TCDD-induced CYP1A1 influences the formation of reactive oxygen species in liver cells

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TCDD-induced CYP1A1 influences the formation of reactive oxygen species in liver cells Stefanie Knerr 1, Johanna Schaefer 1, Angela Mally 2, Wolfgang Dekant 2, Dieter Schrenk 1 1 Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Kaiserslautern, Germany 2 Department of Toxicology, University of Wuerzburg, Wuerzburg, Germany Introduction 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) 1. TCDD acts through a mechanism involving the aryl hydrocarbon receptor (AhR), present in humans and animals 2. The activated AhR, a nuclear transcription factor, forms in the presence of TCDD an active heterodimer with the aromatic hydrocarbon nuclear translocator (ARNT) protein and induces (or suppresses) the transcription of numerous genes, including cytochrome P450 1A1 (CYP1A1) 3. It has been hypothesised that TCDD may be indirectly genotoxic via generation of reactive oxygen species (ROS) by inducing CYP1A enzymes. This may lead to DNA damage via direct interaction or via generation of reactive metabolites from endogenous compounds, such as estradiol 4, 5. We determined the formation of ROS with 2´,7´-dihydrodichlorfluorescein diacetate (H2DCFDA) after incubation of the human cell line HepG2 and primary rat hepatocytes with TCDD. Additionally, the amount of 8-oxo-2´-deoxyguanosine (8-oxo-dG), a marker for oxidative DNA damage, was measured after incubation of cells with TCDD using HPLC-MS/MS. Materials and Methods Cell culture: Wistar rats were obtained from Charles River (Sulzfeld, Germany), and were kept under standard conditions. Adult animals at body weight of 150–180 g were anesthetized, and hepatocytes were prepared as previously described 6. Hepatocytes were seeded at a density of 60,000 cells per well for the H2DCFDA-assay on collagen-coated 24-well plates, and at a density of 6 million cells per plate on collagen-coated 90 mm petri dishes for quantification of 8-oxo-dG. Cells were washed and incubated two hours after seeding with 10 nM TCDD and incubated for 48 hours under standard conditions. The human hepatoma cell line HepG2 was seeded at a density of 30,000 cells per well for the H2DCFDA-assay on collagen-coated 48-well plates and at a density of 600,000 cells per plate on 90 mm petri dishes for quantification of 8-oxo-dG. Cells were washed and incubated 24 hours after seeding with 10 nM TCDD and incubated for 48 hours under standard conditions. H2DCFDA-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 7. 48 hours after incubation, the cell cultures were washed with phosphate buffered saline and incubated with 100 µM H2DCFDA. Fluorescence was determined in 5 minute intervals for 30 minutes at 485/538 nm in a microplate reader (Fluoroskan Ascent FL; Labsystems). Quantification of 8-oxo dG: DNA isolation: 48 hours after incubation, cell cultures were washed with phosphate buffered saline. Isolation of genomic DNA was performed using the high salt method 8. DNA hydrolysis: Solutions containing 50 µg of DNA in 100 µl water were incubated with nuclease P1 (Calbiochem) in a sodium acetate/ zinc chloride buffer, pH 4.8, for 60 min at 37 °C. Tris buffer, pH 8.0, and alkaline phosphatase from calf intestine (Sigma) were then added for an additional 30 minutes of incubation at 37 °C. Proteins and eventually incompletely hydrolyzed DNA were separated by centrifugation for 15 minutes at 10000 g through Amicon Ultrafree®-filters (Millipore). The resulting hydrolyzed DNA nucleoside solution was injected into the HPLC-MS/MS system. HPLC-MS/MS: The HPLC-MS/MS system consisted of an Agilent 1100 LC binary pump and autosampler (Agilent 110 Autosampler) with an API 3000 mass spectrometer (Applied