to increase its activity (Granick, 1966; May *et al*., 1995; Poland and Glover, 1973). BVRA is a cytosolic enzyme, which is not generally considered rate-limiting in heme degradation; on the contrary, mammals normally have a remarkable excess capacity for biliverdin IXα reduction (Maines, 1990; McDonagh, 2001). However, because in this case its substrate accumulates, we examined the expression of BVRA for possible derailments. Finally, the effect of TCDD on uroporphyrinogen decarboxylase (UROD) expression was studied, since it is well known that TCDD inhibits UROD activity causing porphyria but the exact mechanism of inhibition is still unknown. In addition to the liver, the mRNA expressions of HO-1, ALAS1 and BVRA were studied in the spleen. This was prompted by the fact that the spleen is the major organ participating in heme catabolism. Hepatic expressions of cytochrome P450 (CYP) isoforms 1A1 and 1A2, which belong to the classical genes regulated by the AHR, were used as positive controls of induction.

In the present studies, we used rat lines B and A that originate from crossbreedings of the most TCDD-sensitive and the most TCDD-resistant rat strains Long-Evans (*Turku/*AB [L-E]; LD50 10-20 µg/kg TCDD) and Han/Wistar (*Kuopio* [H/W]; LD50 >9600 µg/kg TCDD), respectively (Tuomisto *et al*., 1999). Of these, line B (LD50 400-800 µg/kg TCDD; genotype $Ahr^{wt/wt}$ $B^{hw/hw}$, wt denoting a wild-type allele from the L-E strain and hw denoting an allele from the H/W strain) exhibits biliverdin accumulation after TCDD exposure. In line A (LD50 >10000 µg/kg TCDD; genotype $Ahr^{hw/hw}$ $B^{wt/wt}$), the phenomenon has never been detected. The intermediately TCDD-resistant line B was our

main interest, but we also wanted to see whether the effects are the same or whether they differ in more resistant or sensitive rats and if the AHR genotype has an influence on the outcome. Therefore, L-E and line A rats were examined in addition to line B rats.

MATERIALS AND METHODS

Animal husbandry

All rats were obtained from the breeding colony of the National Public Health Institute, Kuopio, Finland. They were housed in groups of 2-4 animals (Study I) or singly (Study II) in stainless-steel wire-mesh cages with pelleted (Study I) or powdered (Study II) R36 feed (Ewos, Södertälje, Sweden) and tap water available *ad libitum*, except for the feed-restricted group in Study II (see below). The temperature in the animal room was 21 ± 1°C, relative humidity 50 ± 10% and lighting cycle 12/12 h light/dark. The study plans were approved of by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government.

Experimental design

Adult female rats of lines B and A were used in Study I. They were 15-20 weeks old at the time of exposure. Rats were randomly divided in groups of 5-7 animals. TCDD was purchased from UFA Oil Institute (Ufa, Russia) and was over 98% pure as analyzed by gas chromatography-mass spectrometry. It was dissolved in corn oil. Rats were dosed with 300 μg/kg TCDD intragastrically (dosing volume 4 ml/kg) and killed 2, 7, 14, 32 or 35 days later by decapitation. Controls received corn oil 4 ml/kg and were decapitated 2 or 35 days later. The dose 300 μg/kg TCDD was chosen because it is large enough to cause the syndrome at a fairly high incidence in line B rats (Niittynen *et al*., 2003). Usually, development of the syndrome takes 3-5 weeks. Thus we wanted to study possible changes in the mRNA expression of key heme metabolic enzymes during this time period, starting from the early time point (2 days) when no macroscopic changes yet

exist, and extending the follow-up to day 35 when the syndrome has usually reached its maximal severity (characterized by mottled appearance and dark green or black color of the liver, hence the name “black liver syndrome”).

In Study II, 10 to 13-week-old male L-E rats were used. They received 100 μg/kg TCDD i.g. or vehicle (corn oil) and were killed 10 days later by decapitation. Since these rats are highly sensitive to TCDD (LD50 ca. 20 μg/kg [Pohjanvirta *et al*., 1993]), we had to use a lower dose than that employed in lines A and B. In order to ensure that the effects seen were specific to TCDD toxicity and not simply caused by the body weight loss accompanying TCDD exposure, Study II also included a feed-restricted (FR) control group, in which the rats were fed according to a predesigned regimen intended to mimic the food intake patterns of L-E rats treated with a lethal dose of TCDD. These rats were offered *ad libitum*, 16, 14, 11, 8, 6, 4, 4, 2 and 1 g of the powdered R36 feed on days 0 through 9, respectively (day 0 denotes the day when the rats were dosed). Each experimental group (control, TCDD and FR) consisted of 6 animals.

In both studies, spleen and a piece of liver were rapidly removed after decapitation of the rats, flash-frozen in liquid nitrogen and stored at –80° C for subsequent analysis.

