BIOMARKERS, 1999, VOL. 4, NO. 3, 214–228

Induction of cytochrome P450 1A and DNA damage in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Received 20 July 1998, revised form accepted 21 September 1998

Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on hepatocytes isolated from immature rainbow trout (*Oncorhynchus mykiss*) by collagenase perfusion were investigated with respect to induction of cytochrome P450 1A (CYP1A) enzyme activities and protein contents as well as DNA damage. Exposure of primary rainbow trout hepatocytes to TCDD resulted in increased CYP1A contents, as determined by immunoblotting, enhanced activities of 7-ethoxyresorufin-O-deethylase (EROD) and increased DNA damage as determined by the comet assay. By means of electron microscopy, no symptoms of cytotoxicity could be observed except for slight increases of lysosomal components and the smooth endoplasmic reticulum. Whereas CYP1A contents constantly increased over the duration of the entire experiment, EROD activities remained constant from day 3 of exposure to 1 nM TCDD; maximum induction of CYP1A activities was reached with 0.1 nM TCDD after 5 days. DNA damage increased in a time- and dose-dependent fashion until day 3. After 5 days, DNA damage was less pronounced, and the number of damaged nuclei declined in all TCDD concentrations. Since TCDD has been shown to not directly react with DNA, metabolism of TCDD or TCDD-induced changes in other metabolic pathways are suspected to result in the production of DNA-reactive (endogenous) substances.

Keywords: cytochrome P450, isolated hepatocytes, rainbow trout, dioxin, DNA damage.

Introduction

A major aspect of aquatic toxicology is the investigation of fundamental biochemical and cellular processes involved in the interaction between xenobiotics and organisms (Stegeman and Hahn 1994). Pollutant effects in animals may be detected by measuring a variety of changes in physiological and biochemical processes such as the induction of cytochrome P450 1A (CYP1A), which is a frequent reaction of fish exposed to xenobiotics such as polycyclic aromatic hydrocarbons, dioxin-like substances or certain chlorinated hydrocarbons (Goksøyr 1995, Guiney *et al.* 1997, Hahn and Stegeman 1992, Jensen *et al.* 1991, Sijm *et al.* 1993, Sleiderink *et al.* 1995, Stegeman *et al.* 1992). CYP1A induction has therefore commonly been used as a biomarker for environmental pollution by PAHs, chlorinated hydrocarbons or dioxins (Bucheli and Fent 1995, Goksøyr 1995, Goksøyr and Förlin 1992, Goksøyr and Husøy 1998, Goksøyr *et al.* 1991, Hahn *et al.* 1996, Masfaraud *et al.* 1990, Sleiderink *et al.* 1995, van der Weiden *et al.* 1993).

Induction of CYP1A can also be observed in piscine cell culture systems (Braunbeck *et al.* 1996, Collodi *et al.* 1994, Cravedi *et al.* 1996, Devaux *et al.* 1991, Miller *et al.* 1993a, b, Monod *et al.* 1998, Pesonen *et al.* 1988, Scholz *et al.* 1997,

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
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Vaillant *et al.* 1989), and in an attempt to reduce the number of animals required for toxicological experiments *in vivo*, *in vitro* systems with fish hepatocytes have been developed as important tools in aquatic toxicology (Baksi and Frazier 1990, Braunbeck 1998, Braunbeck *et al.* 1996). As an advantage over most classic permanent fish cell lines, isolated hepatocytes preserve several biochemical and cellular properties including inducibility of CYP1A (Braunbeck *et al.* 1996, Vaillant *et al.* 1989). Usually, hepatocytes from rainbow trout (*Oncorhynchus mykiss*) have been preferred due to the large number of *in vivo* studies conducted with this species (for references, see Braunbeck 1998).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is known to be one of the most toxic and mutagenic/carcinogenic substances present in the environment. Due to its lipophilic properties (bioconcentration factor in fish: 15,000–25,000; Frakes *et al.* 1993), TCDD readily enters organisms and accumulates in adipose tissues and cell membranes (Huff *et al.* 1991). TCDD has been shown to be the most potent inducer of the CYP1A-system (Buchmann *et al.* 1993, Forth *et al.* 1996, Guiney *et al.* 1993, Hahn *et al.* 1996, van der Weiden *et al.* 1993) by binding to an intracellular receptor, the Ah-receptor (TCDD-receptor), which is then translocated by the arnt protein to the nucleus of the cell. This complex binds to specific sites on the DNA, the xenobiotic response elements (XRE), and promotes the transcription of several genes including CYP1A (Goksøyr and Husøy 1998).

TCDD itself is only poorly metabolized, and even increased levels of CYP1A do not metabolize TCDD at elevated rates (Okey 1990, Park *et al.* 1996). The only metabolites identified so far are three glucuronic acid conjugates in bile accounting for at maximum 20 % of the total TCDD applied (Kleeman *et al.* 1986, 1988). The rest remains unchanged in the organism (Forth *et al.* 1996). Induced CYP1A, however, metabolizes other (endogenous) substances, which in turn may be responsible for toxic and carcinogenic effects including interactions with DNA. In contrast, TCDD itself does not directly interact with DNA (Forth *et al.* 1996). Thus, e.g., exposure of rats to TCDD lead to an increase of single strand breaks in the DNA (Wahba *et al.* 1988a, 1989a, b). Whereas much is known about the toxic and carcinogenic action of TCDD in mammals, little information has been made available for fish (Berends *et al.* 1997, Buchmann *et al.* 1993, Clemons *et al.* 1994, Firestone *et al.* 1996, Frakes, *et al.* 1993, Henry *et al.* 1997, Mizell and Romig 1997, Sijm *et al.* 1993, van der Weiden *et al.* 1993).

