TCDD EXPOSURE RESULTS IN DIFFERENTIAL EXPRESSION OF α2U-GLOBULIN IN YOUNG MALE RATS

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Abstract
Three groups of five week old male rats were treated for 7 days daily with 0, 15 or 150 ng 2,3,7,8-TCDD/kg bodyweight. A fourth group received 105 ng/kg bw once, at day 7. Animals were sacrificed at day 8. Livers showed increased EROD-activity. Microarray analysis showed increased gene expression of cytochromes P450 1A1, 1A2, 1B1 and 3A62, as well as glutathione-S-transferase pi-2, UDP-glycosyltransferase 1A2 and aldehyde dehydrogenase 3A1. Remarkably, gene expression of α2u-globulin was down-regulated at the dose of 15 ng/kg bw, but up-regulated at the daily dose of 150 ng/kg bw and the single dose of 105 ng/kg bw. A similar study with daily dose levels of 0, 15, 50 and 150 ng/kg bw showed decreased liver levels of α2u-globulin protein at 15 and 50 and increased levels at 150 ng/kg bw. It is hypothesized that the expression of this protein reflects an effect of TCDD on testosterone and/or estradiol levels, being different at low and high doses.

Introduction
Dioxins and dioxin-like PCBs remain a threat to the consumer due to the small margin of exposure/safety. For a good estimation of the potential risks, it is essential to understand the potential effects of dioxins in the consumer, which appear to be rather subtle and best compared to those of certain hormones. Although it is evident that the effects of dioxins are mediated through binding to the Ah-receptor and subsequent up- or down regulation of a number of genes, it is still unclear how this leads to the effects observed in animals, like those on the immune system, sperm production, learning behaviour, endometriosis and liver tumours. The current exposure limit for TCDD in the EU is based on the effects on sperm production of rats exposed *in utero*. For this reason, we decided to study the effects of TCDD on young male rats, using microarray analysis for identifying potential markers involved in the earlier observed/described effects. In addition, we investigated the potential differences between a continuous exposure and a peak exposure, which may be more applicable to the consumer. At present, it is assumed that gradual accumulation, rather than short exposure is of importance for dioxins, as expressed by the use of weekly (TWI) or monthly tolerable intakes.

Materials and methods

Animals and study design
Pregnant rats were obtained from Harlan (Horst, The Netherlands) and kept at a housing temperature of 22˚C and at a relative humidity of 30-70%. Lighting cycle was 12h light and 12h dark. Animals were provided with standard feed (Arie Blok, Woerden, The Netherlands) and tap water *ad libitum*. Young rats were weaned, weighed and in the first study 16 male animals were randomly allocated to 4 treatment groups at postnatal day 24. In the first study, rats were exposed to 0, 15, 50 or 150 ng 2,3,7,8-TCDD/kg bodyweight daily, for 7 days. Animals were weighed daily in order to accurately calculate their individual dose. 2,3,7,8-TCDD was diluted with n-nonane and mixed with 1 ml of custard. Control groups received only the vehicle n-nonane, mixed with 1 ml of custard. Experiments were started at 5 weeks of age.

In the second study 42 male animals were randomly allocated to 7 treatment groups at postnatal day 24. Three treatment groups were exposed to 0, 15 or 150 ng 2,3,7,8-TCDD/kg bodyweight daily for 7 days. One group received the same overall dose as the low dose group, 105 ng/kg bw, but given as a single dose at day 7. Animals from these groups were sacrificed at day 8. Two other treatment groups were exposed on a daily basis to 0 or 15 ng 2,3,7,8-TCDD/kg bodyweight for 7 days, one group received one dose of 105 ng/kg at day 7, followed by one week without treatment. These three groups were sacrificed on day 15.

Microarray analysis and PCR confirmation
Hepatic lobes were snap-frozen in liquid nitrogen and stored at -80˚C. After homogenizing, RNA was isolated and 1µg amplified. In the first study livers were pooled per treatment group, in the second study lobes from each rat were treated individually. RNA from the control group sacrificed at day 8, was pooled, labeled with Cy3 and

used as a reference for the groups sacrificed at day 8. The control group sacrificed at day 15 was used as a reference for the experimental treatment groups killed at day 15. RNAs of each of the treated and control animals were labeled with Cy5. The labeled cRNA was purified and 1 µg of each sample hybridized to 44K whole genome 60-mer rat oligo microarrays (G4130A, Agilent). Slides were read with an Agilent scanner at 532/568 nm (Cy3) and 633/668 nm (Cy5), and spot intensities quantified using Feature Extraction 8.5. Quality control was performed with R, using the limmaGUI interface, before normalizing the data in Genemaths XT, correcting for random and systematic error. To identify regulated genes, one way ANOVA was performed to test for differences between groups and to obtain information about within- and between-group variability for each gene.