RNA-isolation and RT-PCR

Total RNA was isolated from homogenized liver and spleen samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Isolated total RNAs were subjected to DNAse1 –treatment with the DNA-*free* kit (Ambion, Austin,

TX). 1.2 μg (or 0.6 μg) of this RNA was then used for reverse transcription in a 20 μl (or 10 μl) reaction that was performed with Omniscript reverse transcriptase (Qiagen, Hilden, Germany) using random hexanucleotides (Roche, Mannheim, Germany) as primers. The reaction mixture was incubated at 37 ºC for 1.5 h. Resulting cDNAs were diluted in a 1:6 ratio with water and used in subsequent analysis.

Cloning of ALAS1, HO-1, UROD and BVRA gene fragments

About 160 to 280-nt-long fragments of ALAS1, UROD, HO-1 and BVRA double-stranded cDNAs were cloned using line A rat cDNA as a template. PCR was performed with HotStarTaq DNA polymerase (Qiagen) on TGradient-thermocycler (Biometra, Göttingen, Germany). The "touchdown" method was applied: the annealing temperature was set at 63 °C for the first cycle and then decreased by 1 °C / cycle down to 57 °C. A total of 40 cycles were run. The primers used are shown in Table 1. ALAS1, UROD, HO-1 and BVRA primers were designed to span at least one intron. In each case, only a single strong amplicon was generated and it was invariably of correct size.

PCR products were purified from the agarose gel using Sigma GenElute Gel Extraction Kit (Sigma). They were cloned by blunt-end ligation into pCR-Script SK(+) Amp plasmids (Stratagene, La Jolla, CA). XLB-1 supercompetent cells were used in transformations (Stratagene). The plasmids were purified by Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The inserts were sequenced with an A.L.F.express DNA sequencer (Amersham-Pharmacia Biotech, Uppsala, Sweden) using Thermo Sequenase CY5 Dye Terminator Kit (Amersham-Pharmacia Biotech).

Previously prepared CYP1A1, CYP1A2 and β-actin plasmids (Korkalainen *et al*., 2004; Korkalainen *et al.*, 2005) were also used for external standards in mRNA quantitation.

Real-time quantitative PCR

The mRNA expression levels of selected heme metabolic enzymes, as well as those of CYP1A1, CYP1A2 and β-actin were analyzed using QuantiTect SYBR Green PCR Kit (Qiagen) and Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Mortlake, NSW, Australia). The 20 µl reaction mixture contained cDNA derived from 15 ng of total RNA, 0.5 µM each primer and 10 µl QuantiTect. The PCR was initiated with an incubation step of 15 min at 95 °C to activate HotStarTaq DNA polymerase. The "touchdown" method was applied: the annealing temperature was set at 63 °C for the first cycle and then decreased by 1 °C / cycle down to 57 °C. The cycling procedure was denaturation at 95 °C for 20 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s with fluorescence acquired. A total of 45 cycles were run. A melting curve was run at 55-99 °C to verify the specificity of PCR products. Standard curves were generated to determine the absolute amount of template in each reaction. To this end, plasmid DNAs were linearized and quantified by spectrophotometry. Standard curve PCRs were carried out using 1-4 replicates of each dilution of plasmid. The used dilutions varied between 10^{-4}-10^{-11} (relative to original plasmid at a concentration of 200 ng/µl). The amounts of templates in the samples from control and TCDD-treated animals were calculated by setting their crossing points to the standard curves ($R>0.99$; reaction efficiencies 0.88-0.99). A negative control containing all the components of the reaction mixture but water replacing the template was included in each run. Line B liver samples were analyzed in

duplicate in two separate runs: one run consisting of the groups control (day 2), TCDD (day 2) and TCDD (day 7), and the other of the groups control (day 35), TCDD (day 14) and TCDD (day 32). Because the results of duplicates showed good reproducibility, the rest of the samples were analyzed only as single measurements as this enabled us to run all the samples from one strain/line and one tissue at the same time.

Basal expression level of HO-1 mRNA

The basal hepatic mRNA expression levels of HO-1 were compared between line A and B control rats. The first comparison was made by utilizing the β-actin normalized values from initial real-time quantitative PCR analysis. The second analysis was made by preparing new cDNA from A- and B-control rats, and analyzing the amounts of β-actin and HO-1 again. Here, cDNA dilution ratio 1:2 was used because of the low expression level of HO-1 mRNA detected in the first analysis.

Statistical analysis

Groups of each strain/line were compared using the one-way analysis of variance (ANOVA). If this test showed a significant difference, the least significant difference (LSD) test was used as a post-hoc test. In case of non-homogenous variances (according to Levene´s test, $p<0.05$), the non-parametric Kruskal-Wallis ANOVA was used and followed by the Mann-Whitney *U* test. p-value <0.05 was considered statistically significant. In Study I, the controls were from two time points, 2 and 35 days after vehicle. Using Student's *t*-test we verified that there was no significant difference between these two control groups ($p>0.05$). Therefore, they were pooled to form one

control group (n=10). The only exception was spleen BVRA in line B, for which day-2 and day-35 control groups differed significantly from each other ($p = 0.011$) and were thus kept separate.

