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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induced cytochrome P450s alter the formation of reactive oxygen species in liver cells

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was classified by the International Agency for Research on Cancer as a carcinogen in humans. It acts through an aryl hydrocarbon receptor-mediated mechanism, inducing the transcription of numerous genes, including various cytochrome P450s (CYPs – CYP1A1, 1A2, 1B1). Induction of CYPs may lead to genotoxicity by generating reactive oxygen species (ROS) which can damage DNA directly and/or *via* the generation of reactive metabolites. We determined ROS formation with the 2′,7′-dihydrodichlorofluorescein diacetate fluorescence assay after incubation of HepG2 hepatoma cells or primary rat hepatocytes with TCDD. The amount of 8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA was measured using HPLC-MS/MS, the amount of CYP1A1 protein by Western blotting. The catalytic activity of CYP1A enzymes was determined as 7-ethoxyresorufin-*O*-deethylase (EROD) activity. Incubation of cells with TCDD for 48 h caused increased levels of ROS in primary rat hepatocytes as well as increased levels of 8-oxo-dG in DNA compared to untreated cells. In the HepG2 cell line no significant effects were observed for both ROS formation and 8-oxo-dG levels. Both effects were in good agreement with the extent of induction of CYP1A1 protein and EROD activity, suggesting that CYP1 induction is a major source of ROS formation in TCDD-treated hepatocytes.

Keywords: Cytochrome P450 / DNA-damage / Reactive oxygen species / 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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1 Introduction

Cytochrome P450s (CYP) constitute a superfamily of enzymes crucial for the oxidative, peroxidative, and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes and xenobiotics, including many therapeutic drugs and environmental pollutants [1]. Three genes, *CYP1A1*, *CYP1A2*, and *CYP1B1*, are members of the CYP1 family [1]. All of them share the main features of regulation; they are all transcriptionally controlled by the aryl hydrocarbon receptor – aryl hydrocarbon receptor nuclear translocator (AhR-ARNT) pathway [2]. The cytoplasmic AhR is a member of the basic-helix-loop-helix PAS (Per/ARNT/Sim) family of nuclear transcription factors. Upon ligand activation it forms a heterodimer with the ARNT protein and activates transcription of certain genes including *CYP1* genes by binding to xenobiotic-responsive elements (XRE) located in the 5′-flanking region of those genes [3, 4].

In 1997, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) [5]. The environmental pollutant TCDD, one of the most potent AhR agonists, acts through an AhR-mediated mechanism [6]. Park *et al.* [7] suggested that TCDD may be indirectly genotoxic in mouse hepatoma cells *via* generation of reactive oxygen species (ROS) by inducing CYP1 enzymes. However, the authors did not measure DNA damage but the excretion of 8-oxo-guanine into the medium. Carcinogenic effects of TCDD may be caused, in part, by the capacity of the induced CYP enzymes to leak oxidants and thus promote the formation of ROS and oxidative DNA damage. A postu-

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Abbreviations: AhR, aryl hydrocarbon receptor; **ARNT**, aryl hydrocarbon receptor nuclear translocator; **CYP**, cytochrome P450; **DCF**, dichlorofluoresceine; **EROD**, 7-ethoxyresorufin-*O*-deethylase; **H_2DCFDA**, 2′,7′-dihydrodichlorofluorescein diacetate; **8-oxo-dG**, 8-oxo-2′-deoxyguanosine; **ROS**, reactive oxygen species; **TCDD**, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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lated mechanism of production of activated oxygen is the autoxidation of the oxycytochrome P450 complex, generating superoxide [8, 9]. The superoxide anion can dismutate and, as a by-product, generate hydrogen peroxide. If transition metals are present to catalyze the one-electron reduction of hydrogen peroxide, hydroxyl radicals will be produced, leading to cellular damage of biomolecules [10, 11]. DNA damage can result from direct interaction with ROS or from generation of reactive metabolites from endogenous compounds, such as estradiol [11, 12]. Such a mechanism could be relevant for the finding that TCDD treatment leads to increased oxidative DNA damage in female but not in male rats [11]. In fact, enhanced ROS formation has been shown in liver microsomes isolated from rats treated with PCB 77 [13]. However, such an effect has never been shown in isolated hepatocytes in combination with the analysis of oxidative DNA damage.