In order to study the interaction of TCDD-induced cytotoxicity, induction of CYP1A as well as induction of DNA strand breaks, the response of primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*) to *in vitro* exposure to TCDD was investigated. Changes in CYP1A contents and activity were measured by means of immunoblotting and biochemical determination of 7-ethoxyresorufin-O-deethylase (EROD) activity. For detection of DNA damage, single and double strand breaks were recorded using the single cell gel electrophoresis (comet-assay; Devaux *et al.* 1997, Fairbairn *et al.* 1995, McKelvey-Martin *et al.* 1993, Singh *et al.* 1988).

Material and Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing 200 to 400 g were purchased from a commercial fish hatchery (Wellheim, Germany) and kept at 14 °C in a 600 l continuous flow through system (daily exchange rate: 4×) with a dark/light cycle of 12/12 h. The animals were fed daily with commercially available trout diet.

Isolation of hepatocytes

Perfusion of liver and isolation of hepatocytes were performed according to Braunbeck and Storch (1992). In brief, fish were anaesthetized with ethyl-4-aminobenzoate (Sigma, Deisenhofen, Germany) and superficially disinfected with 70 % ethanol. Abdominal and heart cavities were exposed, and a 21-gauge butterfly canula (o.d. 0.8 mm, i.d. 0.6 mm; Braun, Melsungen, Germany) was inserted into the major tributary to the hepatic portal vein. The liver was cleared of blood by *in situ* preperfusion with well-aerated Ca^{2+}- and Mg^{2+}-free PBS-medium (136.9 mM NaCl, 2.7 mM KCl, 23.8 mM $NaHCO_3$, 6.5 mM KH_2PO_4, 2 mM EDTA) adjusted to pH 7.5. Preperfusion was carried out for 3–5 min at room temperature at an initial flow rate of 3 ml min^{-1}. During preperfusion, the liver was removed from the body and transferred to a perfusion apparatus, and perfusion medium was changed to MEM-Hanks (Sigma, 20 mM HEPES). Digestion of connective tissues was accomplished by addition of 0.25 mg ml^{-1} collagenase H from *Clostridium histolyticum* (Clostridiopeptidase A, 150 mU mg^{-1}, Boehringer, Mannheim, Germany) for 15 min at room temperature at a flow rate of 3 ml min^{-1} and subsequently for 15 min at a flow rate of 4 ml min^{-1} with recirculating medium. Liver cells were dissociated from the hepatic stroma mechanically and suspended by two pairs of forceps. The crude cell suspension was filtered through 250 and 70 µm gauzes into 50 ml sterile centrifuge tubes (Greiner, Frickenhausen, Germany) and centrifuged for 7 min at 110 g (14 °C). After resuspension in collagenase-free MEM-Hanks medium, cells were washed twice and taken up in MEM-Hanks medium supplemented with a 1 % mixture of penicillin (10,000 IU, 6 mg ml^{-1})/streptomycin sulphate (12.6 mg ml^{-1}; Sigma), 4 % fetal calf serum (Sigma) and 20 mM HEPES (Sigma). Cell density was adjusted to 1×10^6 cells ml^{-1}; viability was estimated by exclusion of 0.18 % trypan blue in saline (Patterson 1979). Two ml of the cell suspension per well were plated in 24-well plates (Greiner). Hepatocytes were cultured at 14 °C.

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

TCDD was purchased from Alexis (Grünfeld, Germany) as a solution of 10 µg in 1 ml toluene. A 2 nM stock solution of TCDD was prepared in MEM containing 28 mg l^{-1} toluene and 0.5 % DMSO as solvents. Twenty-four hours after isolation, hepatocytes were incubated with 0.01 nM, 0.1 nM and 1 nM TCDD for 24 h, 72 h and 120 h (6 wells per treatment). Samples for detection of genotoxicity were taken after 1, 24, 72 and 120 hours. Controls were incubated with medium only or medium containing the solvents (28 mg l^{-1} toluene and 0.5 % DMSO). The medium was changed every other day by replacing 50 % of the medium. After the incubation period, hepatocytes were harvested and transferred to homogenization buffer (2 M sucrose, 20 mM MOPS, pH 7.4, 10 mM /1 % EDTA/ethanol, 0.1 M phenylmethylsulphonylfluoride in isopropanol, 130 mg l^{-1} ε-amino caproic acid, 0.3 M mercaptoethanol, 670 mg l^{-1} dithiothreitol). The suspension was centrifuged at 400 g. The supernatant was discarded, 50 µl of fresh homogenization buffer were added, and samples were frozen in liquid nitrogen and stored at –80 °C until further processing.