In HO-1 analysis, some samples had HO-1 mRNA content less than the detection limit (line A: 3, 2 and 2 samples from postexposure days 2, 7 and 14, respectively; L-E-strain: two samples in FR group). These samples were given the lowest detectable value in their group, after it was considered not to skew the final result. When comparing the basal expression levels of HO-1 mRNA between A and B control rats, Student's *t*-test or, in case of nonhomogenous variances, Mann-Whitney *U* test were used.

RESULTS

Macroscopic observations

As expected, no black livers were seen in the earlier time points (2, 7 and 14 days). The last time point was intended to be 35 days. However, it appeared that the exposed line B rats were likely to succumb before this time point and were thus killed prematurely. Out of the seven rats in line B day-35 group, 4 had a severe syndrome, (found dead / killed on day 32 or 34; from 2 of these, we got the samples), two did not (found dead on day 27 and 33) and one could not be determined (found dead on day 20). The incidence of 57 % (4/7) black livers is in good agreement with our previous results (Niittynen *et al*., 2003).

In line A rats there was no mortality. No black livers were observed either, but on day 35 5 out of 6 line A rats had yellowish liver, which might suggest accumulation of bilirubin in the liver. Bilirubin increase has been reported in resistant rat strains (line A and H/W rats), although it is far less severe than in more sensitive strains (Unkila *et al*., 1994; Tuomisto *et al*., 1999).

mRNA expression levels

Overall, the effects of TCDD on the studied mRNA expression levels were similar between line A and line B rats (Fig. 2), in both liver and spleen. Also Study II with L-E rats gave similar results (Fig. 3), except for splenic HO-1.

β-Actin, CYP1A1 and CYP1A2

β-Actin is often used for normalization of mRNA expression levels. However, we observed a time-dependent increase in its hepatic expression due to TCDD in line B and L-E rats (Figs. 2 and 3). In line A, the increase was seen only at the latest time-point (day 35). The strain difference suggests that the increase in β-actin expression might associate with liver toxicity, which is a strain-dependent effect of TCDD. In spleen, β-actin expression remained largely stable. Due to the potential of TCDD to affect hepatic β-actin expression, we did not normalize the values to this house-keeping gene but instead to starting material: in every case, the same amount of total RNA (1.2 μg in 20 μl reaction or 0.6 μg in 10 μl reaction) was used for cDNA synthesis. β-Actin normalization was only used when the basal levels of HO-1 expression between lines A and B were compared.

CYP1A1 and CYP1A2, which were used as positive controls of induction, showed the characteristic induction in liver after TCDD (Figs. 2 and 3; because of their current role as controls their expressions were not determined in spleen). Maximal CYP1A1 induction was about 6700-fold (line B, day 14), whereas maximal CYP1A2 induction was 25-fold (line B, day 2). Thus, there is a large quantitative difference in the induction of these two enzymes by TCDD. In addition, it seems that CYP1A1 induction may be more pronounced in line B than in line A (means of maximal fold inductions 6700 and 2300, respectively).

HO-1

HO-1 was not induced by TCDD in liver (Fig. 2). Rather, in both line B and line A, hepatic HO-1 expression showed a downward tendency on day 2, mean mRNA levels being 38 % and 22 % of control, respectively. However, the differences were not statistically significant. In addition, some line A samples from post-exposure days 2, 7, and 14 had HO-1 mRNA concentration below the detection limit. This prompted us to compare the basal HO-1 expression levels between lines A and B and, interestingly, the basal hepatic HO-1 expression level in control rats was 9 times greater in line B than in line A ($p<0.001$). To verify this result, new cDNA was made from the controls for second analysis of β-actin and HO-1 expression. At this time, HO-1 expression in line B control rats was 2.5 times greater than in line A control rats ($p<0.001$).

In L-E rats, no statistically significant changes were seen in HO-1 expression 10 days after TCDD exposure (100 μg/kg) or feed restriction when compared with control (Fig. 3). However, TCDD-treated and feed-restricted groups differed significantly from each other ($p=0.016$).

In spleen, HO-1 mRNA expression was decreased after TCDD exposure in both rat lines A and B (Fig. 2). However, in L-E rats no significant deviation from control value was seen (Fig. 3), in fact, there was a slight upward tendency in the TCDD-exposed group. Gender, TCDD dose, TCDD sensitivity and AHR genotype are factors that may underlie the difference between the rat strains/lines.

