Toxicology and Applied Pharmacology 262 (2012) 167–176

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice

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ARTICLE INFO

Article history:
Received 29 February 2012
Revised 25 April 2012
Accepted 26 April 2012
Available online 4 May 2012

Keywords:
2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDD
Gender differences
Acute toxicity
AH receptor
Sex hormones

ABSTRACT

The acute toxicity of the ubiquitous environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) varies widely among species and strains. Previous studies in rats have established that females are approximately 2-fold more sensitive to TCDD lethality than males. However, there is a surprising gap in the literature regarding possible gender-related sensitivity differences in mice. In the present study, by using three substrains of TCDD-sensitive C57BL/6 mice and transgenic mice on this background, we demonstrated that: 1) in contrast to the situation in rats, female mice are the more resistant gender; 2) the magnitude of the divergence between male and female mice depends on the substrain, but can amount to over 10-fold; 3) AH receptor protein expression levels or mutations in the primary structure of this receptor are not involved in the resistance of female mice of a C57BL/6 substrain, despite their acute LD_{50} for TCDD being over 5000 μg/kg; 4) transgenic mice that globally express the rat wildtype AH receptor follow the mouse type of gender difference; 5) in gonadectomized mice, ovarian estrogens appear to enhance TCDD resistance, whereas testicular androgens seem to augment TCDD susceptibility; and 6) the gender difference correlates best with the severity of liver damage, which is also reflected in hepatic histopathology and the expression of pro-inflammatory cytokines, especially IL-6. Hence, the two closely related rodent species most often employed in toxicological risk characterization studies, rat and mouse, represent opposite examples of the influence of gender on dioxin sensitivity, further complicating the risk assessment of halogenated aromatic hydrocarbons.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD or simply "dioxin") is a ubiquitous environmental contaminant and one of the most potent toxicants known. Characteristically, however, its acute lethality exhibits exceptionally wide variation both among species (LD_{50} values ca. 1 and 5100 μg/kg for the guinea pig and hamster, respectively) and within species (LD_{50} values ca. 10 and >9600 μg/kg for two strains of rat) (Henck et al., 1981; Pohjanvirta et al., 1993; Schwetz et al., 1973; Unkila et al., 1994). In TCDD-sensitive rat strains, gender has also proved to be an important determinant of TCDD sensitivity, with males being approximately 2-fold more resistant than females to its acute lethality (Pohjanvirta et al., 1993). The gender difference extends to some other forms of TCDD impacts as well, including the induction of hepatic microsomal enzymes (Lucier et al., 1973; Walker et al., 1995) and liver tumor formation in chronic exposure (Kociba et al., 1978).

Practically all the adaptive and toxic effects of TCDD are mediated by a ligand-activated transcription factor, the aryl hydrocarbon receptor (AHR) (Okey, 2007). The AHR is a member of the bHLH/PAS protein superfamily. Ligand binding causes a transformation of the cytosolic receptor to a form capable of entering the nucleus, where it heterodimerizes with another bHLH/PAS protein, ARNT. The AHR/ARNT dimer binds to specific enhancer sites (dioxin response elements, DREs) in the regulatory regions of *CYP1A1* and other genes regulated by the AHR, eventually leading to altered expression of these genes (Beischlag et al., 2008; Ma, 2012). Besides this canonical pathway, the AHR has been found to employ alternative signaling routes. For example, in certain cell lines, ligand-activated AHR alone, independently of ARNT, is able to repress estrogen-regulated transcription (Labrecque et al., 2012), and by increasing intracellular calcium activate protein kinases and ultimately cyclo-oxygenase 2 (Matsumura, 2012). Likewise, DRE binding has been shown not to be involved in the induction by ligand-activated AHR of acute-phase response genes in vitro (Patel et al., 2009), or in the suppression of genes involved in cholesterol synthesis in vivo (Tanos et al., 2012).

The structure of the AHR seems to be an important determinant of dioxin sensitivity. For example, the roughly 10-fold difference between C57BL/6 and DBA/2 mouse strains to most biological effects of TCDD is due to a single amino acid dissimilarity in the ligand-

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doi:10.1016/j.taap.2012.04.032

binding domain of the AHR (Poland et al., 1994). Likewise, the main reason for the 1000-fold higher resistance of Han/Wistar (*Kuopio*) (H/W) rats compared with Long/Evans (*Turku/AB*) rats to the acute lethality and some other endpoints of TCDD toxicity is a splicing defect-based alteration at the C-terminal transactivation domain of the AHR in H/W rats (Pohjanvirta et al., 1998; Tuomisto et al., 1999). Transgenic mouse lines expressing the wildtype isoform of rat AHR are highly sensitive to dioxin toxicity, whereas those expressing the H/W type receptor are exquisitely resistant (Pohjanvirta, 2009).

While we were studying the sensitivities of various transgenic mouse lines to TCDD toxicity, we happened to observe that female mice of our C57BL/6 colony were surprisingly refractory to the acute lethality of TCDD, but this resistance did not extend to male mice of the same strain (Pohjanvirta, 2009). Intrigued by this finding, we decided to more closely examine the phenomenon. We discovered that this reversed gender difference compared with rats is a general characteristic of TCDD-sensitive mice, and apparently dependent on both estrogens and androgens.