Electron microscopy

Cytotoxic changes of hepatocytes were investigated at an ultrastructural level. For this purpose, cells were seeded and cultured on Thermanox™ coverslips (Nunc, Wiesbaden, Germany) in 24-well plates. Hepatocytes were fixed with ice-cold 2.5 % glutardialdehyde solution in 0.1 M cacodylate buffer (pH 7.6) containing 4 % polyvinylpyrrolidone (Merck, Darmstadt, Germany) and 0.05 % $CaCl_2$ (Merck) for at least 1 hour at 4 °C. After repeated rinsing in cacodylate and Teorell-Stenhagen buffers (TS buffer; Le-Hir *et al.* 1979), pH 10.0, cells were incubated with 5 mM 3,3´-diaminobenzidine (DAB) in 10 mM TS buffer and 0.5 % H_2O_2 as a substrate at 37 °C for 60 min. Hepatocytes were rinsed again three times each with TS and cacodylate buffers and postfixed with 1 % osmium ferrocyanide (1:1 mixture of 2 % aqueous OsO_4 and 3 % $K_4[Fe(CN)_6]$; Karnovsky 1971) for 60 min at 4 °C. After osmication, samples were rinsed in cacodylate and 0.05 M maleate buffers three times each. *En bloc*-staining was performed by incubation with 1 % uranyl acetate in maleate buffer overnight. The following day, cells were dehydrated in a graded series of ethanol (75, 85, 95 and 100 % three times each for 10 min) and infiltrated with Spurr's medium (Spurr 1969). For polymerization, Thermanox coverslips were removed from the culture wells and placed upside down on a beem capsule completely filled with Spurr´s medium. After polymerization, coverslips were removed. Ultrathin sections of 40–70 nm thickness were cut on a Reichert OM-U 2 ultramicrotome. Sections were stained with lead citrate (Reynolds 1963) for 2 min, and at least six grids per treatment were evaluated in a Zeiss EM 10 electron microscope.

Biochemistry

7-Ethoxyresorufin-O-deethylase (EROD) activity was measured in triplicate with a spectrofluorometer (Perkin Elmer, Überlingen, Germany). In brief, 100 µl 50 µM 7-ethoxyresorufin solution in methanol and 20 µl of cell homogenate were added to 1870 µl phosphate buffer (80 mM

Na_2HPO_4, 20 mM KH_2PO_4, 11.18 g l^{-1} KCl, pH 7.4). The reaction was started by adding 10 µl 8 mg ml^{-1} NADPH solution in phosphate buffer. The increase in emission at 586 nM due to resorufin formation was recorded.

Immunoblotting

Monoclonal antibodies specific of cytochrome P450 1A (CYP1A) from rainbow trout were raised in mouse myeloma cells in collaboration with the Environmental Research Centre (Leipzig, Germany), the University of Leipzig and the Max Delbrück Centre (Berlin, Germany; Scholz *et al.* 1997). Cells were sonicated, and protein contents were determined according to Bradford (1976). A homogenate equivalent to 15 µg protein was mixed 1:1 with SDS sample buffer (200 mM Tris, pH 6.8; 40 % glycerine; 0.3 % dithiotreitol; 4 % lauryl sulphate; 0.025 % bromphenol blue) and boiled for 10 min. SDS-PAGE was performed according to Laemmli (1970) using a 3.8 % stacking gel and 10 % separating gel. Electrophoresis was performed at 15 mA per gel in the stacking phase and 25 mA per gel in the separating phase. After electrophoresis, gels and polyvinylidendifluoride (PVDF) membranes (Immobilon™, Serva, Heidelberg, Germany) were transferred to blotting buffer (20 mM Tris; 150 mM glycine; 20 % methanol) and blotting was performed in a semi-dry blotting chamber (Biorad, Munich, Germany) at 0.8 mA cm^{-2} for 40 min. Membranes were washed three times in 0.15 M PBS buffer (0.05 % Tween 20) for 10 min and then blocked with 5 % non-fat dry milk for 2 h. The membranes were incubated for 24 h with the first antibody, washed in 0.15 M PBS buffer and incubated for 24 h with a horseradish peroxidase-coupled anti-mouse secondary antibody. Membranes were stained with 1,4-chloronaphthol using hydrogen peroxide as a substrate. After drying, staining intensities of the bands were analysed by means of densitometry (E.A.S.Y.™ software; Herolab, Wiesloch, Germany). All blots were carried out in triplicate.

Genotoxicity assay

The comet assay was performed according to Singh *et al.* (1988). Five thousand viable hepatocytes per slide were embedded in 0.7 % low melting agar (FMC BioProducts, Rockland, USA) on frosted slides (Langenbrinck, Emmendingen, Germany) coated with two layers of 0.5 % and 1 % normal melting agar (FMC), respectively. Lysis of hepatocytes was accomplished by incubation (1 h at 4 °C) in 2.5 M NaCl supplemented with 100 mM EDTA, 10 mM Tris, 1 % sodium sarcosinate, 10 % DMSO and 1 % triton X–100 and adjusted to pH 10.0 with NaOH. Electrophoresis was performed in a horizontal electrophoresis chamber using 300 mM NaOH containing 1 mM EDTA as buffer. The alkaline environment causes a loss of hydrogen bonds in the DNA and therefore leads to single strand DNA fragments in the gels. To avoid unspecific strand breaks, electrophoresis was performed in the dark for 20 min at 300 mA. After electrophoresis, gels were washed three times with 0.4 M Tris (pH 7.5). Finally, DNA fragments were stained with ethidium bromide (20 µg/ml), and the lengths of comet tails of 50 cells per treatment were measured directly using a fluorescence microscope (green filter 510 B: 560 nm) equipped with automatic image analysis software Comet™ 3.0 (Kinetic Images, Liverpool, UK). The lengths of the comets were used as a direct indicator of the extent of DNA fragmentation (Devaux *et al.* 1997, McKelvey-Martin *et al.* 1993, Singh *et al.* 1988).