ALAS1, UROD and BVRA

The most dramatic effect in this study was the repression of hepatic ALAS1 by TCDD (Figs. 2 and 3). In line B, already on postexposure day 2 its mRNA expression was 45 % of the control value, and on day 7 only 18 %. The effect was similar in lines A and B and also in L-E rats, so it is not dependent on AHR genotype or TCDD sensitivity, and thus can be classified as type I dioxin effect (for definition of type I and type II effects, see Simanainen *et al*., 2002). In feed-restricted L-E rats, ALAS1 expression increased, indicating that the repression is specifically related to TCDD and not caused by the body weight loss. TCDD did not influence splenic ALAS1 mRNA expression in any rat strain/line.

UROD had the most stable expression pattern in this study: TCDD did not influence hepatic UROD mRNA expression in any rat strain/line, nor did feed restriction in L-E rats (Figs. 2 and 3). UROD was not determined in spleen, because the porphyrinogenic effect of TCDD is generally considered to be due to inhibition of UROD activity in liver.

In both rat lines A and B, hepatic BVRA expression was slightly induced on postexposure days 7 and 14 (1.5 - 1.9-fold increase; Fig. 2). In line A, the induction was still at the same level on day 35. Also in L-E rats BVRA expression was increased after TCDD-exposure (1.6-fold on postexposure day 10; Fig. 3), but decreased in feed-restricted rats. This indicates that the effect is caused by TCDD and not unspecifically due to body weight loss. In spleen, BVRA mRNA expression did not significantly change in any rat strain/line (Figs. 2 and 3).

DISCUSSION

In the present study, we show that TCDD affects the mRNA expression of ALAS1 and BVRA in rat liver in all strains/lines tested, and splenic HO-1 expression in line A and line B rats but not in L-E rats. Concomitantly, TCDD does not influence hepatic UROD expression. The findings reflect in part the status of the ongoing heme metabolism, since ALAS1 and HO-1 are rate-limiting enzymes regulated mainly at the transcriptional and mRNA level. For a more complete view of the hepatic and splenic heme metabolism after TCDD exposure, also the protein amounts and activities of the enzymes should be examined, as they are not necessarily concordant with the amount of mRNA. However, the current approach is supported by the nature of action of TCDD and AH receptor, the general consensus being that all the major effects of TCDD are mediated by its modulation of gene expression via AHR. It is emphasized that the results presented here apply only to the mRNA expression of selected heme metabolic enzymes. The possibility remains that TCDD might lead to changes in the activity of a critical heme metabolic enzyme via some other mechanism.

HO-1

HO-1 is an inducible enzyme sensitive to oxidative stress (Maines, 1999). Female rats have been demonstrated to develop oxidative stress after TCDD exposure (Stohs *et al*., 1990). One marker of oxidative stress, namely lipid peroxidation, has been shown to be a type II (genotype-dependent) effect of TCDD, as L-E rats were more sensitive to it than H/W rats (Pohjanvirta *et al*., 1990). On the basis of this, it is somewhat peculiar that HO-

1 induction was not seen in the liver. In line A and B spleen, HO-1 mRNA expression was even repressed by TCDD. The reason for this is unknown.

In light of these results, it seems unlikely that the primary reason for biliverdin accumulation would be an exaggerated action of HO-1 in liver. There is still the possibility that HO-1 mRNA may have peaked before the earliest time-point (2 days) used in this study. However, considering the fact that marked biliverdin accumulation is not observed until about 4 weeks postexposure, it seems implausible that an early and temporary HO-1 induction could be responsible for that accumulation. Depending on the experimental model, half-life of HO-1 protein has been determined to vary between 2.3 - 15 h (Srivastava *et al*., 1993; Wu *et al*., 2004).

Interestingly, in TCDD-resistant rat line A, the basal expression level of HO-1 mRNA in liver was somewhat lower than in intermediately TCDD-resistant line B. This raises the possibility that HO-1 expression is partly dependent on AHR genotype.

ALAS1

We observed a clear repression of ALAS1 by TCDD in liver in all three strains. Repression was specific to liver, as the expression did not change in spleen. Recently, Fletcher *et al.* (2005) also observed ALAS1 repression by TCDD in microarray studies on Sprague-Dawley rats. The traditional view has rather been that TCDD induces ALAS1, as, for example, TCDD is a potent inducer of ALAS1 activity in chick embryo (Poland and Glover, 1973). However, in rats, ALAS1 induction by TCDD is not well

established. For example, Kitchin and Woods (1978) and Woods (1973) demonstrated that TCDD (2 μg/kg or 25 μg/kg) did not alter ALAS1 activity in rat liver after up to 28 days or 1 day, respectively. Marked increase in ALAS1 activity was seen only after chronic exposure to TCDD (16 weeks, 1 μg/kg/week; Goldstein *et al*., 1982). This suggests that ALAS1 induction is a secondary effect of TCDD in rats.

