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Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on heme oxygenase-1, biliverdin IXα reductase and δ-aminolevulinic acid synthetase 1 in rats with wild-type or variant AH receptor

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) causes hepatic accumulation of biliverdin and its monoglucuronide in moderately TCDD-resistant line B rats, but not in highly TCDD-resistant line A rats. In the mammalian heme degradation process, heme is cleaved to biliverdin by the rate-limiting enzyme heme oxygenase-1 (HO-1). Subsequently, biliverdin IXα reductase (BVRA) catalyzes the reduction of biliverdin to bilirubin. In heme biosynthesis, the rate-limiting enzyme is δ-aminolevulinic acid synthetase 1 (ALAS1). The effect of TCDD on HO-1, BVRA and ALAS1 was studied at the levels of mRNA (all three enzymes), protein expression (HO-1), and enzymatic activity (BVRA, liver only) in order to determine whether the accumulation of biliverdin could be due to their altered expression. In both lines A and B, 300 μg/kg TCDD transiently repressed hepatic HO-1 mRNA on day 2 but induced HO-1 protein expression at later time-points; however, the impact emerged earlier (day 14 vs. day 35) in line B rats. In spleen, TCDD repressed HO-1 mRNA and protein expression in lines A and B through days 2–35, but did not affect its mRNA levels in TCDD-sensitive L-E rats (10 days after 100 μg/kg). In all rat strains/lines, there was a strong repression of ALAS1 and a moderate induction of BVRA mRNA in liver, but mostly not in spleen. Hepatic BVRA activity was increased in lines A and B on day 14. At 5 weeks, it was still elevated in line A but reduced to 51% of control in line B. The results suggest that hepatic heme degradation is induced by TCDD in rats; however, this does not alone explain the accumulation of biliverdin in line B rats. Other factors such as the late repression of BVRA found here and possibly oxidative stress may be important contributors to biliverdin accumulation in these rats.

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1. 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-Hoï et al., 1972; Cantoni et al., 1987; Kociba et al., 1976; Pohjanvirta and Tuomisto, 1994). In addition, we have shown in our previous studies (Niittynen et al., 2003, 2007) that TCDD causes hepatic accumulation of the heme degradation product biliverdin and its derivative, biliverdin monoglucuronide, in line B rats. This rat line is moderately TCDD-resistant, and it originates from crosses between highly TCDD-sensitive Long-Evans (*Turku/AB*; L-E; LD_{50} 10–20 μg/kg TCDD) and extremely TCDD-resistant Han/Wistar (*Kuopio*; H/W; LD_{50} > 10,000 μg/kg TCDD) rats (Tuomisto et al., 1999).

The exceptional TCDD resistance of H/W rats is related to a mutated aryl hydrocarbon receptor (AHR) allele (AHR^{hw}) and to another, unknown gene *B* allele (B^{hw}) (Pohjanvirta, 1990; Tuomisto et al., 1999). The AHR^{hw} allele has been shown to harbor a point mutation that results in an abnormal C-terminus transactivation domain and a smaller AHR protein in H/W rats compared with the wild-type receptor present in TCDD-sensitive strains such as Sprague–Dawley and L-E (~98 kDa vs. 106 kDa, respectively) (Elferink and Whitlock, 1994; Pohjanvirta et al., 1998, 1999). The identity of gene *B* has not yet been determined, but it may encode a protein participating in the AHR signaling pathway. In order to segregate the two H/W-type TCDD resistance genes into separate rat lines, the H/W and L-E rats were crossbred (Tuomisto et al., 1999). Two new resultant rat lines were named lines A and B. Line A has the mutated AHR^{hw} allele and the wild-type *B* allele

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(genotype $AHR^{hw/hw}$ $B^{wt/wt}$). Line B has the wild-type AHR^{wt} allele but is homozygous for the H/W-type allele of gene *B* (genotype $AHR^{wt/wt}$ $B^{hw/hw}$). Lines A and B exhibit highly different LD_{50} values for TCDD: >10,000 μg/kg and 830 μg/kg in males, respectively, and >2000 μg/kg and 410 μg/kg in females, respectively (Tuomisto et al., 1999). Thus, line A appears to be as resistant as H/W and line B is intermediately resistant.

In the mammalian heme degradation process, heme is cleaved to biliverdin by the rate-limiting enzyme heme oxygenase. The isoform heme oxygenase-1 (HO-1) is often induced in various cellular stress states, such as oxidative stress, and is considered a cytoprotective enzyme (Alam and Cook, 2003). Subsequently, biliverdin is rapidly and quantitatively reduced to bilirubin by biliverdin reductase A (BVRA; McDonagh, 2001) and transferred to liver for conjugation and excretion. For a yet unknown reason, TCDD causes pronounced hepatic accumulation of biliverdin and biliverdin monoglucuronide in line B rats (Niittynen et al., 2003, 2007). The phenomenon is probably related to elevation of serum bilirubin, a well-known effect of TCDD reported in many rat strains/lines, including line B (Buu-Hoï et al., 1972; Kociba et al., 1976; Tuomisto et al., 1999; Zinkl et al., 1973). However, although TCDD induces vast increases in serum bilirubin in L-E rats (Pohjanvirta et al., 1995; Simanainen et al., 2002; Unkila et al., 1994), no definite cases of biliverdin accumulation have been detected in L-E rats after exposure to even lethal doses of TCDD (macroscopic observations in our laboratory, unpublished). Further, the syndrome has never been recorded in rats expressing only the H/W type AHR (genotype $AHR^{hw/hw}$) suggesting that a wild-type AHR with a fully functional transactivation domain may be necessary for the biliverdin accumulation (Niittynen et al., 2003, 2007). The apparent dependence on genetic factors makes biliverdin accumulation an intriguing endpoint of TCDD toxicity from a mechanistic point of view.

It was hypothesized that either increased formation of biliverdin due to induction of HO-1 or decreased elimination of biliverdin due to decreased activity of BVRA might be the immediate reason for its accumulation (Fig. 1). As AHR mediates most effects of TCDD through changes in gene expression (Okey, 2007), we first examined the effect of TCDD on mRNA expression of HO-1 and BVRA in liver and spleen; the latter organ was included as it is the major organ participating in heme degradation. Subsequently, also hepatic and splenic HO-1 protein expression and hepatic BVRA activity were analyzed. In addition, the effect of TCDD on mRNA expression of the nonspecific form of δ-aminolevulinic acid synthetase (ALAS1) was studied. ALAS1 is the first and rate-limiting step in heme biosynthesis in nonerythroid cells (May et al., 1995; Fig. 1). TCDD and some other cytochrome P450 inducers have been shown to increase its activity (Goldstein et al., 1982; Granick, 1966; May et al., 1995; Poland and Glover, 1973). The ultimate goal of this study was to look for such changes in gene/protein expression or enzymatic activity that might be related to the pathogenesis of hepatic biliverdin accumulation. Line B was our main interest, but we also wanted to see whether the effects are the same or whether they differ in more TCDD-sensitive or -resistant rats, in which the syndrome of biliverdin accumulation has never been observed, and further, whether the AHR genotype has an influence on the outcome. Therefore, L-E and line A rats were examined in addition to line B rats. In summary, the purpose of the study was to examine whether the increased biliverdin concentrations were associated with either augmented synthesis of heme (elevated ALAS1 activity) or of biliverdin (enhanced HO-1 activity), or with decreased metabolism of biliverdin (reduced BVRA activity).