Results

Cytological alterations

Reaggregation of hepatocytes during the culture period indicated good viability. Control hepatocytes were characterized by centrally located nuclei surrounded by small stacks of rough endoplasmic reticulum (RER), some mitochondria and peroxisomes (Figures 1A, B). Smooth endoplasmic reticulum (SER) was present in only very small amounts close to the nucleus (Figure 1A). Extensive glycogen fields could consistently be found in the periphery of the hepatocytes. After 5 days in culture, a limited number of cells displayed glycogenosomes, i.e., a regular array of straight or circular cisternae of the endoplasmic reticulum alternating with rows of glycogen α-particles (Figure 1B); it should be noted that these cells appeared otherwise free of any symptoms of pathology. Ultrastructure of control hepatocytes thus closely resembled that of hepatocytes described in earlier studies (Braunbeck 1993, Braunbeck and Storch 1992).

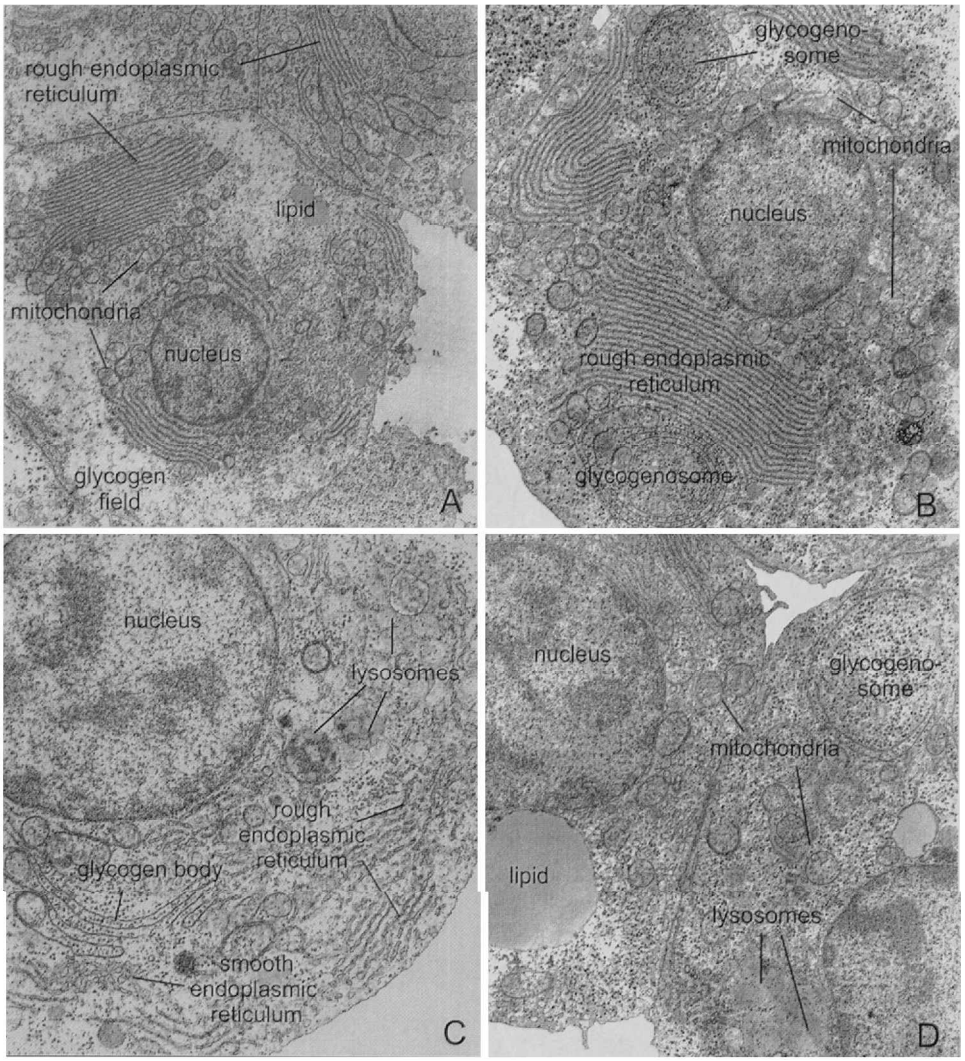

Figure 1. The ultrastructure of control isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes is characterized by a central nucleus surrounded by mitochondria, peroxisomes, stacks of rough endoplasmic reticulum and glycogen areas; lipid inclusions were scant (Figures 1A, B). If compared to hepatocytes kept for 3 d in culture (Figure 1A), no significant alterations can be recorded after 5 d in culture (Figure 1B). Hepatocytes exposed to 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 3 (Figure 1C) and 5 days (Figure 1D) showed only minor morphological changes comprising formation of glycogen bodies and a slight proliferation of smooth endoplasmic reticulum.

No effects were seen after either 3 day exposure to 0.01, 0.1 and 1 nM TCDD (Figure 1C) or 5 day exposure to 0.01 and 0.1 nM TCDD. In cells exposed to 1 nM TCDD for 5 days (Figure 1D), however, the rough endoplasmic reticulum showed a slight increase in volume and rate of vesiculation. Moreover, hepatocytes showed an increased accumulation of lipid and glycogenosomes, if compared to corresponding controls (Table 1). Ultrastructural features of hepatocytes exposed to TCDD thus indicate that there were no severe cytotoxic effects of TCDD even at the highest test concentration of 1 nM.