The important regulator of ALAS1 expression and activity is the heme molecule (Cable *et al*., 2000; Granick 1966; Srivastava *et al*., 1988; Yamamoto *et al*., 1988). Heme negatively regulates ALAS1 activity and expression by decreasing mRNA half-life (Hamilton *et al*., 1991), reduces transfer of the enzyme to mitochondria (Hayashi *et al*., 1972) and may also inhibit its gene transcription at least in rats (Srivastava *et al*., 1988). Thus, one possibility for the observed ALAS1 repression could be an increase in hepatocellular free heme content due to TCDD exposure. However, no data could be found about the effect of TCDD on hepatic free heme concentration. On one hand, the increases in serum bilirubin (observed in many rat strains) and hepatic biliverdin (line B) might be accompanied by an increase in the amount of their precursor, heme, in liver. On the other hand, HO-1 should be readily induced if free heme concentration increases, but we observed no induction. This does not necessarily disprove the hypothesis, since HO-1 induction is fairly short-lived and may be prone to post-induction repression (Alam *et al*., 2003). Also, according to Kitchin (1983), only 1/10 as much heme is needed to repress ALAS1 as is needed to induce HO-1. Our results warrant further work on whether TCDD in fact increases free heme content in the liver.

Decrease in hepatic ALAS1 mRNA expression or enzymatic activity has been observed in various situations of tissue injury, such as carbon tetrachloride induced hepatotoxicity (Nakahira *et al*., 2003), rat model of septic multiple organ dysfunction syndrome (Suzuki *et al*., 2000) and after heavy metal administration (Kaliman *et al.*, 1998). A common denominator in these examples is increased free heme concentration in liver, its putative source varying from cytochrome P450s to hemolysis.

In addition to heme, other possible reasons for ALAS1 repression must be considered. To the best of our knowledge, ALAS1 does not have a dioxin responsive element (DRE) in its promoter. However, TCDD might repress ALAS1 directly by some unknown mechanism. Interestingly, Guberman *et al*. (2003) suggest that activator protein-1 (AP-1) complex has an inhibitory effect on ALAS1 expression, and TCDD has been shown to induce AP-1 (Puga *et al*., 2000). Also insulin has been shown to inhibit ALAS1 gene expression in rat hepatocytes (Scassa *et al*., 1998), but TCDD causes hypoinsulinemia (Pohjanvirta and Tuomisto, 1994). Kitchin (1983) reported that bilirubin reduces ALAS1 activity but in the studies of Piper *et al*., (1986) and Kohashi *et al*., (1984) ALAS1 activity increased in rats with hyperbilirubinemia caused by biliary obstruction. No data could be found that would suggest bilirubin to affect ALAS1 mRNA expression in any manner.

UROD

The mRNA expression of the other heme synthetic – but normally not rate-limiting - enzyme, UROD, was not influenced by TCDD. TCDD is one of the most potent

porphyrinogenic agents known (Cantoni *et al*., 1986; Smith *et al*., 1985), which effect was first detected in rats by Kociba *et al*. (1976). The main accumulating porphyrin is uroporphyrin as hepatic UROD activity is inhibited (Cantoni *et al*., 1987; Jones and Sweeney, 1977; Smith *et al*., 1985). However, the exact mechanism by which inhibition occurs is unknown. Our results support the hypothesis that the inhibition is unrelated to gene expression and takes place at the level of enzymatic activity. Indeed, there are a number of studies pointing to a formation of an inhibitor of UROD by TCDD (Cantoni *et al*., 1986; De Verneuil *et al*., 1983; Smith *et al*., 1985). Also, it has been suggested that the inactivation of UROD may be due to an oxidative reaction that destroys the enzyme (Cantoni *et al.*, 1987; Smith *et al*., 1985).

BVRA

The mRNA expression of BVRA was enhanced slightly and similarly (1.5-1.9-fold) by TCDD in all rat strains. There are not many agents known to affect the expression of BVRA, and it is not considered a rate-limiting enzyme. However, BVRA message has been shown to be induced 6 h after hyperthermia about 2-fold in rat kidney (McCoubrey *et al*., 1995). In human renal carcinoma, a 175 % increase in transcript level was observed (Maines *et al*., 1999). Interestingly, the induction levels reported in the above-mentioned studies are similar to our observations although the causative agent is different. A possible molecular route for increased BVRA mRNA could be induction of AP-1 by TCDD (Puga *et al*., 2000), as BVRA gene has an AP-1 binding site in its promoter (McCoubrey *et al*., 1995). AP-1 is a family of transcription factors that are activated by changes in cellular redox state (Pinkus *et al*., 1996; Puga *et al*., 2000).

According to Baranano *et al*. (2002), bilirubin is oxidized to biliverdin during intracellular oxidative stress. BVRA then reduces biliverdin back to bilirubin, forming a catalytic cycle that provides antioxidant cytoprotection. If this cycle truly is an important part of the cellular antioxidant defense, then it could be hypothesized that under conditions when bilirubin oxidation is increased due to ROS, also reduction of biliverdin should be enhanced in order to maintain cellular homeostasis, and this might be the underlying reason, or part of it, for BVRA induction.