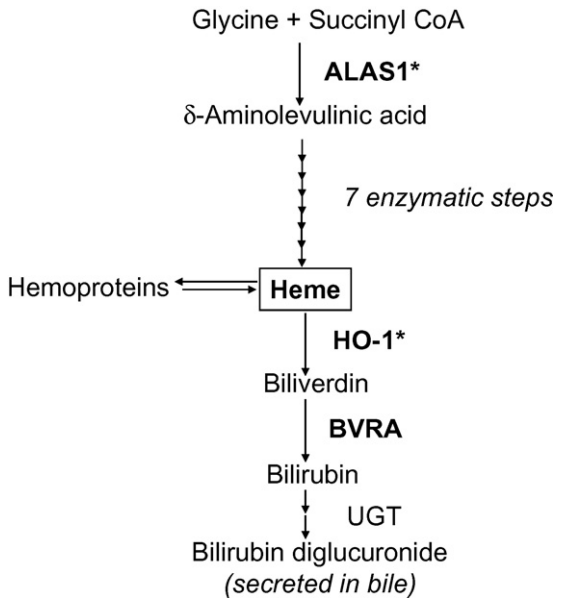

Fig. 1. Heme biosynthetic and degradation pathways showing the enzymes whose mRNA or protein expression or enzymatic activity after TCDD exposure was studied (in bold). ALAS1 = δ-aminolevulinic acid synthetase 1, BVRA = biliverdin IXα reductase, HO-1 = heme oxygenase-1, UGT = UDP-glucuronosyltransferase. Asterisk (*) denotes a rate-limiting step.

2. Materials and methods

2.1. Chemicals

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. Biliverdin (80% purity) was purchased from Chromatrin, Dublin, Ireland. Tris (99.8%) was obtained from Sigma–Aldrich Chemie, Steinheim, Germany. Sucrose, bovine serum albumin (BSA, minimum 98%) and β-NADPH (97%) were purchased from Sigma–Aldrich Co., St Louis, MO. In BVRA activity measurements, stock solutions of biliverdin and β-NADPH were prepared in 20 mM sodium phosphate buffer ($NaH_2PO_4 \cdot H_2O$; J.T. Baker, Deventer, Holland), pH 7.2. In HO-1 protein expression analysis, phosphate buffered saline (PBS, pH 7.4) was prepared using dipotassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$, Merck), $NaH_2PO_4 \cdot H_2O$ (J.T. Baker) and sodium chloride (NaCl, FF-Chemicals Ab, Yli-Ii, Finland). PBS ingredients were of analytical grade. Nonidet P40 Substitute (NP40) was purchased from Fluka Chemie, Buchs, Switzerland. Hydrochloric acid (HCl, 37%) and sodium hydroxide (analytical grade) used for adjusting the pH of the solutions were from Merck, Darmstadt, Germany and FF-Chemicals Ab, respectively. In all work involving DNA or RNA, Sigma's water for molecular biology was used. In all other analysis, purified water (Milli-Q Water purification system, Millipore, Bedford, MA) was used.

2.2. 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 (Experiment I) or singly (Experiment II; see the next section for descriptions of Experiments I and II) in stainless-steel wire-mesh cages with pelleted (Experiment I) or powdered (Experiment II) R36 feed (Lactamin, Stockholm, Sweden) and tap water available *ad libitum*, except for the feed-restricted group in Experiment II. 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. The procedures were conducted in accordance with the Guidelines of the European Community Council directives 86/609/EEC.

2.3. Experimental design

Adult female rats of lines A and B were used in Experiment I. They were 15–20 weeks old at the time of exposure. Rats were randomly divided in groups of 5–7 animals. They were dosed with 300 μg/kg TCDD by gavage (dosing volume 4 ml/kg) and killed 2, 7, 14, 32 or 35 days later by decapitation (n = 6–7). Controls received corn oil 4 ml/kg and were decapitated 2 or 35 days later (n = 5–6). The dose 300 μg/kg TCDD was chosen because it is large enough to cause the syndrome of hepatic biliverdin accumulation 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 mRNA/protein expression or catalytic activity of selected enzymes of heme metabolism during this time period, starting from an early time point (2

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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 swelling, mottled appearance and dark green or black color of the liver, hence the name "black liver syndrome").

In Experiment II, 10–13-week-old male L-E rats were used. They received 100 μg/kg TCDD by gavage or vehicle (corn oil) and were killed 10 days later by decapitation. Since these rats are highly sensitive to TCDD (LD_{50} 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, Experiment 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 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 experiments, 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. In Experiment I, the rest of the liver was used for immediate cytosol preparation (see below). When rats died prematurely, they were inspected at necropsy, but no samples were collected.

2.4. 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 °C for 1.5 h. Resulting cDNAs were diluted in a 1:6 ratio with water and used in subsequent analysis.

2.5. Cloning of ALAS1, HO-1 and BVRA gene fragments

169 to 284-nt-long fragments of ALAS1, 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. They were ordered from Sigma-Genosys Ltd., Cambridgeshire, UK. ALAS1, 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 as well as a purified PCR product of GAPDH (Korkalainen et al., 2004, 2005; Pohjanvirta et al., 2006; Table 1) were also used for production of external standard curves in mRNA quantitation.

2.6. Real-time quantitative PCR

The mRNA expression levels of ALAS1, HO-1, BVRA, CYP1A1, CYP1A2, GAPDH 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} and 10^{-11} (relative to original plasmid at a concentration of 200 ng/μl). The Rotor-Gene 2000 version 4.6 software was employed to obtain the threshold cycle (C_t) for best fit (least squares method) of the standard curve. The C_t values were then converted to concentrations by the formula:

$$\text{Calculated concentration} = 10^{(\text{slope} \times C_t + \text{intercept})}$$

The instrument analysis software was always allowed to carry out dynamic tube corrections. Reaction efficiencies were between 85 and 99%. All *R*-values were >0.996. 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 of a single tissue from a given strain/line at the same time.

2.7. Selection of a housekeeping gene for normalization of the mRNA expression data

Initially, we intended to use β-actin for normalization of the mRNA expression data. However, its hepatic expression increased in response to TCDD in L-E and line B rats when exposure lasted 7 days or more (Pohjanvirta et al., 2006). In line A, the increase was seen only at the latest time-point (day 35). In the study of Pohjanvirta et al. (2006), 18 commonly applied housekeeping genes were analyzed in order to find a widely applicable housekeeping gene for acute toxicity studies of TCDD. *GAPDH* proved to be one of the most stable genes and was therefore used here for normalization of the gene expression data derived from liver. β-Actin mRNA was used for normalization of gene expression data derived from spleen, as it was indifferent to TCDD in that organ.