Table 1. Semiquantitative evaluation of ultrastructural changes in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) after exposure to 1 nm TCDD.

		0.01 nm		0.1 nm		1 nm TCDD	
	Control	3d	5d	3d	5d	3d	5d
Rough endoplasmic reticulum							
Vacuolation of cisternae	–	–	–	–	–	+	+
Fenestration of cisternae	±	±	±	±	±	–	–
Smooth endoplasmic reticulum							
Overall amount	±	±	±	±	±	+	+
Lysosomes							
Overall amount	+	+	+	+	+	++	++
Formation of glycogenosomes	+	+	+	+	+	++	++
Formation of glycogen bodies	±	±	±	±	±	+	+
Lipid							
Overall amount	–	–	–	–	–	+	+

Data are based on the inspection of at least 6 grids per treatment with approx. 6 sections per grid. Abbreviations: – = absent; ± = moderately developed; + = strongly developed; ++ = very strongly developed.

7-Ethoxyresorufin-O-deethylase activities

After 24 h of exposure (i.e., 48 h after isolation), control hepatocytes showed measurable EROD activities, which, however, decreased to unmeasurable levels after 4 days post-isolation (Figure 2). In the solvent control, the activity remained stable until the end of the experiment, however at very low levels (6.9–8.5 pmol resorufin min^{-1} mg^{-1} protein). After 1 day of exposure to TCDD, hepatocellular EROD activities were significantly increased over controls at all TCDD concentrations tested. The activities steadily rose with a positive dose-response relationship at all concentrations until day 3 of exposure and reached 23, 100 and 120 pmol min^{-1} mg^{-1} protein in cells exposed to 0.01, 0.1 and 1 nM TCDD, respectively (Figure 2). After 5 days, a further 10 % increase in activity could be recorded in hepatocytes exposed to 1 nM TCDD (131 pmol min^{-1} mg^{-1} protein), which, however, was statistically no more significant. In contrast, maximum induction of CYP1A activity was reached after exposure to 0.1 nM TCDD after 5 days (164 pmol min^{-1} mg^{-1} protein; Figure 2).

Cytochrome P450 1A (CYP1A) contents

Relative CYP1A contents as determined by immunoblotting demonstrated a time- and dose-dependent response (Figure 3). In contrast to the biochemical determination of CYP1A activities (Figure 2), no inhibitory effects of higher TCDD concentrations on CYP1A induction could be observed at the protein level (Figure 3). As early as 24 h of exposure, increased CYP1A contents could be shown in hepatocytes after exposure to all TCDD concentrations. Likewise, at all TCDD concentrations, relative amounts of CYP1A monotonously increased during the entire duration of the experiment.

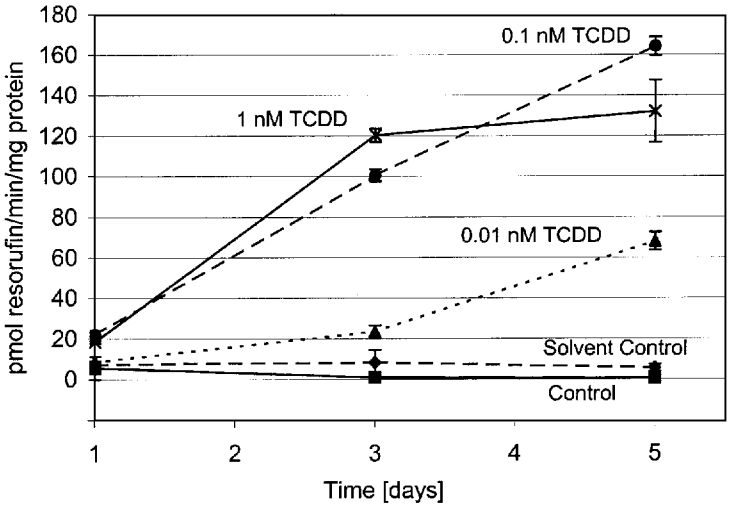

Figure 2. 7-Ethoxyresorufin-O-deethylase (EROD) activity in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Whereas the activity in controls slightly decreases, activities remain stable in the solvent controls and significantly increase after exposure to TCDD. After 5 d of exposure, highest activity is reached with 0.1 nM TCDD, whereas hepatocytes exposed to 1 nM TCDD reach a constant activity level after 3 days of exposure.

Genotoxicity

In the majority of control hepatocytes, comets did not exceed 10 μm in length (Figure 4). Due to isolation stress, however, both controls and solvent controls were not free of DNA damage as visualized by a number of cells displaying a tail in the comet assay: In 11 cells out of 50, tail lengths increased to at maximum 50 and 40 μm after 1 h and 5 d, respectively (Figure 4).

In contrast, after only 1 h of exposure to TCDD, a slight increase of tail length to > 60 μm could be recorded in hepatocellular nuclei at the highest concentration of 1 nM TCDD (Figure 4A).

A closer analysis of the extent of DNA damage by the comet length reveals that part of DNA damage observed in TCDD-exposed hepatocytes might be due to stress imposed by the combination of the solvents toluene and DMSO; however, damage was clearly increased, when TCDD was added (Figure 4).