Conclusions

In liver, TCDD unexpectedly did not induce HO-1 and had no effect on UROD expression, but it strongly repressed ALAS1 and slightly increased BVRA expression. In spleen, TCDD decreased HO-1 expression in lines A and B. On this basis, the primary reason for biliverdin accumulation does not seem to be a TCDD-induced disturbance in mRNA expression of HO-1, BVRA or ALAS1, as the observed effects were in the opposite direction to what would be expected if they were the cause of the syndrome. However, ALAS1 repression and BVRA induction are new effects of TCDD and the molecular mechanisms behind them are worth investigating. These effects did not depend on the AHR genotype, thus they belong to type I dioxin effects. However, mutated AHR might be related to the somewhat lower basal hepatic HO-1 mRNA expression recorded in line A rats. In addition, it remains to be determined why HO-1 was not induced despite the oxidative stress previously shown to be caused by TCDD in L-E rats.

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TABLE 1. The primers used in the study.

Gene	Accession No.	Forward primer	Reverse primer	Size of product (bp)
ALAS1	J03190	GCGCAATGTCAAGCTTATGA	TGGGTAATTAATGGCCTGGA	169
UROD	Y00350	GGACAGTGGCTCCAAAGAAA	TTAGCAATGTAGCGCTGTGG	157
HO-1	J02722	CACAAAGACCAGAGTCCCTCA	AGAAAAGAGAACCCAGGCAAG	284
BVRA	NM-053850	CATGTCCTCGTGGAATACCC	AGCTGTGAAGCGAAGAGACC	186
CYP1A1	NM-012540	CCATATGCTTTGGCAGACGTTA	TCAAACCCAGCTCCAAAGAG	361
CYP1A2	NM-012541	TGATAACTTTGTGCTGTTTCTGC	GGTTGACCTGCCACTGGTTTA	473
β-Actin	NM-031144	CACGGCATTGTAACCAACTG	GAGCGCGTAACCCTCATAGA	297

LEGENDS FOR THE FIGURES

FIG. 1. Heme metabolic pathway showing the key enzymes whose mRNA expression was studied here. ALAS1 = δ-aminolevulinic acid synthetase 1, BVRA = biliverdin IXα reductase, HO-1 = heme oxygenase 1, UGT = UDP-glucuronosyltransferase, UROD = uroporphyrinogen decarboxylase. Asterisk (*) denotes a rate-limiting step.

FIG. 2. The effect of TCDD (mean+SEM) on the mRNA expression of key heme metabolic enzymes in liver and spleen of female line B and line A rats as a function of time. Pooled control, consisting of day-2 and day-35 control groups, was given the value of 1. TCDD dose was 300 μg/kg. Day 0: n=10; in other groups: n=5 (exceptions: line B, day 32: n=2; line B spleen BVRA, day 0: n=5;). Solid symbols: $p<0.05$ compared with control. UROD, CYP1A1 and CYP1A2 were only analyzed in liver (see text). *Note*: The BVRA results of line B spleen are presented in comparison with day-35 control group instead of a pooled control, because the day-2 and day-35 control groups differed significantly from each other. The statistical significances remained the same also when TCDD-exposed groups were compared with the day-2 control group.

FIG. 3. The effect of TCDD (mean±SEM) on the mRNA expression of key heme metabolic enzymes in liver and spleen of male L-E rats. Rats were given 100 μg/kg TCDD and studied 10 days later. N=6, the asterisks denote statistically significant differences (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$) compared with control. FR=feed-restricted control. UROD, CYP1A1 and CYP1A2 were only analyzed in liver (see text). In this figure, the absolute amounts of mRNAs are reported rather than fold inductions

used in Fig. 2. This way it is possible to see the differences between mRNA expression levels of different enzymes and between liver and spleen. Note the logarithmic scale in the graph for CYP1A1.

FIG. 1.