We determined the formation of ROS with 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) after incubation of HepG2 human hepatoma cells or primary rat hepatocytes with TCDD compared to untreated cells. Additionally, the amount of 8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA, a hallmark of oxidative DNA damage, was measured after incubation of HepG2 cells or primary rat hepatocytes with TCDD using HPLC-MS/MS.

2 Materials and methods

2.1 Materials

All chemicals were of reagent grade and were purchased from commercial suppliers. TCDD was from Promochem (Wesel, Germany), H_2DCFDA from Molecular Probes (Invitrogen, Karlsruhe, Germany). DMEM, fetal bovine serum, and penicillin/streptomycin were from PAA (Cölbe, Germany). The polyclonal anti CYP1A1 antibody (sc-20772) and the secondary goat antirabbit antibody (sc-2004) were from Santa Cruz (Heidelberg, Germany). HepG2 hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2 Cell culture and treatment

Wistar rats were obtained from Charles River (Sulzfeld, Germany), and were kept under standard conditions. Adult animals at a body weight of 150–180 g were anesthetized, and hepatocytes were prepared as previously described [14]. Viabilities of hepatocytes were >90% as determined by trypan blue exclusion. Cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Hepatocytes were seeded at a density of 60 000 cells *per* well for the H_2DCFDA assay on collagen-coated 24-well plates, or at a density of 6 million cells *per* plate on collagen-coated 90 mm petri dishes for quantification of 8-oxo-dG, Western blotting, and measurement of EROD activity. Cells were washed and incubated 2 h after seeding with 1 nM TCDD and incubated for 48 h under standard conditions.

The human hepatoma cell line HepG2 was seeded in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at a density of 30 000 cells *per* well for the H_2DCFDA assay on collagen-coated 48-well plates or at a density of 600 000 cells *per* plate on 90 mm petri dishes for quantification of 8-oxo-dG, Western blotting, and measurement of EROD activity. Cells were washed and incubated 24 h after seeding with 1 nM TCDD and incubated for 48 h under standard conditions.

2.3 H_2DCFDA assay

Chemically reduced and acetylated forms of 2′,7′-dichlorofluorescein (DCF) are nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Oxidation of these probes can be detected by monitoring the increase in fluorescence with a microplate reader [15].

Forty-eight hours after incubation, the cell cultures were washed with PBS and incubated with 100 µM H_2DCFDA. Fluorescence was determined at 5-min intervals for 30 min at 485/538 nm in a microplate reader (Fluoroskan Ascent FL; Labsystems, Frankfurt, Germany).

2.4 Quantification of 8-oxo-dG

2.4.1 DNA isolation

Forty-eight hours after incubation, cell cultures were washed with PBS. Isolation of genomic DNA was performed using the high salt method [16]. For DNA hydrolysis, solutions containing 100 µg of DNA in 100 µL of water were incubated with 10 U nuclease P1 (Sigma, Steinheim, Germany) in a sodium acetate/zinc chloride buffer, pH 4.8, for 60 min at 37°C. Tris buffer (100 mM; pH 8.0) and 7.5 U alkaline phosphatase from calf intestine (Roche, Mannheim, Germany) were then added for an additional 30 min of incubation at 37°C. Proteins were separated by centrifugation for 15 min at 10 000 × *g* through Amicon Ultrafree®-filters (Millipore, Eschborn, Germany). The resulting hydrolyzed DNA solution was injected into the HPLC-MS/MS system.

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2.4.2 HPLC-MS/MS

The HPLC-MS/MS system consisted of an 1100 LC binary pump (Agilent, Waldbronn, Germany) and autosampler (Agilent 110 Autosampler) with an API 3000 mass spectrometer (Applied Biosystems, Darmstadt, Germany). HPLC separations were achieved using a AQ 12S051502 QT, 150 × 2.1 mm column (YMC Europe, Schermbeck, Germany). The samples were separated by gradient elution with 10 mM ammonium acetate pH 4.3 (solvent A) and methanol (solvent B) using the following conditions: 98% A for 5 min, followed by a linear increase to 85% A within 10 min, followed by a constant 85% A for 10 min at a flow rate of 300 µL/min. 8-oxo-dG was detected in the positive-ion mode at a vaporizer temperature of 400°C and a TurboIon®Spray voltage of +4.0 kV. Spectral data were recorded with N_2 (CAD = 4) as collision gas, a declustering potential of 26 V, and a collision energy of 19 V. Data acquisition was performed in MRM mode monitoring the transition of *m/z* 284–168 and *m/z* 284–140 for 8-oxo-dG. Transitions for unmodified 2′-deoxyguanosine (*m/z* 268–152) were also monitored. Linear HPLC-MS/MS calibration curves with external 8-oxo-dG standard were obtained over the range 0.1–10 pg/µL.