After 72 hours of TCDD exposure, the number of undamaged cells was low even in the solvent control. The longest comets (tails), however, were always measured at the highest concentration of TCDD. After 120 hours, the number of damaged cells decreased, but the pattern of tail length in the exposures remained constant (Figure 4). The positive dose-response relationship in the induction of DNA fragmentation became most obvious, when the lengths of the comets of all individual cells analysed were summed up (Figure 5): Although the solvents accounted for the induction of some genotoxicity, increasing TCDD concentrations resulted in a dose-dependent increase of comet formation.

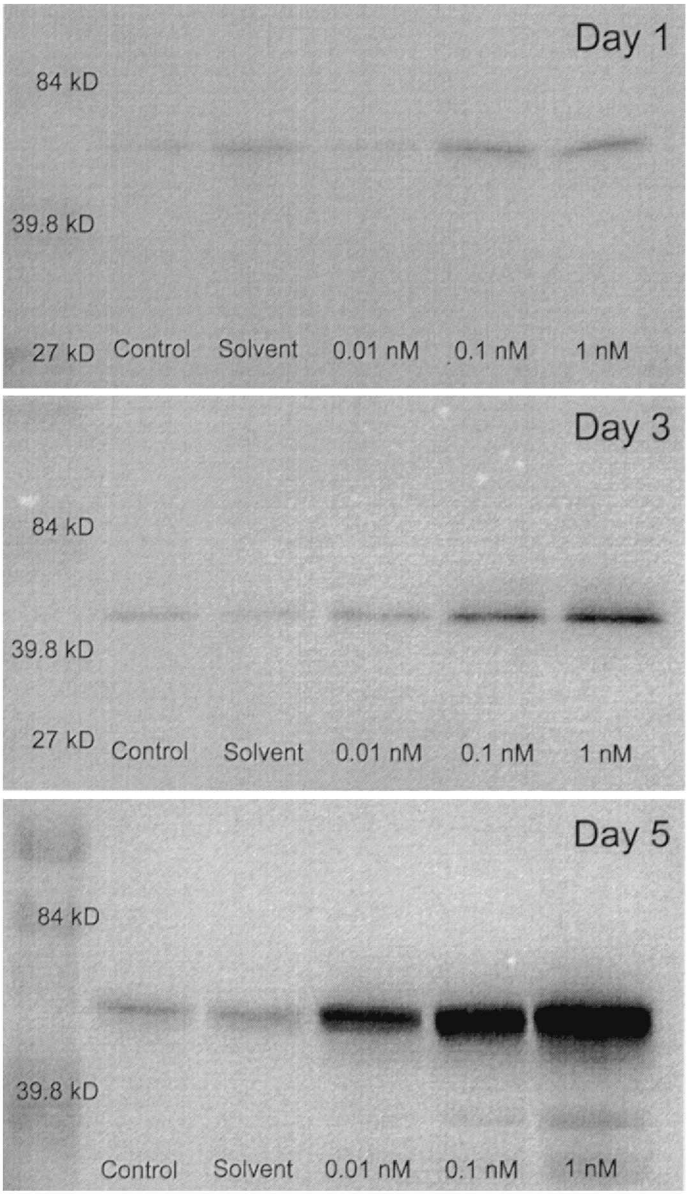

Figure 3. Induction of cytochrome P450 1A (CYP1A) contents in hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) by exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Data as determined by Western blotting using a monoclonal antibody raised against cytochrome P450 1A isolated and purified from rainbow trout (Scholz *et al.* 1997), followed by densitometrical analysis.

Discussion

The purpose of the present study was to investigate the correlation between the induction of cytochrome P450 1A (CYP1A) and DNA fragmentation as a mechanism of genotoxicity, which can be visualized by the comet assay (Devaux *et al.* 1997, Fairbairn *et al.* 1995, McKelvey-Martin *et al.* 1993, Singh *et al.* 1988).