For quantitative RT-PCR, RNA from 3 randomly chosen animals per group, was isolated, treated with DNAse I and cDNA synthesized using 1 µg RNA. Primers were selected using Beacon Designer 5 to obtain an amplicon of ~200 bp length with a T_m of 60˚C. After design, all primers were run through NCBI Blast in order to check for specificity. ARBP was selected as housekeeping gene as its expression levels varied between 0.9 and 1.1 in all samples. Expression levels were normalized using the ARBP-expression levels in individual samples.

EROD assay

In order to measure the activity of cytochrome P450 1A, the ethoxyresorufin-O-deethylase activity (EROD) was determined in liver microsomes.

Analysis of TCDD in livers and abdominal fat

TCDD was determined in livers and fat using HRGC/HRMS following purification on a Powerprep system. Thus far only part of the samples has been analysed.

Determination of alpha-2u-microglobulin by Western blot

Liver homogenates were suspended in lysis buffer and run on a SDS/PAGE gel. After transfer to a blot, the alpha-2u-globulin was detected using a purified polyclonal antibody obtained from R&D Systems, a secondary antibody and chemoluminescence.

Results and Discussion

Treatment of rats in the first study with dose levels of 0, 15, 50 and 150 ng/kg bw/day for 7 days resulted in TCDD liver levels of 0.00, 0.73, 2.05 and 5.01 ng/g ww, and levels in abdominal fat of 0.00, 0.53, 0.94 and 1.77 ng/g. In study 2, thus far only levels in abdominal fat were determined, being 0.00 ± 0.01, 0.74 ± 0.06 and 4.88 ± 0.92 ng/g for the blanc, the 7 days 15 ng/kg bw/d and the 7 days 150 ng/kg bw/d groups. The rats receiving a single dose of 105 ng/kg bw showed a level of 0.26 ± 0.05 ng/g, being 3-fold lower than the rats receiving the same dose in 7 days. After an additional 7 days without treatment the difference was still there but smaller, being 0.52 ± 0.13 and 0.32 ± 0.04 ng/g.

Figure 1 shows that the treatment with TCDD resulted in a clear dose-related increase in the EROD-activity in liver. A similar 2-fold difference between the 15 and 150 ng/kg bw/d groups was observed in the first study. However, the single dose of 105 ng/kg bw showed a much lower response than the repeated dose of 7x 15 ng/kg bw/day. After one week after the last treatment the activity clearly decreased, again showing the difference between the single and repeated dose.

A dose related increase was also seen in the expression of genes encoding for a number of cytochrome P450 enzymes, as determined by microarray analysis (Table 1). In the case of CYP1A1, the variation between the animals in the high dose group was quite high, showing fold-increases of 34, 41, 141, 77, 55 and 274. Remarkable was the apparent increased expression of CYP3A62, which to our knowledge is not known to be inducible by TCDD. The activity of 3 other biotransformation enzymes, glutathione-S-transferase pi-2, UDP-glycosyltransferase 1A2 and aldehyde dehydrogenase 3A1 also showed the previously reported increase in gene expression (Table 1). The effect on TCDD-inducible poly(ADP-ribose) polymerase (Tiparp) was only observed at the high dose level. Another gene that showed a clear dose related increase was selenium binding protein 2, which is in line with the increased protein levels reported in livers of male rats by Pastorelli *et al.* (2006).
An interesting observation and to our knowledge only briefly mentioned by Fletcher *et al.*, was the effect of TCDD on the expression of a number of genes encoding for different proteins belonging to the family of alpha2u-globulins (Table 1). Levels of all different members were decreased in the low dose group (15 ng/kg/d), and increased in the high dose-group (150 ng/kg/d). This observation was confirmed by a quantitative rt-PCR-