2.5 7-Ethoxyresorufin-*O*-deethylase (EROD) assay

Analysis of EROD activity and protein content was measured in well plate format as described by Kennedy and Jones [17, 18] using a Fluoroskan fluorescence plate reader with an excitation wavelength of 544 nm and an emission wavelength of 590 nm for resorufin and 390 nm excitation to 460 nm emission for fluorescamin.

2.6 Western blotting

For the preparation of total cell homogenates, cell cultures were washed with ice-cold saline, and the cells were harvested by scraping off in saline. After centrifugation at 3000 × *g* for 10 min the pellet was resuspended in homogenization buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2.6H_2O$, 0.05% NaN_3, 0.1% DTT, and 0.1% PMSF) and homogenized with a sonifier for 10 s. Twenty micrograms protein of each sample was fractionated electrophoretically on a 10% SDS-gel. The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Billerica, USA) using the semidry blotting method. After blocking overnight at 4°C in 5% milk powder dissolved in Tris-buffered saline with 0.1% Tween 20 (TBS-T), the membrane was washed with TBS-T, probed with anti-CYP1A1 antibodies for 120 min at room temperature, and, after washing, incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The resulting complexes were visualized using the enhanced chemiluminescence detection method according to the manufacturer (Western Lightning Kit, Perkin-Elmer, Munich, Germany) measured with a LumiImager™. To estimate the level of CYP1A1 protein a densitometric analysis was performed using the LumiImager's quantification software.

2.7 Statistical analysis

Statistical analysis was performed using the GraphPad InStat Version 3.00 for Windows. Results are shown as mean + SD from at least three independent experiments. Statistical significance of the difference between the control and the experimental group was determined by paired *t*-test/one-tailed *p* value, one asterisk indicating significant differences from the control group.

3 Results

3.1 H_2DCFDA assay

For the H_2DCFDA assay the human hepatoma cell line HepG2 and primary rat hepatocytes were incubated with 1 nM TCDD for 48 h under standard conditions. Incubation of cells with TCDD caused statistically significant (paired *t*-test; one-tailed *p* value) increased levels of ROS in primary rat hepatocytes, measured as increase in DCF-mediated fluorescence. Compared to untreated cells, DCF-mediated fluorescence was more than five-fold higher in TCDD-treated cells. In contrast, no increased levels of ROS were observed after treatment of HepG2 cells with TCDD (Fig. 1).

3.2 Quantification of 8-oxo-dG

For quantification of 8-oxo-dG, a hallmark of oxidative DNA damage, the human hepatoma cell line HepG2, and primary rat hepatocytes were incubated with 1 nM TCDD for 48 h under standard conditions. Incubation of cells with TCDD caused statistically significant (paired *t*-test; one-tailed *p* value) increases in 8-oxo-dG levels in genomic DNA of primary rat hepatocytes compared to untreated cells. In TCDD-treated primary rat hepatocytes the amount of 8-oxo-dG *per* 10^6 dG (mean ± SD) was 19.37 ± 5.87, in untreated cells 11.66 ± 4.52. In the HepG2 cell line the levels were also increased after TCDD treatment (6.12 ± 3.83 8-oxo-dG *per* 10^6 dG in TCDD-treated cells *vs.* 3.47 ± 2.25 in untreated cells). However, these results did not reach statistical significance (Fig. 2).