2.8. Concentration of the HO-1 protein

Hepatic and splenic concentration of HO-1 protein was determined in selected samples using the Rat Heme Oxygenase-1 EIA Kit (Precoated; Takara Bio Inc., Otsu Shiga, Japan). The kit is a solid phase EIA Kit based on a sandwich method utilizing two mouse monoclonal anti-rat heme oxygenase-1 antibodies to detect rat heme oxygenase-1 by a two-step procedure. The assay was carried out on a precoated 96-well plate. A piece (about 50–90 mg) of deep-frozen liver or spleen was weighed and homogenized in extraction buffer (1% NP40 in PBS) for 30 s using Ultra Turrax-homogenizer (T25 basic, IKA Labortechnik). The homogenates were centrifuged (21,000 × *g*, 10 min, 4 °C), after which the supernatants were collected and stored at −80 °C until analysis. 1:20 (liver) and 1:2000 (spleen) dilutions of supernatants, prepared in the kit's sample diluent, were used for analysis of HO-1 concentration. The analysis was carried out according to the manufacture's instructions. Standard series consisted of 0.125, 0.25, 0.5, 1, 2, 4 and 8 ng/ml dilutions of HO-1 protein. Both the samples and the standards were analyzed in duplicates. After the completion of the assay, the plate was read at 450 nm using a microplate reader (Labsystems IEMS Reader MF, Labsystems Oy, Helsinki, Finland). As the most dilute standards (0.125–0.5 ng/ml) did not reproducibly differ from the blank (kit's sample diluent), they were ignored. The *R*-values of the standard series were 0.994 and 0.992 for liver and spleen analyses, respectively. Nearly all values for the samples were encompassed by the standard series, except for a few liver samples, which exceeded the highest standard. They were re-analyzed as 1:160 dilutions, and at that time, values for the samples were encompassed by the standard series ($R = 0.991$). Total protein concentrations of the supernatants were analyzed as explained below. The concentration of HO-1 protein in supernatants was normalized to total protein concentration, and the result was expressed as the amount of HO-1 protein in ng per mg total protein.

2.9. BVRA activity

BVRA activity was determined in liver cytosols of lines A and B rats. Livers were perfused *in situ* for 4 min with 0.9% NaCl (Natrosteril, Orion, Finland) right after decapitation of the rat. The first ca. 30 s of perfusion was done using room-temperatured NaCl followed by cold NaCl for the rest of the time. Perfusion was made through portal vein – inferior vena cava. A piece of liver (~2–4 g) was homogenized in 4 volumes of filter-sterilized ice-cold buffer (10 mM Tris and 0.25 M sucrose, pH 7.5 with HCl) using Teflon-pestled Potter-Elvehjelm glass homogenizer in a Heidolph homogenizer device. Homogenates were centrifuged for 20 min at 11,100 × *g*, at 4 °C. The supernatant was transferred into an ultracentrifuge tube and centrifuged for 1 h at 100,000 × *g*, 4 °C. The resulting cytosols were flash-frozen in liquid nitrogen and stored at −80 °C.

BVRA activity was measured at room temperature (23 °C) using Shimadzu UV-1601 spectrophotometer (Fennolab, Fenno Medical Oy, Kyoto, Japan) and Starna Brand 1 cm cuvets (Optiglass Ltd, Hainault, Essex, UK). BVRA activity was identified as a spontaneous increase in absorbance at wavelength 460 nm, reflecting the formation of bilirubin in the solution. The initial composition of the reaction (volume = 3.08 ml) was 100 mM Tris (pH 8), 10 μM biliverdin, and 150 μl of cytosol. Immediately after mixing these components, the reaction was followed for 10 × 1 min to record the blank rate. After this, β-NADPH was added to the solution to a concentration of 100 μM and the reaction was followed again for 10 × 1 min to record the BVRA activity. Total volume of the reaction was 3.1 ml. Total protein concentrations of the cytosols were analyzed as explained below.

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Table 1
The primers used in the study

Gene	GenBank accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of product (bp)
ALAS1	J03190	GCGCAATGTCAAGCTTATGA	TGGGTAATTAATGGCCTGGA	169
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
GAPDH	NM-017008	ATCCCGCTAACATCAAATGG	GTGGTTCACACCCATCACAA	170

The mean of recordings between 6 and 10 min was used as the value for blank rate and the mean of recordings between 7 and 10 min after addition of β-NADPH as the value for BVRA activity (dA/min). The amount of bilirubin formed in a minute was calculated using the molar absorption coefficient of 52,500 l mol^{-1} cm^{-1} (Rigney et al., 1989). This was then divided by the total protein concentration of the cytosol to get the BVRA activity expressed as the amount of bilirubin formed (nmol/min/mg total protein).

2.10. Total protein concentration

Total protein concentrations were analyzed using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA). 1:50 dilutions of the cytosols (BVRA activity assessment) and 1:40 and 1:20 dilutions of hepatic and splenic supernatants (HO-1 protein concentration assessment), respectively, were prepared for the analysis. Dilutions of BSA at concentrations of 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg/ml were used as standards. Assays were made on 96-well plates. 20 μl of water (blank), standard or diluted sample was applied in the wells and 200 μl of the Protein Assay Dye Reagent was added on top. The plate was gently mixed for 5 min, and the absorbances of the wells were recorded with a microplate reader at wavelength 595 nm (Labsystems iEMS Reader MF, Labsystems Oy, Helsinki, Finland). The samples and standards were measured in triplicates. The Labsystems Genesis V2.12 software was employed to create standard curves. All regression coefficients were >0.991. The signals of the samples were converted to protein concentrations by the formula: concentration = (absorbance − intercept)/slope.

2.11. 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 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*-Values less than 0.05 were considered statistically significant. In Experiment I, the controls were from two time-points, 2 and 35 days after vehicle administration (referred to as C2 and C35 control groups, respectively). Using Student's *t*-test, or in case of non-homogenous variances, Mann–Whitney *U* test, we verified that for most variables analyzed, there was no significant difference between these two control groups ($p > 0.05$). Therefore, they were pooled to form one control group ($n = 10–12$). In a few cases, however, C2 and C35 groups differed significantly from each other ($p < 0.05$) and were thus kept separate.

In the real-time quantitative RT-PCR analysis, some samples had HO-1 mRNA content less than the detection limit (line A: 3, 2 and 2 samples from days 2, 7 and 14, respectively; L-E-strain: two samples in FR group). These samples were given the lowest detectable value of the variable under study.

3. Results

3.1. Macroscopic observations

As expected, the used doses of TCDD resulted in significant impacts on body weight in both line B and L-E rats, but only a mild effect was observed in TCDD-resistant line A rats (Table 2). Line B rats were exposed to a dose of TCDD close to their LD_{50} value. All rats in Experiment I survived for 2 weeks, but thereafter five out of seven line B rats succumbed prematurely and no samples were obtained from them, but they were examined at necropsy and macroscopic changes recorded. The remaining two rats of the group were sampled 3 days earlier than intended (day 32 instead of 35).

There were changes in liver appearance in lines A and B rats at the latest time point (Table 2). In line B, the incidence of 57% (4/7) of livers with accumulation of dark green/black pigment is in good agreement with our previous results (Niittynen et al., 2003). Thirty-five days after TCDD exposure, 5 out of 6 line A rats had a yellowish liver, which might result from accumulation of bilirubin in the liver. Increased serum bilirubin levels have been reported in resistant rat strains (line A and H/W rats), although they are far lower than those in more sensitive strains (Unkila et al., 1994; Tuomisto et al., 1999).

3.2. mRNA expression levels

By and large, the effects of TCDD on the mRNA expression levels analyzed were similar in lines A and B rats (Figs. 2–4), in both liver and spleen. Also Experiment II on L-E rats yielded largely concordant results with the lines A and B rats (Figs. 2–4), except for splenic HO-1 and BVRA.