Materials and methods

Animals and animal husbandry. Adult male and female C57BL/6 mice (original strain C57BL/6J) were obtained from the National Public Health Institute (today the National Institute for Health and Welfare), Kuopio, Finland. To specifically refer to this substrain, we hereafter call it C57BL/6Kuo. Intact C57BL/6NTac mice and intact, ovariectomized or castrated C57BL/6JBomTac mice were purchased from Taconic Europe (Lille Skeneved, Denmark). Transgenic C57BL/6 mice expressing rat wildtype AHR were produced as described earlier (Pohjanvirta, 2009). In the great majority of the experiments, the mice were employed at the age of 9–15 weeks; for the transgenic mice the age range was wider, with the oldest animals being 23 weeks old. During the experiments, the mice were housed in Macrolon cages with pelleted Altromin 1314 feed (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and tap water available ad libitum. To prevent aggressive social behavior (typical of male mice housed together) from biasing the results, the mice were kept singly in the course of the sensitivity experiments. The temperature in the artificially illuminated animal room was 21 °C, relative humidity 50 ± 10% and lighting cycle 12/12 h light/dark. The study plans were approved 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.

Experimental design. 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 and administered to mice by gavage (10 ml/kg). For mice receiving the highest doses (4000 or 5000 μg/kg), TCDD was given in two boluses (each 10 ml/kg) ~8 h apart. In the sensitivity experiments, the mice were monitored at least twice daily and weighed two or three times per week. The mice that showed clinical signs consistent with imminent death within the next 24 h (apathy, listlessness, loss of reactivity, immobility) were euthanized by cervical dislocation. At the termination of these experiments, the surviving mice were killed by the same method. For ethical reasons, the number of mice was kept to the minimum required for establishing coarse LD_{50} levels.

Two additional experiments were conducted to compare the biochemical and morphological changes occurring in the early stages of TCDD intoxication in male and female mice differing widely in their TCDD sensitivities. In a dose–response experiment, adult male and female C57BL/6Kuo mice were exposed to 125, 250, 500, or 1000 μg/kg TCDD in corn oil (10 ml/kg) and killed on day 4 (males: n = 4; females: n = 5). In a time-course experiment, adult male and female C57BL/6Kuo mice were treated with 500 μg/kg TCDD in corn oil (10 ml/kg) and killed at 1, 3, or 6 days post-exposure (males: n = 4 for control and n = 5 for TCDD at all time-points; females: n = 3 for control, days 1 and 6; n = 4 for control, day 3; n = 5 for TCDD at all time-points). In both studies, the mice were euthanized with carbon dioxide immediately followed by cardiac exsanguination. Heparinized plasma was separated, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. The liver, thymus and spleen were removed, weighed and sampled. Samples of all these three tissues were taken to −80 °C via liquid nitrogen, and a slice from the liver was additionally fixed in buffered 4% formalin for subsequent histological analysis.

Clinical chemistry. The following variables were determined in all plasma samples: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), total bilirubin, glucose, free fatty acids (FFA) and triglycerides. Additionally, alkaline phosphatase (ALP) was analyzed in plasma samples from males or females alone in the dose–response and time-course studies, respectively. The activities of plasma ALAT (EC 2.6.1.2), ASAT (EC 2.6.1.1) and ALP (EC 3.1.3.1) were measured according to the recommendations of the International Federation of Clinical Chemistry (Anonymous, 1983, 2002a, 2002b). Spectrophotometric methods were used for the determination of total bilirubin (Malloy and Evelyn, 1937), glucose (Trinder, 1969) and triglycerides (Wahlefeld, 1974). An enzymatic, colorimetric method was used for the determination of free fatty acids (FFA; NEFA-C, Waco Chemicals GmbH, Neuss, Germany). The analyses were performed with a clinical chemistry analyzer (Konelab 30i, ThermoFisher Scientific, Vantaa, Finland).

AHR sequencing. Virtually the entire coding sequence of the *AhR* gene was analyzed from a female C57BL/6Kuo mouse. Hepatic cDNA was prepared as described above. The full AHR open reading frame (except for the stop codon) along with most of the 5′ leader sequence was amplified in 6 partly overlapping stretches (each 600–750 bp long) by PCR employing Phusion DNA Polymerase (Finnzymes, Espoo, Finland). The primers used and the amplicon lengths can be found in Supplementary Table 1B. The DNA fragments were purified from agarose gel with the GenElute Gel Extraction Kit (Sigma-Aldrich), concentrated with the DNA Clean & Concentrator-25 kit (Zymo Research, Orange, CA, USA), and sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit on the ABI3130XL Genetic Analyzer (both from Applied Biosciences, Foster City, CA, USA).

AHR Western analysis. AHR protein levels were determined by the quantitative Odyssey (Li-Cor Biosciences, Lincoln, NE, USA) near-infrared Western analysis methodology with β-actin employed for normalization following the instructions of the instrument manufacturer. Contrary to mRNA expression, TCDD did not affect β-actin protein abundance (data not shown). A total of 90 μg homogenate protein was pipetted into each well of 10% Precise Tris-Glycine Gels (Thermo Fisher Scientific). The primary antibodies for the AHR and β-actin were polyclonal and were purchased from BioMol (today a subdivision of Enzo Life Sciences, Lausen, Switzerland) and Novus Biologicals (Littleton, CO, USA), respectively. The IRDye 800CW-labeled secondary antibody was from Li-Cor Biosciences.

Expression analyses. The hepatic mRNA expression levels of AHR were measured to determine whether the suppression of hepatic AHR protein levels revealed by Western assays was of transcriptional origin. Moreover, mRNA abundances of tumor necrosis factor-α (TNFα), the macrophage cell-surface protein F4/80, the chemokines KC (= mouse CXCL1) and CXCL2, and the vascular cell adhesion molecule for blood inflammatory cells VCAM1, as well as interleukins 1β (IL-1β), 6 (IL-6) and 10 (IL-10) were measured to further characterize the gender-related differences in the hepatic inflammatory reaction revealed by histopathological analyses. These determinations were performed by real-time RT-qPCR with the Rotor-Gene 3000 instrument (Corbett Research,