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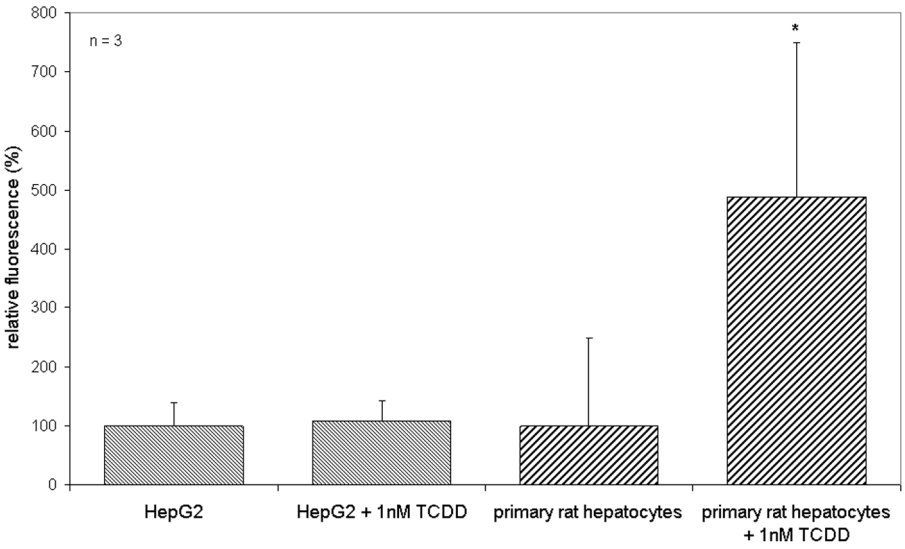

Figure 1. Effects of 1 nM TCDD on the formation of ROS, measured as increase in oxidized DCF in HepG2 cells and in primary rat hepatocytes. Cells were incubated with TCDD over a period of 48 h. TCDD was dissolved in DMSO. Bars represent mean + SD from $n = 3$ independent experiments; the asterisk indicates significant difference (paired *t*-test; one-tailed *p* value) from the vehicle-treated control.

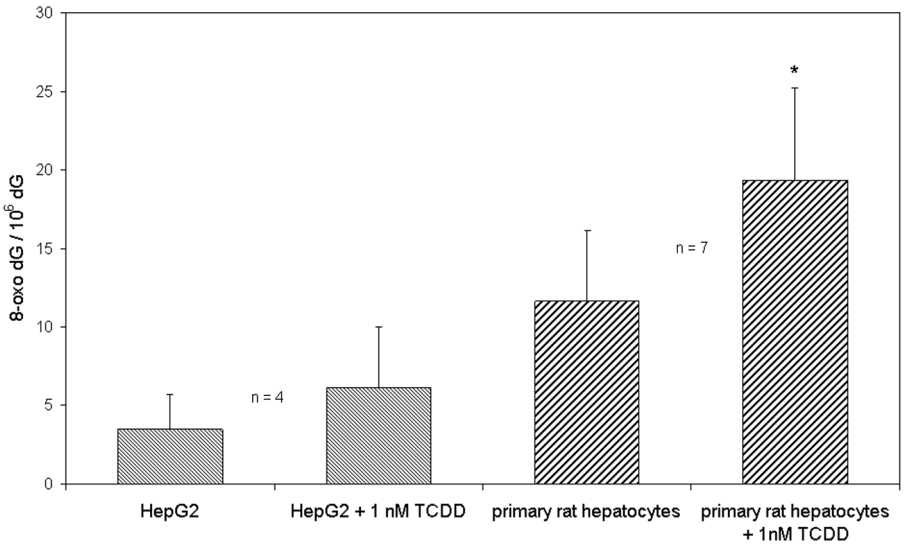

Figure 2. Effects of 1 nM TCDD on the formation of 8–oxo-dG, a hallmark of oxidative DNA damage, in HepG2 cells and in primary rat hepatocytes. Cells were incubated with TCDD over a period of 48 h. TCDD was dissolved in DMSO. Bars represent mean + SD from $n = 4$ and $n = 7$ independent experiments, respectively; the asterisk indicates significant difference (paired *t*-test; one-tailed *p* value) from the vehicle-treated control.

3.3 EROD assay

In addition, we determined the catalytic activity of CYP1A enzymes in HepG2 cells and primary rat hepatocytes. Our aim was to check if increased levels of ROS or 8-oxo-dG, in TCDD-treated samples, correlate with an increased enzymatic activity of CYP1A, measured as EROD activity. In TCDD-treated primary rat hepatocytes EROD activity (mean ± SD) was 329.9 ± 70.5 pmol · $min^{-1}mg^{-1}$ protein, in untreated cells 0.07 ± 0.52 pmol · $min^{-1}mg^{-1}$, protein and in vehicle control (DMSO) 1.3 ± 0.78 pmol · $min^{-1}mg^{-1}$ protein. In the HepG2 cell line CYP1A activity was also induced but to a much lower extent than in primary rat hepatocytes. EROD activity in TCDD-treated HepG2 cells was 26.6 ± 1.2 pmol · $min^{-1}mg^{-1}$ protein (*vs.* 0.3 ± 0.1 pmol · $min^{-1}mg^{-1}$ protein in untreated cells and 0.4 ± 0.2 pmol · $min^{-1}mg^{-1}$ protein in vehicle controls) (Fig. 3).