3.3. CYP1A1 and CYP1A2

CYP1A1 and CYP1A2 mRNAs, which were used as positive controls of induction, showed the characteristic elevation in liver after TCDD exposure (data not shown). Maximal CYP1A1 induction was about 4650-fold (line B, day 7), whereas maximal CYP1A2 induction was 18–26-fold (line B, day 7; relative to C35- and C2-groups, respectively). 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 ± S.D. of maximal fold inductions 4652 ± 891 and 2788 ± 619, respectively).

3.4. ALAS1, HO-1 and BVRA

TCDD significantly decreased hepatic expression of ALAS1 mRNA (Fig. 2). In line B, ALAS1 mRNA expression was 37% of control value already on day 2, and fell further down to 14% by day 14. 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. In feed-restricted L-E rats, ALAS1 mRNA expression increased, implying that the repression is caused by TCDD and not secondary to body weight loss. TCDD did not influence splenic ALAS1 mRNA expression in any rat strain/line.

HO-1 mRNA was not induced by TCDD in liver (Fig. 3). Rather, in both lines A and B, hepatic *HO-1* expression showed a downward tendency on day 2 with mean mRNA levels being 17% ($p < 0.05$) and 22% ($p < 0.05$, compared with the C2 group) of control, respectively. In spleen, HO-1 mRNA expression was decreased after TCDD exposure in both rat lines A and B throughout the observation period (Fig. 3). In contrast, no significant departure from control value was seen in L-E rats (Fig. 3). In fact, there was a slight upward tendency in the TCDD-exposed group.

Interestingly, there seemed to be a rather large difference in the basal expression level of hepatic HO-1 mRNA between lines A and B rats (Fig. 3; panels "Line A, liver" and "Line B, liver"; control groups). Technical matters may at least partly account for this, since lines A and B samples were analyzed in different real-time quantitative PCR runs and also cDNAs were prepared separately. However, we

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Table 2
Changes in body weight and liver appearance in the experimental groups

Group	Experiment	Body weight change (%)[a]	Changes in liver appearance compared with control
Line A, 2-day control	I	−0.9 ± 1.8	
Line A, 35-day control	I	5.3 ± 3.1	
Line A, TCDD 300 μg/kg, 2 days	I	−1.3 ± 1.0	
Line A, TCDD 300 μg/kg, 7 days	I	−6.1 ± 2.6	
Line A, TCDD 300 μg/kg, 14 days	I	−7.2 ± 3.0	
Line A, TCDD 300 μg/kg, 35 days	I	−0.2 ± 2.9	Yellowish liver in 5 out of 6 rats
Line B, 2-day control	I	1.8 ± 1.3	
Line B, 35-day control	I	6.2 ± 4.2	
Line B, TCDD 300 μg/kg, 2 days	I	−1.5 ± 1.4	
Line B, TCDD 300 μg/kg, 7 days	I	−15.6 ± 4.7	
Line B, TCDD 300 μg/kg, 14 days	I	−15.2 ± 5.4	
Line B, TCDD 300 μg/kg, 32 days	I	−11.5 ± 3.1	Dark green/black liver in 4 out of 7 rats
L-E, control, 10 days	II	4.3 ± 3.0	
L-E, TCDD 100 μg/kg, 10 days	II	−31.6 ± 2.2	
L-E, feed-restricted control, 10 days	II	−18.7 ± 3.2	

[a] Percent of initial body weight (mean ± S.D.).

wanted to clarify whether there indeed are differences in the basal expression level of HO-1 mRNA between the rat strains/lines. To this end, new cDNAs were prepared from liver samples of line A, line B and L-E control rats. Subsequently, HO-1 and β-actin mRNA levels were analyzed (here it was possible to use β-actin for normalization, since no TCDD was involved). In this analysis, all samples from different strains/lines were prepared and analyzed at the same time in order to minimize confounding factors. It was found out that in

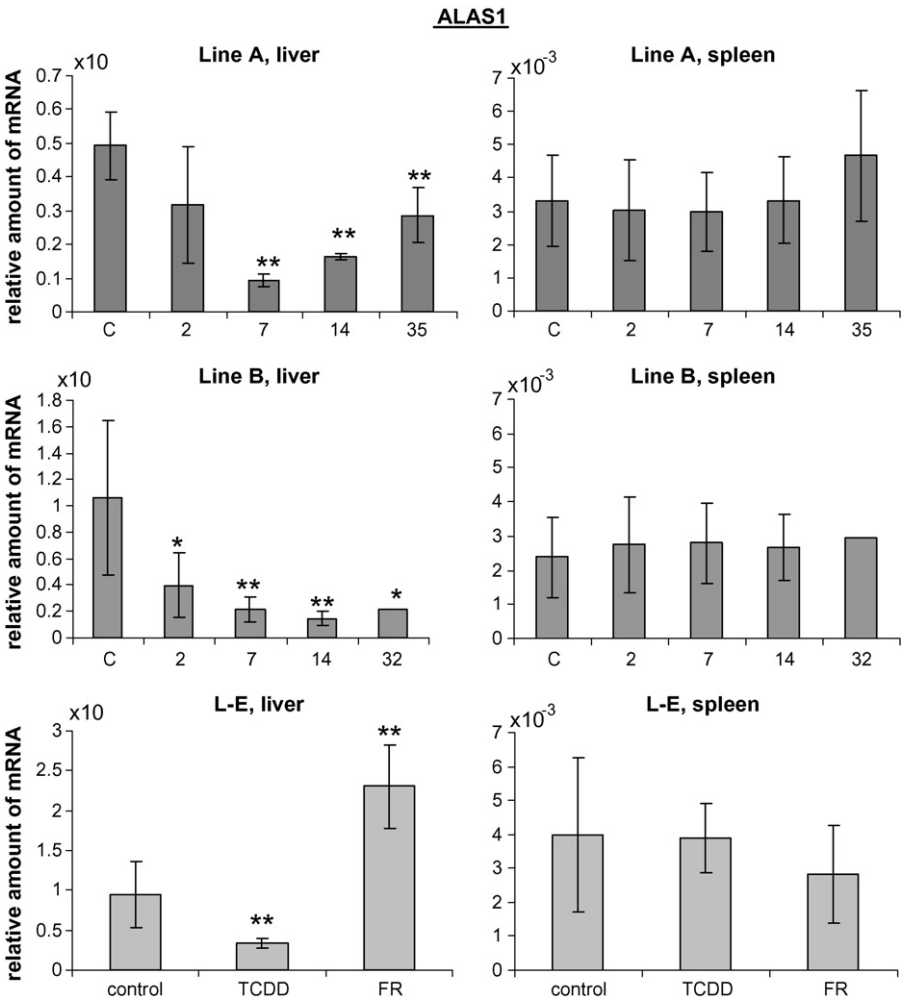

Fig. 2. Effect of TCDD (mean ± S.D.) on the hepatic (relative to GAPDH) and splenic (relative to β-actin) mRNA expression of ALAS1 in female lines A and B rats 2–35 days after exposure (n = 5–10, except for line B day 32 where n = 2; S.D. is not shown for this group) and male L-E rats 10 days after exposure (n = 6). TCDD doses were 300 μg/kg for lines A and B rats and 100 μg/kg for L-E rats. In the panels for lines A and B rats the number under the x-axis denotes the day post-exposure (C = control). FR = feed-restricted control. The asterisks denote statistically significant differences from the control at the level of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