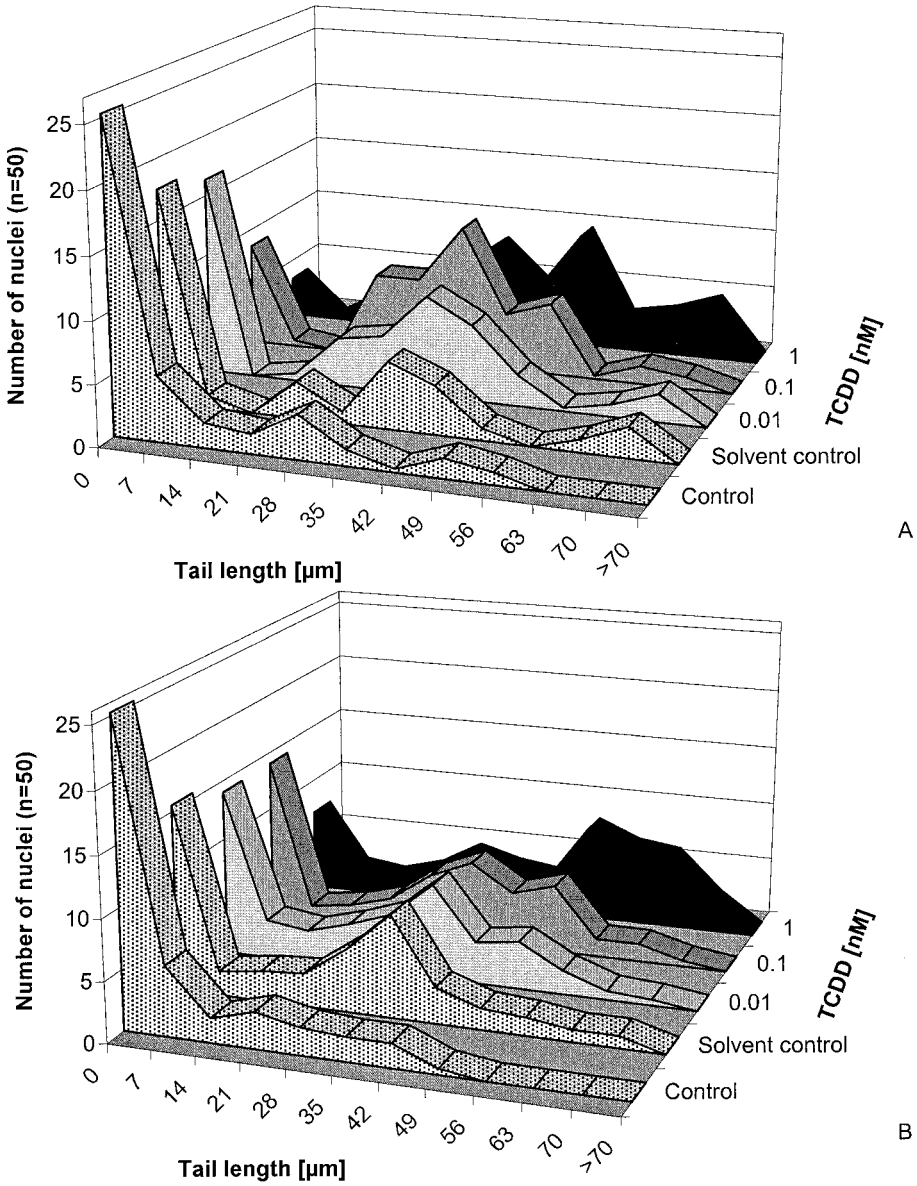

Figure 4. Distribution of comet lengths in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as revealed by single-cell gel electrophoresis after the comet assay. (A) After 1 h exposure to TCDD, a gradual increase in tail length can be observed with increasing TCDD concentrations, and the number of undamaged nuclei decreases in a dose-dependent fashion. (B) After TCDD exposure for 120 h, the shift in tail length is more pronounced than after a 1 h exposure.

Results document an increase of both CYP1A contents after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in parallel to dose-dependent DNA damage in isolated rainbow trout hepatocytes. DNA damage seems to be highly selective, since almost no cytotoxic effects could be shown at the ultrastructural level, a method which has repeatedly been demonstrated to be extremely sensitive for

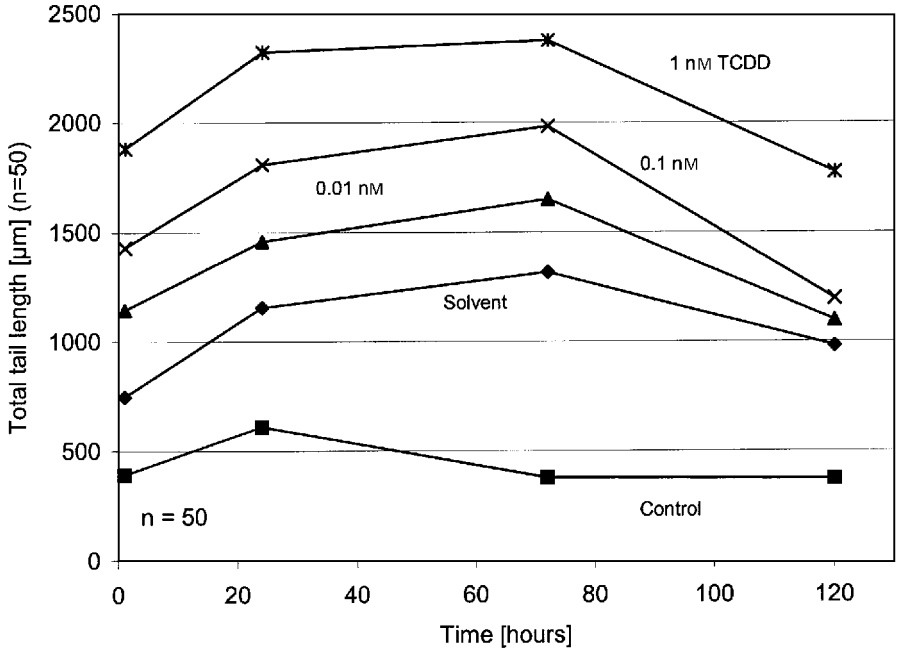

Figure 5. Sum of tail lengths from comets in all rainbow trout (*Oncorhynchus mykiss*) hepatocytes measured per TCDD concentration. Total tail length is smallest in controls and increases in a consistently dose-dependent fashion with increasing concentrations of TCDD.

sublethal effects of chemicals (Braunbeck 1998). Whereas CYP1A contents as determined by immunoblotting increased monotonously with dose and time, EROD activities reached maximum values after 120 h in hepatocytes exposed to 0.1 nM TCDD. Results thus corroborate observations by Pesonen and Andersson (1991) and Miranda *et al.* (1993), who found highest EROD activities in primary rainbow trout hepatocytes and in a liver cell line derived from zebrafish (*Danio rerio*) exposed to 0.1 nM TCDD.