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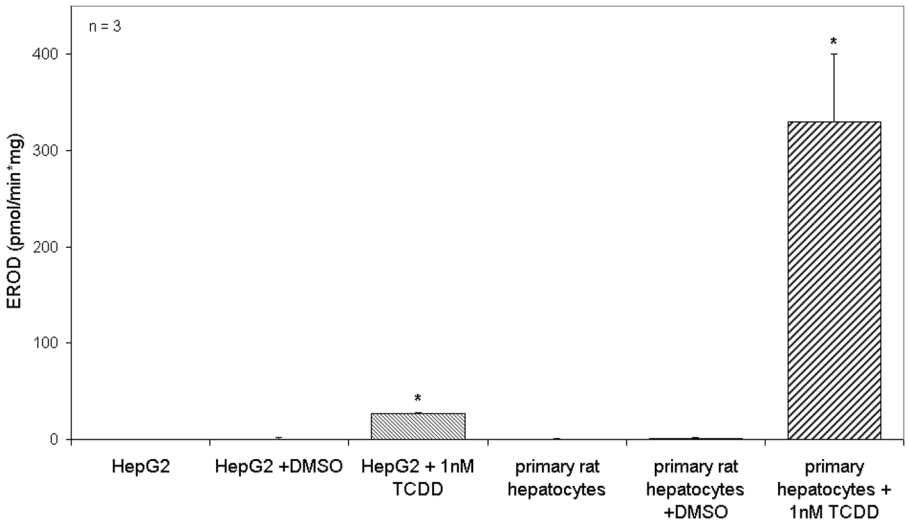

Figure 3. Effects of 1 nM TCDD on EROD activity in HepG2 cells and in primary rat hepatocytes. Cells were incubated with TCDD over a period of 48 h. TCDD was dissolved in DMSO. Bars represent mean + SD from $n = 3$ independent experiments; asterisks indicate significant difference (paired *t*-test; one-tailed *p* value) from the negative control.

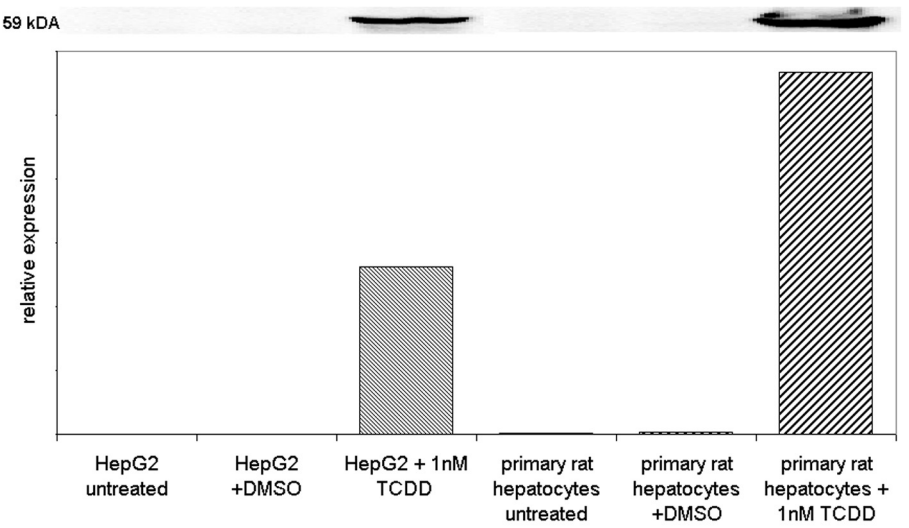

Figure 4. Representative Western blot analysis of the effects of TCDD on immunoreactive protein levels (using polyclonal anti-CYP1A1 antibody) in HepG2 cells and in primary rat hepatocytes. Cells were incubated with 1 nM TCDD, negative controls without vehicle (DMSO) or with DMSO only over a period of 48 h. Western blots were performed with 20 μg protein sample *per* lane.