Biosystems). HPLC separations were achieved using a AQ 12S051502 QT, 150 x 2.1 mm column (YMC). The mobile phase consisted of 10 mM ammoniumacetate pH 4.3 and methanol. Electrospray ionization was carried out in positive ion mode using nitrogen as the nebulising gas. Linear HPLC-MS/MS calibration curves with external 8-oxo-dG standard were obtained over the range 0.1–10 pg/µl. For HPLC-MS/MS analysis in multiple reaction monitoring (MRM) mode transitions m/z = 284.1/186.3 (8-oxo-dG) were recorded. Transitions for unmodified 2´-deoxynucleosides were also monitored; dG (m/z = 268.1/152.1) Results and Discussion Figure 1. 8-oxo-dG levles and H2DCFDA mediated fluorescence of primary rat hepatocytes, treated with TCDD (10-8 M), compared to untreated control. Error bars represent standard deviation. Figure 2. 8-oxo-dG levles and H2DCFDA mediated fluorescence of HepG2 cells, treated with TCDD (10-8 M), compared to untreated control. Error bars represent standard deviation. Incubation of cells with TCDD (10 nM) 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 or much lower effects were observed for both the H2DCFDA assay and 8-oxo-dG levels. Possibly

the formation of ROS and 8-oxo-dG depends on the potency of TCDD as inducer of CYP1A enzymes in the HepG2 cell line and in primary rat hepatocytes. Zeiger et al. were able to show that primary rat hepatocytes are more than 20 times more sensitive towards TCDD in terms of CYP1A-mediated 7-ethoxyresorufin-O-deethylase (EROD) activity compared to HepG2 cells, which are of human origin 9. These findings suggest that the formation of ROS and 8-oxo-dG depends on the induction potency of CYP1A enzymes in the cells. However the effects did not reach statistical sinificance ($p \leq 0.05$). Additionally, these findings seem to support the hypothesis that increased levels of liver tumors, found in rats, after exposure to TCDD, are a consequence of increased ROS formation, resulting in DNA damage which eventually causes permanent mutations and cancer. Acknowledgements We are grateful to thank Mrs. Monika Groß for the technical assistence. References 1 IARC (International Agency for Research on Cancer). 1997. Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans. IARC Monogr Eval Carcinog Risks Hum 69. 2 Steenlan K, Bertazzi P, Baccarelli A, Kogevinas M. 2004. Dioxin revisited: Developments since the 1997 IARC classification of dioxin as a human carcinogen. Environmental Health Perspectives 112 (13): 1265-1268. 3 Whitlock P. 1999. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 39:103–125. 4 Wyde ME, Wong VA, Kim AH, Lucier GW, Walker NJ. 2001. Induction of hepatic 8-oxo-deoxyguanosine adducts by 2,3,7,8-tetrachlorodibenzo-p-dioxin in Sprague-Dawley rats is female-specific and estrogen-dependent. Chem Res Toxicol 14: 849-855. 5 Whitlock JP Jr, Chichester CH, Bedgood RM, Okino ST, Ko HP, Ma Q, Dong L, Li H, Clarke-Katzenberg R. 1997. Induction of drug-metabolizing enzymes by dioxin. Drug Metab Rev. 29 (4):1107-27. 6 Schrenk D, Karger A, Lipp HP, Bock KW. 1992. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and ethinylestradiol as co-mitogens in cultured rat hepatocytes. Carcinogenesis. 13(3):453-6. 7 Molecular Probes. 2004. Product information; reactive oxygen species (ROS) detection reagents. 8 http://www.riedlab.nci.nih.gov/publications/2442.2601_DNA_Prep_TC4_High_Salt.pdf Section of Cancer Genomics, Genetics Branch, NCI National Institutes of Health. 2004. DNA preparation from cell cines, high salt method. 9 Zeiger M, Haag R, Hockel J, Schrenk D, Schmitz HJ. 2001. Inducing effects of dioxin-like polychlorinated biphenyls on CYP1A in the human hepatoblastoma cell line HepG2, the rat hepatoma cell line H4IIE, and rat primary hepatocytes: comparison of relative potencies. Toxicol Sci. 2001 63(1):65-73.