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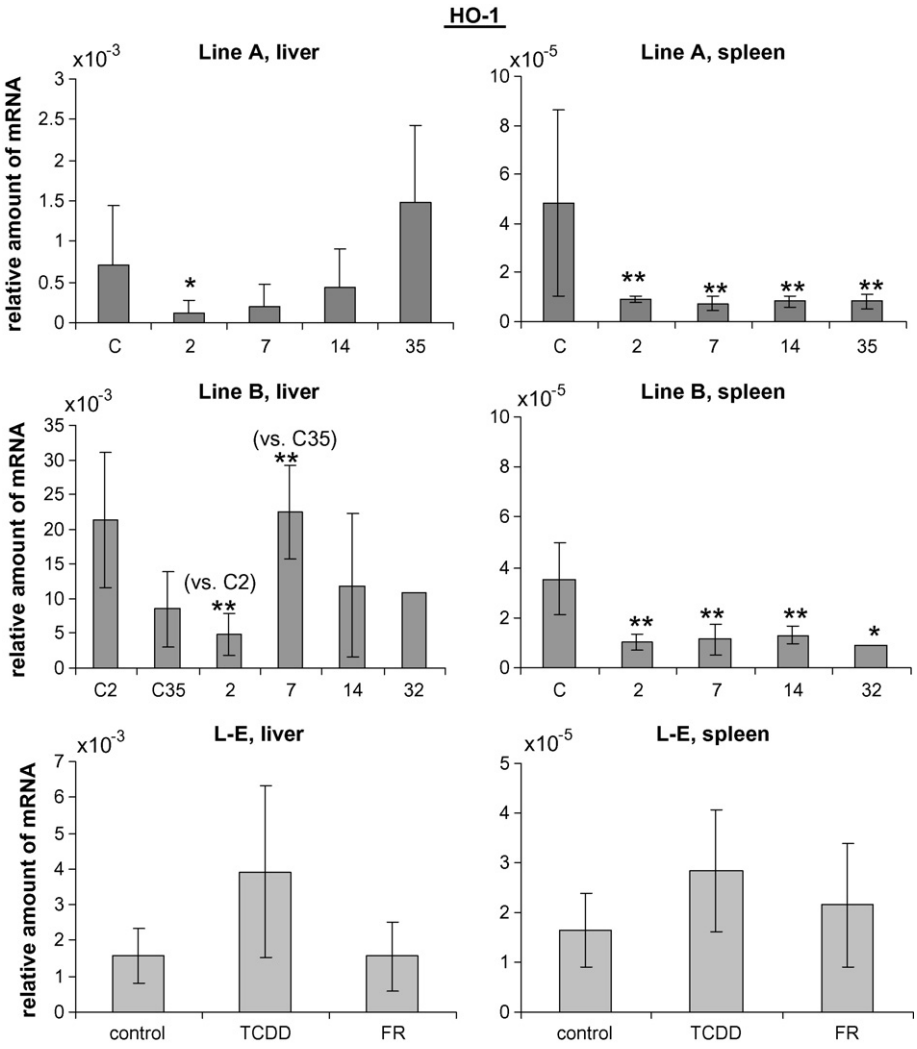

Fig. 3. Effect of TCDD (mean ± S.D.) on the hepatic (relative to GAPDH) and splenic (relative to β-actin) mRNA expression of HO-1 in female lines A and B rats 2–35 days after exposure and in male L-E rats 10 days after exposure. Experimental conditions and symbols are as in Fig. 2. C consists of control groups from time-points of 2 and 35 days (C2 = day-2 control; C35 = day-35 control). If C2 and C35 differed significantly from each other as assessed by the *t*-test, they were kept separate; otherwise a pooled control (C) was used.

line B ($n = 10$) and L-E ($n = 6$) control rats the expression level of HO-1 mRNA was 2.5-fold ($p < 0.001$) and 1.7-fold ($p < 0.05$), respectively, higher relative to line A ($n = 10$). This implies that there indeed may be differences in the basal expression level of *HO-1* among rat strains/lines.

Hepatic expression of BVRA mRNA increased somewhat in all rat strains/lines after TCDD exposure. In line A, the effect was seen on days 14 and 35 (2.4- and 3.3-fold increase, respectively; Fig. 4). In line B, a slight increase (1.75-fold) was seen on day 7 (Fig. 4). BVRA mRNA expression increased also in L-E rats after TCDD-exposure (2.2-fold on day 10; Fig. 4), but was unchanged in feed-restricted rats. This implies that the effect is related to TCDD exposure and not simply due to body weight loss. In spleen, BVRA mRNA expression did not significantly change due to TCDD exposure in lines A or B but increased 1.7-fold in L-E rats on day 10 (Fig. 4).

3.5. Expression of HO-1 protein

For technical reasons, expression of HO-1 protein was analyzed only in selected samples. The later time-points (14 and 32–35 days) were regarded as most appropriate, since the accumulation of biliverdin is macroscopically seen at the earliest about 3 weeks after TCDD exposure. Samples from day 2 were also analyzed (line B only), since there was a repression of HO-1 mRNA on that day (Fig. 3).

In line A, TCDD had no influence on expression of HO-1 protein on day 14, but induced its expression clearly on day 35 (Fig. 5). The mean induction was 6.5-fold, however, individual differences were remarkable with the maximum individual induction being 14.5-fold. In line B, expression levels of HO-1 differed in C2 and C35 control groups, the concentration in the latter being only 58% of that in the former (Fig. 5). Therefore, the exposed groups were compared separately with C2 and C35. A significant decrease in HO-1 expression (45% of the C2) was found on day 2. This is in accordance with HO-1 mRNA expression (Fig. 3). At later time-points, HO-1 protein expression was induced: on day 14 it was 2.8- ($p = 0.114$) and 4.8-fold ($p = 0.016$) relative to C2 and C35, respectively. Similarly to line A, individual differences were very large. On day 32, the mean value of HO-1 protein concentration was 2.4- and 4.1-fold higher relative to C2 and C35, respectively, but the induction was not statistically significant due to the small number of rats in the TCDD group. In contrast to HO-1 mRNA levels, there was no significant difference ($p = 0.094$) between the hepatic concentrations of HO-1 protein in lines A and B control rats on post-vehicle day 35 (day 2 could not