analysis on the PGCL-1 isoform in livers of 3 rats per group (Figure 2A). A quite similar expression pattern was observed for caldesmon and ubiquitin D. Perhaps even more remarkable was the fact that in this case the rats fed the single dose of 105 ng/kg bw showed an increased expression of these genes and thus resembled the effect in the 150 ng/kg bw/d dose group. For the biotransformation enzymes and the selenium binding protein 2 mentioned above the effects in this single dose group resembled the 15 ng/kg bw/d dose group. In the first study with dose levels of 15, 50 and 150 ng TCDD per kg bw/d, a smaller array was used, not containing the cDNA's for the alpha2u-globulins. However, preliminary results with immunoblots of livers homogenates from this first study show decreased protein levels in the 15 and 50 ng/kg/d dose groups and increased levels in the highest dose group (Figure 3), thus confirming the results from the second study. The gene for these proteins did not show any dioxin responsive elements and it seems that the effects must be indirect. Alpha-2u globulins are so-called lipocalins, involved in the binding and transport of low-molecular weight compounds. They are excreted in the urine of rodents and the levels are known to be depressed by treatment with estrogens and increased by treatment with androgens. Therefore, the observed effects may reflect an effect of TCDD on the levels of these hormones in the animals. Another interesting observation was the fact that the single dose did not affect the CYP1B1 levels, in contrast to the repeated dose levels. This was confirmed by PCR-analysis (Figure 2B). After 1 week without further treatment, most of the effects on gene expression disappeared (data not shown), with the exception of the cytochrome P450 enzymes 1A1, 1A2 and 1B1. It remains to be determined whether this was accompanied by a clear decrease in the TCDD levels in the liver.

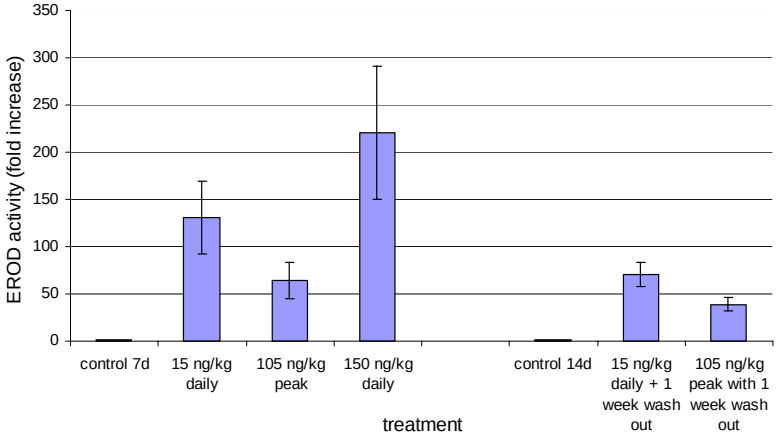

Figure 1. EROD activity in livers of rats from the second study. Results expressed as the fold-increase compared to the controls (mean and SD for n=6).

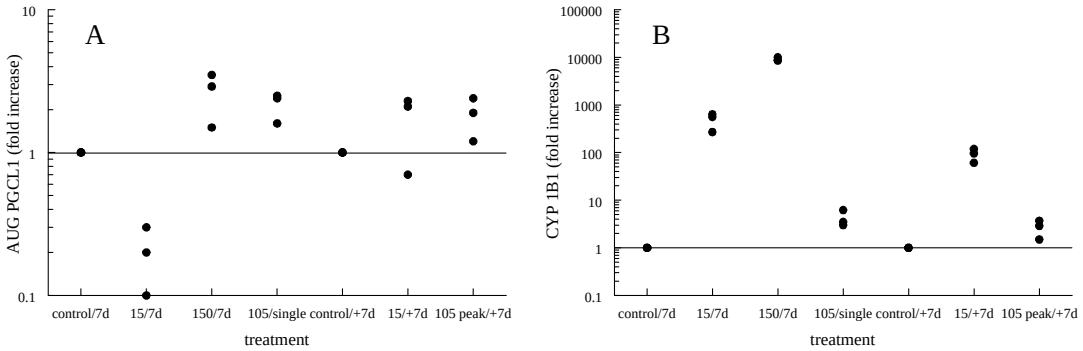

Figure 2. PCR analysis of alpha-2u globulin PGCL1 (A) and cytochrome P450 1B1 (B) in 3 randomly selected rats from each treatment group. Data expressed relative to the two control groups, i.e. without (/7d) or with (/+7d) an additional wash-out period.