3.4 Western blotting

Western blot analysis of the effects of TCDD on immunoreactive protein levels (using polyclonal anti-CYP1A1 antibody) in HepG2 cells and in primary rat hepatocytes was also investigated. Immunoreactive bands at approximately 59 kDa, indicating CYP1A1, were detected only in TCDD-treated HepG2 cells and TCDD-treated primary rat hepatocytes, not in untreated cells or vehicle controls. In the HepG2 cell line we detected lower intensity of immunoreactive CYP1A1 after densitometric analysis compared to primary rat hepatocytes. Figure 4 shows the results of one representative Western blot analysis.

4 Discussion

Liver and thyroid gland are the major target organs of the carcinogenicity of TCDD in rats. Primarily in female rats, TCDD treatment leads to strongly increased numbers of animals with benign and malignant liver tumors [19]. Furthermore, TCDD acts as a potent tumor promoter in rat

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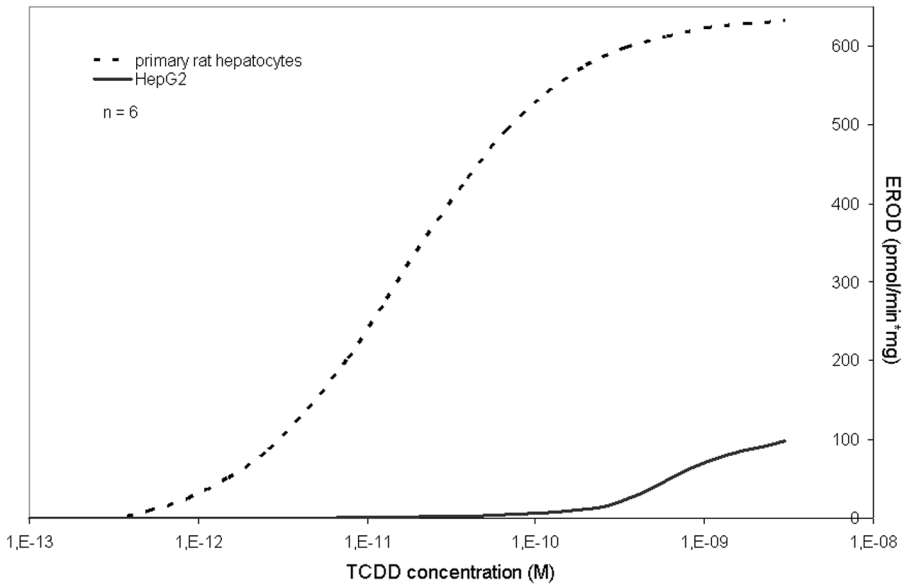

Figure 5. EROD-inducing effects of TCDD in HepG2 cells and primary rat hepatocytes after treatment over 48 h. Data were obtained from six independent experiments. Data were taken from Zeiger *et al.* 2001 [21].

liver [20]. Oxidative DNA damage resulting from TCDD treatment of female rats may play an important role in the development of liver cancer [11]. In particular, the formation of 8-oxo-dG, a hallmark of oxidative DNA damage, was found primarily in female rats and was strictly estrogen dependent. However, the available literature does not show a direct link between induction of CYP1 enzymes and the formation of 8-oxo-dG. In a study by Park *et al.* [7], an increased cellular release of 8-oxo-guanine was shown in TCDD-treated hepatoma cells. However, the interpretation of this finding seems to be difficult. A major conclusion from our study is that in fact the formation of 8-oxo-dG in hepatocellular DNA is related to the potency of TCDD as inducer of CYP1A enzymes in HepG2 cells and in primary rat hepatocytes. Induction of drug-metabolizing enzymes is generally considered as an adaptive but not adverse effect. Our results shed doubts on this general point of view suggesting that CYP1A induction should be considered as potentially adverse and genotoxic in hepatocytes.

Zeiger *et al.* [21] showed that primary rat hepatocytes are more sensitive toward the CYP1A-inducing potency of TCDD than HepG2 cells, which are of human origin [19]. Certainly, primary human hepatocytes would serve as a better comparison than a human hepatoma cell line. However, a lower efficacy of TCDD as an inducer of EROD activity was found earlier [22] in human hepatocytes when compared to rat hepatocytes (Fig. 5).

Our findings strongly suggest that the formation of 8-oxo-dG depends on the induction of CYP1A enzymes in liver cells, and may be mediated by ROS. Further work is needed to clarify the reasons for different effects of TCDD on ROS and 8-oxo-dG formation in HepG2 cells, and the meaning of these findings for human risk assessment of TCDD.

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