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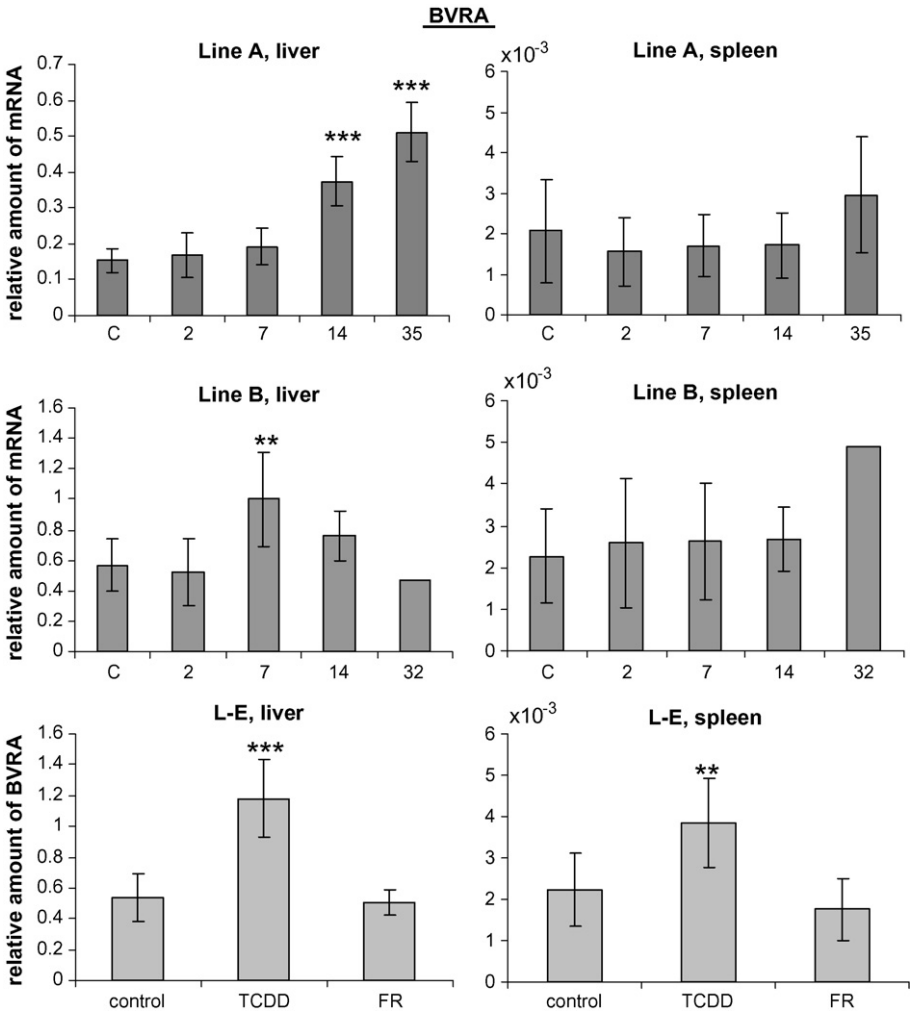

Fig. 4. Effect of TCDD (mean ± S.D.) on the hepatic (relative to GAPDH) and splenic (relative to β-actin) mRNA expression of BVRA in female lines A and B rats 2–35 days after exposure and in male L-E rats 10 days after exposure. Experimental conditions and symbols are as in Fig. 2.

be analyzed for this aspect as HO-1 protein concentration was not assessed in line A samples from day 2).

The Pearson correlations between hepatic HO-1 mRNA and HO-1 protein were 0.608 ($p = 0.036$) and 0.558 ($p = 0.013$) in lines A and B, respectively, indicating a moderate-to-good correlation. In spleen, HO-1 protein concentration decreased by ca. 20% in both lines A and B after TCDD exposure, although in line B only relative to C35 (Fig. 5). The Pearson correlations between splenic HO-1 mRNA and protein concentrations were 0.745 ($p = 0.008$) and 0.657 ($p = 0.003$) in lines A and B, respectively, indicating even stronger concordances than in liver.

3.6. Enzymatic activity of BVRA

TCDD increased the catalytic activity of liver cytosolic BVRA in both rat lines (Fig. 6). In line A, 1.3-, 1.5- and 1.5-fold increases were observed at days 2, 14 and 35, respectively. In line B, a 1.5-fold increase was seen on day 14. However, this increase in line B rats flipped to 50% reduction by day 32.

Interestingly, in line A, TCDD had a significant effect on the blank rate (the rate detected in a reaction mix containing buffer, biliverdin and cytosol, but not NADPH), increasing it more than 2-fold at 2, 7 and 14 days (Fig. 6). A similar but milder effect was seen in line B rats at 2 days. Blank rate should represent the unspecific (non-enzymatic) change in absorbance at 460 nm. However, an enzymatic reaction is also possible with endogenous NADPH (and possibly NADH) in the cytosol. TCDD has been shown to initially increase hepatic NADPH content (Stohs et al., 1990), which might explain the increase in the blank rate after TCDD exposure. At the last time point, the blank rate was again at the control level in line A but only 9% of the control in line B. The latter observation might reflect depletion of NADPH during the possible TCDD-induced severe oxidative stress (Stohs, 1990) or through inappropriately active *de novo* synthesis of fatty acids and/or cholesterol.

4. Discussion

Hepatic accumulation of biliverdin is a novel TCDD-induced phenomenon reported only in some intermediately TCDD-resistant rats, predominantly those of line B, developed in our laboratory (Niittynen et al., 2003). In the present study, the effect of TCDD on the enzymes responsible for the formation and elimination of biliverdin was studied in order to look for possible TCDD-induced derailments in their activity or expression that might play a role in biliverdin accumulation.

4.1. HO-1

Two days after TCDD exposure (300 μg/kg), mRNA expression of HO-1 was repressed in liver and spleen; in the latter organ this

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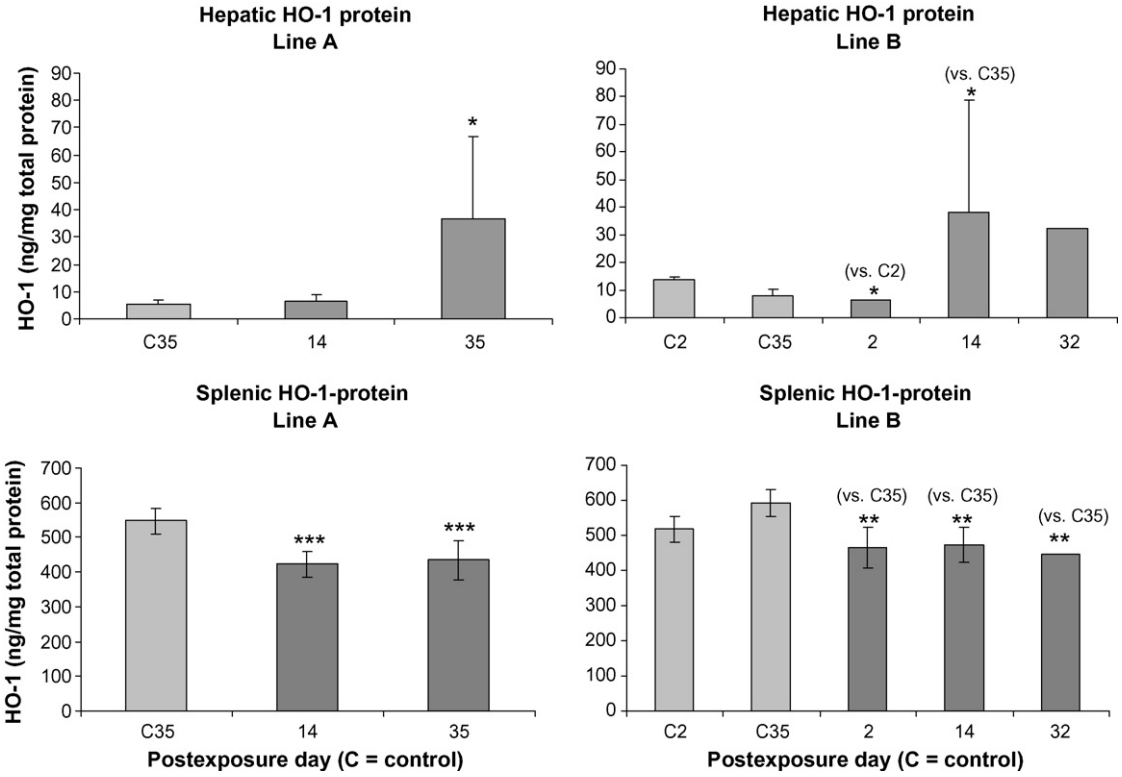

Fig. 5. Effect of TCDD (300 μg/kg; mean ± S.D.) on hepatic and splenic HO-1 protein levels in female lines A and B rats 2–35 days after exposure (n = 4–5, except for line B, day 32, where n = 2; S.D. is not shown for this group). Symbols are as in Figs. 2 and 3.