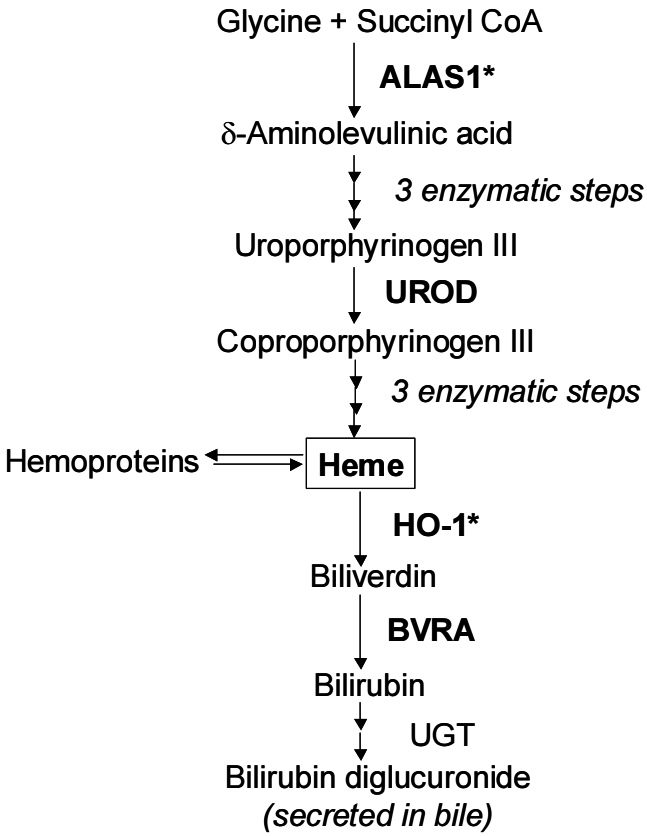

Glycine + Succinyl CoA
ALAS1*
δ-Aminolevulinic acid
3 enzymatic steps
Uroporphyrinogen III
UROD
Coproporphyrinogen III
3 enzymatic steps
Hemoproteins
Heme
HO-1*
Biliverdin
BVRA
Bilirubin
UGT
Bilirubin diglucuronide
(secreted in bile)

FIG. 2.