Table 1. Gene expression of a number of selected genes, as determined by microarray analysis in rat livers, expressed as fold-increase or decrease to the average of the control (mean + SD for n=6).

Gene*	control 7d			15 ng/kg 7d			150 ng/kg 7d			105 ng/kg 1d		
	mean	±	SD	mean	±	SD	mean	±	SD	mean	±	SD
Cyp1a1	1.0	±	0.1	151.2	±	15.0	103.6	±	91.8	137.7	±	16.4
Cyp1a2	1.0	±	0.2	8.6	±	0.4	22.7	±	6.3	6.9	±	0.8
Cyp1b1	1.0	±	0.2	4.7	±	0.7	36.1	±	10.8	0.8	±	0.1
Cyp3a62	1.0	±	0.2	2.5	±	0.4	11.1	±	2.7	1.2	±	0.3
Gstp2	1.0	±	0.2	3.2	±	1.0	6.0	±	1.4	1.9	±	0.7
Ugt1a2	1.0	±	0.2	2.2	±	0.3	5.1	±	1.0	2.0	±	0.4
Aldh3a1	1.0	±	0.2	1.8	±	0.1	19.4	±	5.2	1.3	±	0.1
Selenbp1	1.0	±	0.1	3.0	±	0.2	12.0	±	2.7	2.1	±	0.4
Ubd	1.0	±	0.4	0.3	±	0.0	2.0	±	0.9	1.4	±	0.8
Caldesmon	1.0	±	0.2	0.3	±	0.1	1.8	±	0.3	1.5	±	0.6
Tiparp	1.0	±	0.2	1.1	±	0.1	2.4	±	0.7	1.0	±	0.1
Aug PGCL1	1.0	±	0.2	0.3	±	0.1	4.1	±	0.9	2.6	±	1.4
Aug PGCL3	1.0	±	0.2	0.3	±	0.1	1.6	±	0.3	1.4	±	0.2
Aug PGCL4	1.0	±	0.2	0.3	±	0.1	3.6	±	1.0	2.4	±	1.3
Aug PGCL5	1.0	±	0.2	0.3	±	0.1	4.1	±	0.9	2.8	±	1.4
Aug PGCL7	1.0	±	0.1	0.3	±	0.1	3.2	±	0.8	2.3	±	1.2

* Cyp1a1: cytochrome P450 1A1; Cyp1a2: cytochrome P450 1A2; Cyp1b1: cytochrome P450 1B1; Cyp3a62: cytochrome P450 3A62; Gstp2: glutathione S-transferase pi 2; Ugt1a2: UDP glycosyltransferase 1A2; Aldh3a1: aldehyde dehydrogenase 3A1; Selenbp1: selenium binding protein 2; Ubd: ubiquitin D;Tiparp: TCDD-inducible poly(ADP-ribose) polymerase; Aug: alpha-2u globulin.

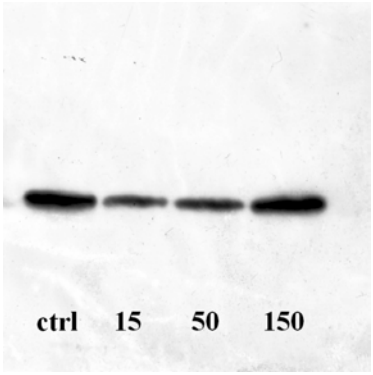

Figure 3. Immunoblot of pooled livers of rats exposed to 0, 15, 50 or 150 ng/kg bw/d for 7 days. Blots were treated with antibodies against rat alpha-2u globulin.

Conclusions

In conclusion, the present study reveals a series of interesting observations, thus far not reported, and may contribute to understanding the effects of TCDD in rats. Eventually, this may help to interpret the effects of TCDD in test animals with respect to their significance for humans.

References

1. Fletcher N, Wahlström, Lundberg R, Nilsson CB, Nilsson KC, Stockling K, Hellmold H, Håkansson. *Toxicol. Appl. Pharmacol.* 2005; 207: 1.
2. Pastorelli R, Carpi D, Campagna R, Alroldi L, Pohjanvirta R, Viluksela M, Hakansson H, Boutros PC, Moffat ID, Okey AB, Fanelli R. *Molecular. Cellular Proteomics* 2006; 5: 882.