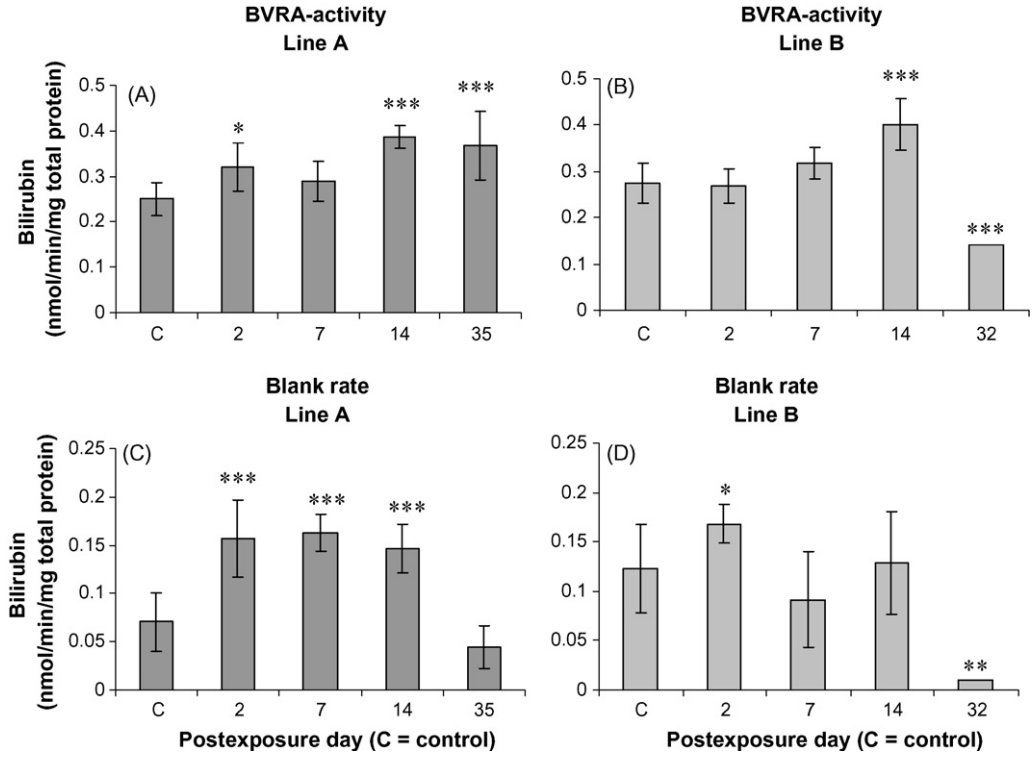

Fig. 6. Effect of TCDD (300 μg/kg; mean ± S.D.) on hepatic BVRA activity in female lines A and B rats 2–35 days after exposure (A and B; n = 5–12 except for line B, day 32, where n = 2). Symbols are as in Fig. 2. The blank rate (before the addition of NADPH) is also presented (C and D). (*Blank rate* is the rate detected in a solution consisting of buffer + biliverdin + cytosol; *BVRA activity* is the rate detected after the addition of NADPH [100 μM] into the reaction solution.)

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repression remained throughout the whole observation period of 35 days. In liver, HO-1 mRNA concentration returned to the control level at later time-points. In accordance with the changes in HO-1 mRNA, also HO-1 protein expression was decreased on day 2 (analyzed in line B). However, HO-1 protein was clearly induced 14 and 32 days after TCDD exposure in line B, and on day 35 in line A. HO-1 is regarded to be regulated mainly at the transcriptional level (Alam and Cook, 2003; Morse and Choi, 2002), and findings from our correlation analyses of HO-1 mRNA and protein expression are in good agreement with this view in both liver and spleen. However, since the mean induction of HO-1 protein is manifold compared with the mean increase in mRNA levels, our results suggest that post-transcriptional mechanisms are also involved in regulation of HO-1 expression in rat liver. This seems to hold also in spleen, since a large reduction in HO-1 mRNA levels resulted in a much smaller reduction in HO-1 protein concentrations (compared as percentages).

Induction of HO-1 protein expression 14–32 or 35 days after TCDD exposure in lines B and A rats, respectively, suggests that degradation of heme may be increased in rat liver due to TCDD exposure. On the other hand, the initial (day 2) repression of HO-1 at both mRNA and protein levels suggests that HO-1 induction is not a direct effect of TCDD on *HO-1* gene. More likely, induction of HO-1 might be caused by some TCDD-triggered change in cellular homeostasis. This kind of change might be increased production of reactive oxygen species (ROS), a known consequence of TCDD exposure (Senft et al., 2002; Stohs, 1990) and an established inducer of HO-1 (Ryter and Choi, 2002). In addition, the substrate heme is a well-known inducer and feed-back regulator of HO-1 (Shibahara, 2003). Therefore, if TCDD increased hepatic free heme level, this might lead to HO-1 induction. However, no studies of TCDD's effect on hepatic free heme concentrations could be found in the literature. Furthermore, both ROS and heme are known to mediate HO-1 induction at the transcriptional level, but here it seems that the main regulation would occur post-transcriptionally. Importantly, hepatic induction of HO-1 protein was seen in both lines A and B rats, although it emerged earlier in line B. This indicates that TCDD can induce hepatic expression of HO-1 protein both in rats with mutated, H/W-type AHR and in rats with wild-type AHR. However, altered AHR may delay the onset of induction.

Another rat strain-associated difference in hepatic HO-1 was the difference in basal level of HO-1 mRNA especially between lines A and B rats. However, there was no significant difference in HO-1 protein levels between lines A and B control rats on post-vehicle day 35. Thus, the observed difference in mRNA level may be of minor importance as to action of HO-1 enzyme and its effects in liver. Yet, this observation suggests that AHR genotype may have some influence on the regulation of *HO-1* expression. It is conceivable that the altered AHR is related to a lower level of basal oxidative stress, which might delay induction of HO-1 in line A rats compared with line B rats. In fact, there are some previous data suggesting that the mutated AHR of H/W rats is associated with a slightly lower basal hepatic lipid peroxidation level (a measure of oxidative stress) in comparison with L-E rats carrying the wild-type AHR; H/W rats further exhibited reduced sensitivity to TCDD-induced lipid peroxidation (Pohjanvirta et al., 1990).

Only hypothetical reasons for repression of splenic HO-1 expression can be given. Two such phenomena are hypoxia and feed-back regulation by intracellular heme. Repression of HO-1 has been demonstrated in cultured human cells due to hypoxia with the potentially beneficial consequences such as reduction of energy expenditure and prevention of local accumulation of CO, iron and bilirubin (Shibahara, 2003). On the other hand, it has been suggested that HO-1 is normally induced in spleen due to large amounts of heme present (Braggins et al., 1986). Therefore, if the amount of heme in spleen reduced then the concentrations of HO-1 protein and mRNA might decrease. Overall, the consequences of TCDD exposure on HO-1 expression seem to be largely different in spleen and in liver. This is not surprising, since HO-1 is often regulated in a tissue-specific manner (Shibahara, 2003). Splenic repression of HO-1 was not observed in L-E rats. Gender, TCDD-dose, TCDD-sensitivity, duration of exposure as well as *AHR*- and gene *B*-genotypes are factors that may underlie the difference between L-E vs. lines A and B rats.