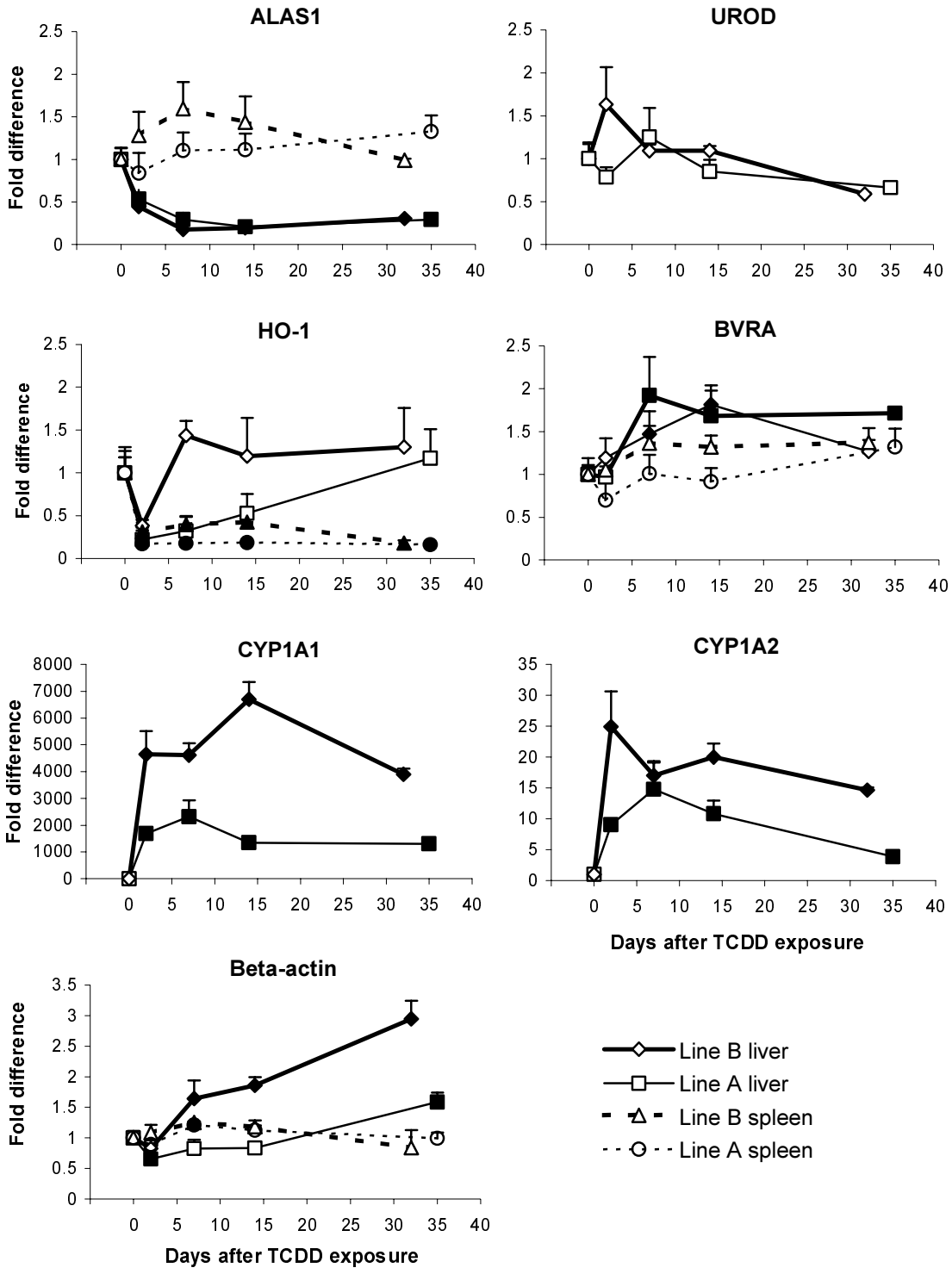
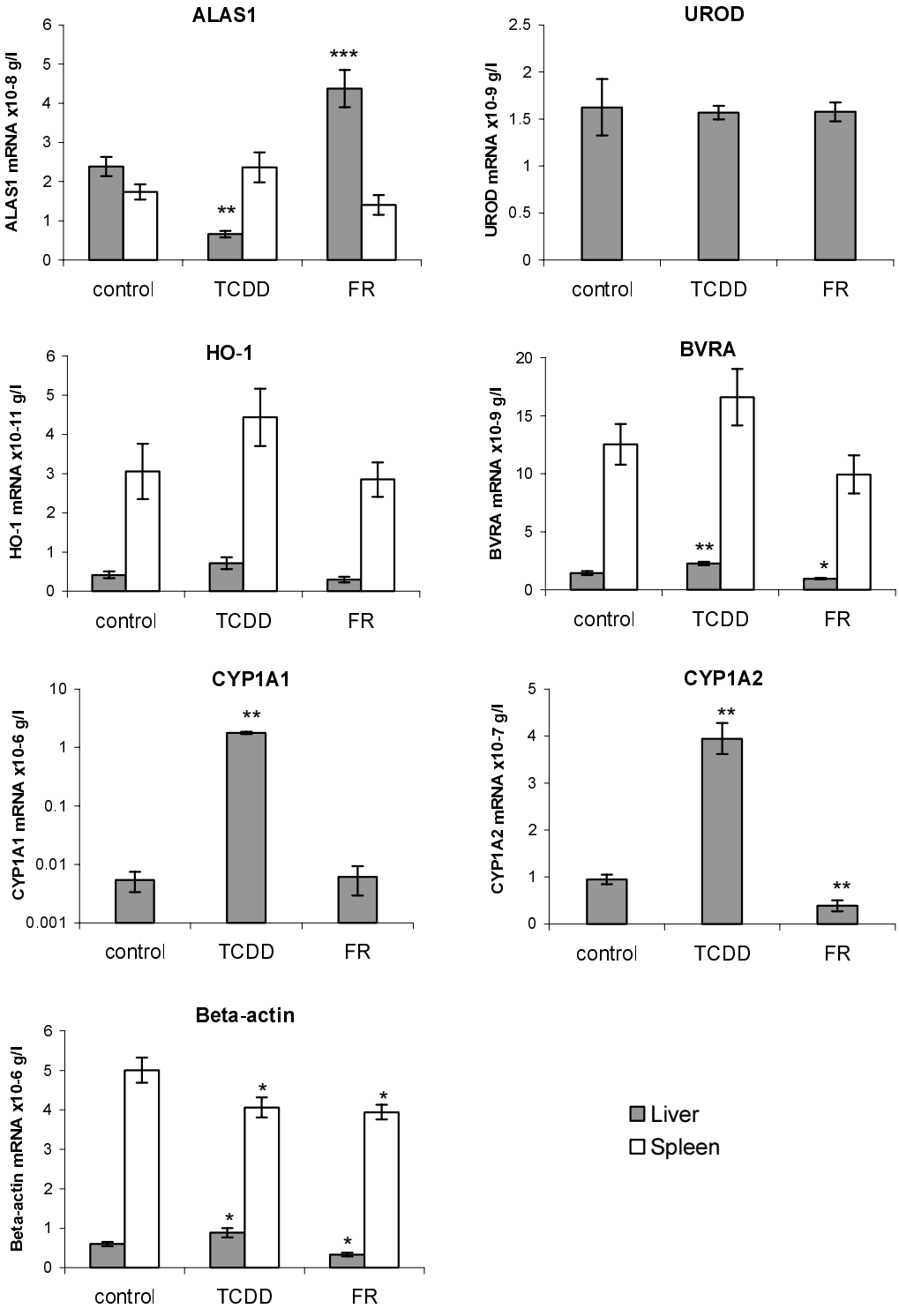

ALAS1
UROD
HO-1
BVRA
CYP1A1
CYP1A2
Beta-actin
Fold difference
Days after TCDD exposure
Line B liver
Line A liver
Line B spleen
Line A spleen

FIG. 3.


ALAS1
ALAS1 mRNA x10-8 g/l
control
TCDD
FR
UROD
UROD mRNA x10-9 g/l
HO-1
HO-1 mRNA x10-11 g/l
BVRA
BVRA mRNA x10-9 g/l
CYP1A1
CYP1A1 mRNA x10-6 g/l
CYP1A2
CYP1A2 mRNA x10-7 g/l
Beta-actin
Beta-actin mRNA x10-6 g/l
Liver
Spleen