So far, there are no data to support the assumption that poor metabolism of TCDD results in elevated intracellular TCDD doses, which would act as direct inhibitor of EROD activity as speculated by Hahn *et al.* (1996). Rather, the synthesis of the haem component in CYP1A is effected from δ-aminolevulinic acid by mitochondrial δ-aminolevulinic acid synthetase *via* uroporphyrinogen (Poland and Kende 1976). In chick embryo hepatocytes, uroporphyrin is formed from uroporphyrinogen (Lambrecht *et al.* 1988); following exposure to TCDD, EROD activities followed a maximum curve, whereas uroporphyrin accumulation consistently increased with TCDD concentrations (for corresponding results in mammals, see De-Matteis and Marks 1996). Likewise, in the fish hepatoma cell line PLHC-1, TCDD-induced uroporphyrin accumulation in parallel to EROD decrease occurred only if the medium was supplemented with δ-aminolevulinic acid (Hahn and Chandran 1996). In contrast, α-naphthoflavone, an inhibitor of CYP1A induction, suppressed uroporphyrin accumulation (Hahn and Chandran 1996). Thus, formation of uroporphyrin might result in a lack of the haem component precursor uroporphyrinogen. As a consequence, the shortage of haem

would lead to a decline in functionally active CYP1A. Detectability of CYP1A protein components by Western blot is, therefore, not a contradiction to the failure to detect EROD activities. Experiments have been initiated to elucidate whether TCDD is capable of inducing uroporphyrin accumulation in isolated rainbow trout hepatocytes as well.

Finally, TCDD has been shown to release iron from liver microsomes from rat (Wahba *et al.* 1988b). Limited iron availability within microsomes may thus be a further reason for the decline in functionally active CYP1A, i.e., EROD activity. On the other hand, prooxidative ferrous ions released from microsomes put oxidative stress on cells as demonstrated by higher lipid peroxidation rates (Al Bayati and Stohs 1987, Al Bayati *et al.* 1987, Kukielka and Cederbaum 1994). Since accumulation of iron in the nucleus has repeatedly been demonstrated (for references, see Cable *et al.* 1998 as well as Hu and Shih 1997), increased oxidative pressure on DNA might be directly related to reduced incorporation of iron into the haem component of CYP1A.

It is assumed that TCDD induces carcinogenesis *via* a promoter-mediated reaction and, hence, is not a directly acting carcinogen (Schulte-Hermann *et al.* 1995). Induction of CYP1A leads to increased metabolism of endogenous substrates; TCDD itself is only poorly metabolized by CYP1A (Okey 1990, Park *et al.* 1996). One possible side reaction of CYP1A may be the production of free superoxide radical anions (Poulos and Ragg 1992). In peritoneal lavage cells of rat, e.g., TCDD exposure results in accumulation of superoxide anions (Alsharif *et al.* 1994). As shown by Stohs (1990), superoxide radical anions may readily interact with cellular macromolecules including DNA and lead to elevated rates of lipid peroxidation (alTurk *et al.* 1988, Braunbeck and Neumüller 1996, Wahba *et al.* 1989a, b). Reactive oxygen species and various oxyradicals have repeatedly been shown to result in unspecific interaction with DNA leading to positive results in the comet assay (Braunbeck and Neumüller 1996, Fairbairn *et al.* 1995, McKelvey-Martin *et al.* 1993). In the hepatoma cell line Hepa1c1c7, superoxide radical anions cause the release of 8-oxoguanine as a specific marker of oxidative DNA damage (Park *et al.* 1996); α-naphthoflavone reduced the excretion rate of 8-oxoguanine. In mammals, DNA damage can therefore directly be related to the presence of CYP1A and EROD activity.

The less pronounced DNA damage after 120 h TCDD exposure is remarkable, since, to date it remains unclear to what extent, if at all, cultured fish hepatocytes are able to repair damaged DNA, especially single strand breaks (Ishikawa *et al.* 1984, Walton *et al.* 1983, 1987). Hepatocytes of fish usually react to prolonged xenobiotic exposure with adaptive mechanisms (Braunbeck 1998, Hofer and Lackner 1995). The nature of such defence mechanisms designed to protect hepatocytes against, e.g., elevated oxidative stress is still widely unclear. If fish cells are unable to repair DNA single strand breaks, an alternative effective defense mechanism has to be directly based on lowering oxidative stress. The most common way of elimination of superoxide radical anions is their direct breakdown by cytosolic superoxide dismutase. Induction of superoxide dismutase activity was observed in white muscle tissues of rainbow trout after treatment with 3,3′,4,4′-tetrachlorobiphenyl in concentrations which also induced EROD activity in the liver (Otto and Moon 1995). It might thus be hypothesised that a superoxide-mediated TCDD-dependent induction of superoxide dismutase might lead to elevated tolerance against oxygen radicals.

In conclusion, the present communication provides data to support a hypothesis, which functionally links apparently isolated phenomena of TCDD exposure such as genotoxicity and induction/suppression of CYP1A/EROD. Further research is required to elucidate the connection between TCDD, CYP1A synthesis and activity, cellular iron regulation, the generation of reactive oxygen species by CYP1A-mediated metabolism as well as DNA damage and repair.

Acknowledgments

The authors wish to express their thanks to Mrs G. Adam for excellent assistance in graphical reproduction, as well as to Mr A. Schnurstein for support in the interpretation of the comet assay results. This study was supported by the Federal Ministry for Education, Science and Technology of the Federal Republic of Germany under contract no. AZ 0310742 and the County of Baden-Württemberg under contract no. AZ II – 7532.25-1-12/1.

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