4.2. BVRA

Hepatic BVRA activity was increased (1.5-fold) in both lines A and B rats 14 days after TCDD exposure suggesting enhanced capacity of hepatic biliverdin reduction. Induction seems to occur at the transcriptional level since also hepatic BVRA mRNA levels were elevated in all three rat strains after TCDD exposure. The next question is, why and how does TCDD induce BVRA? There are only a few previous reports where induction of BVRA has been described in any conditions. McCoubrey et al. (1995) reported about 2-fold induction of BVRA mRNA after hyperthermia in rat kidney and Maines et al. (1999) observed a 175% increase in transcript level in human renal carcinoma. 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 transcription factors by TCDD (Puga et al., 2000), as BVRA gene has an AP-1 binding site in its promoter (McCoubrey et al., 1995).

Potentially interesting is the difference in the BVRA activity between lines A and B rats on day 32/35. At that time-point, line B rats had severe accumulation of biliverdin (enlarged liver containing dark green/black pigment) and the activity of BVRA was diminished down to 51% of control. In contrast, line A rats exhibited increased activity of BVRA on day 35 and their livers were yellowish. It is possible that decreased BVRA activity in line B rats is due to the overt liver damage at that stage. On the other hand, the possibility remains that between days 14 and 32, BVRA activity would start to diminish in line B rats, and this would lead to biliverdin accumulation. Even so, the late reduction in BVRA activity could hardly be the only or primary reason for the syndrome, since it was preceded by induction of BVRA on day 14. A more likely scenario is that accumulation of biliverdin is preceded by increased bilirubin concentration (as bilirubin glucuronides) in hepatocytes due to its diminished clearance (Choe and Yang, 1983; Peterson et al., 1979; Yang et al., 1983) and/or augmented formation (Mitchell et al., 1990), and oxidized back in line B to biliverdin glucuronides due to severe and prolonged oxidative stress caused by TCDD. This is supported by the fact that biliverdin glucuronides, not free biliverdin, seem to constitute the bulk of the pigment (Niittynen et al.; unpublished results). With biliverdin starting to accumulate, BVRA could be partly inactivated due to substrate inhibition, i.e. formation of an enzyme–NADP–biliverdin complex (Rigney and Mantle, 1988).

Better knowledge of the mechanism of increased serum bilirubin levels, a well-known effect of TCDD, might further help ascertain the reason for biliverdin accumulation. Unfortunately, no conclusive evidence exists in the literature as to whether the increase in serum bilirubin after TCDD exposure is due to its decreased secretion or increased formation or both (see e.g. Pohjanvirta and Tuomisto, 1994 and references therein). One possible reason is hepatobiliary dysfunction (Peterson et al., 1979) resulting in cholestasis or decreased secretion of bilirubin to bile. However, we recently found that bile bilirubin levels were increased about 2.6-fold relative to control in TCDD-sensitive rats 7 days after 10 μg/kg TCDD (unpublished data). This does not support the view that decreased secretion of bilirubin from hepatocytes to bile would

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be the key (or at least the sole) reason for increased serum bilirubin levels. Other possibilities are that the (re)uptake of bilirubin at the sinusoidal membrane of hepatocytes is impaired by TCDD or that its hepatic synthesis is enhanced. The latter option is supported by the current results showing induction of HO-1 and BVRA after TCDD exposure. At least part of the increase in bilirubin levels could conceivably be due to its increased *de novo* formation since the hepatic enzymes responsible for its formation are induced. However, while BVRA seems to be as efficiently induced in line A as in line B, bilirubin is much less increased in line A serum than in line B serum after TCDD exposure (Tuomisto et al., 1999). Moreover, BVRA is not a rate-limiting enzyme (Fig. 1). Thus, TCDD may cause problems in bilirubin clearance especially in line B and other rat strains/lines with wild-type AHR. Another possibility is that there might be less substrate (biliverdin) for BVRA available in line A than in line B rats, but the HO-1 data do not support this. Future studies are still needed to fill the gap in our current understanding of the basis for increased serum bilirubin levels after TCDD exposure.

Interestingly, in line A, TCDD increased the blank rate in BVRA activity measurements on days 2, 7 and 14. This is possibly due to an increased amount of endogenous NADPH, the limiting factor of the reaction, as it has been shown that 100 μg/kg of TCDD increased NADPH content in the livers of Sprague–Dawley rats until day 5, whereafter its amount was reduced below the control level (Stohs et al., 1990). However, in line B, only a slight increase in blank rate was observed and only on day 2. Furthermore, line B control rats had a somewhat higher blank rate than line A control rats. The presumed increase in cytosolic NADPH content in line A rats might be a protection mechanism, while the drastic reduction in blank rate in line B rats on day 32 might be an indicator of severe oxidative stress, if it is indeed due to a reduced NADPH content in the liver (Stohs et al., 1990). Altogether these observations suggest differences in the degree of TCDD-induced oxidative stress between lines A and B and/or in the ability of these rat lines to handle the oxidative insult resulting from TCDD treatment. These results on BVRA activity further support the discussion above on the role of AHR genotype in basal oxidative stress status. The altered, H/W-type AHR may afford protection against TCDD-induced oxidative stress and/or decrease the basal level of it.

4.3. ALAS1

Repression of hepatic ALAS1 mRNA expression after TCDD exposure was a consistent finding in all three rat strains, so this effect seems to be independent of the AHR genotype. Repression was specific to liver, as the expression did not change in spleen. A possible reason for ALAS1 repression is increased amount of free heme, which is a well-known feed-back repressor of ALAS1 gene expression in liver (Yamamoto et al., 1988). Heme negatively regulates ALAS1 expression by decreasing mRNA half-life (Hamilton et al., 1991) and may also inhibit ALAS1 gene transcription (Srivastava et al., 1988). Thus, two observations of this study, induction of HO-1 and repression of ALAS1, are conditions which can be caused by free heme. This warrants future studies on TCDD's interference with hepatic free heme content. The pattern of ALAS1 repression and HO-1 induction has been observed in acute liver failure in humans (Fujii et al., 2004) and in the rat model of septic multiple organ dysfunction syndrome (Suzuki et al., 2000). In these cases, it was proposed that the causative agent was increased free heme concentration. In addition to the elevated free heme level, another possible cause for ALAS1 repression has been proposed. Guberman et al. (2003) suggested 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). It is also noteworthy that the repression of ALAS1 found here after a single exposure to relatively high, even lethal dose (depending on the rat strain) of TCDD is at variance with its induction reported after repeated exposure of Sprague–Dawley rats to a low dose (1 μg/kg/week) of TCDD for 16 weeks (Goldstein et al., 1982).

In conclusion, the results suggest that hepatic heme degradation is induced by TCDD in the rat strains/lines studied. However, this does not alone explain the biliverdin accumulation in line B rats. The repression of BVRA activity may be an important contributor, but probably there are also problems in bilirubin secretion and/or severe oxidative stress, which might lead to the oxidation of bilirubin and its glucuronides to biliverdin and respective glucuronides.

Conflict of interest statement

None.

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