# Influence of the Flame Retardant Tetrabromobisphenol-A on the Expression of Cytochrome P450 Isoenzymes in Rat Liver

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## Introduction

As one of the major brominated flame retardants (BFRs) tetrabromobisphenol A (TBBPA) is widely used in flammable plastic materials. There, it is incorporated either as covalently binding BFR or as an additive leading to likely leaching out of goods. Indeed, TBBPA was found in indoor air, environmental and human samples, i.e. mother's milk. Thus a certain degree of risk for human has to be considered.[8]

Some BFRs have been suspected to act as endocrine disrupters and/or affect the development of the unborn. Induction of drug metabolism may play a role in such effects by changing the body's homeostasis of hormones, such as steroids, thyroxine, and others.[8,9,10,11] BFRs are prospected to lead to thyroid hormone deficiencies, neurodevelopmental deficiencies, cancer.[12] Furthermore a variety of inducing agents have been described as tumor promoters in rodent liver. Herein the induction of enzymes of the cytochrome P450 family (CYP) plays a major role.[13]

## Methods and Materials

Wistar rats:
Rats of WU(CPB) strain were bred in the RIVM facilities (Bilthoven, Netherlands), and included in the experiment at 6-8 weeks of age. Light/dark regime was 12/12 h, and standard pelleted rat feed without soy (Hope Farms / Arie Blok Diervoeding, Woerden, the Netherlands) and drinking water were supplied ad libitum. In this study, the rats, sex of both, were orally administered TBBPA mixed with feed in approximate doses in the range of 0, 30, 100, 300 mg/kg body weight per day for 28 days. After the exposure period, animals were euthanised by exsanguination from the abdominal aorta after anesthesia with isoflurane. Necropsies were performed according to the OECD guidelines. The order of necropsies was scheduled to allow clustered immunological

analysis in males, and necropsy of females in the diestrus stage of the estrous cycle. Dissected tissues were snap-frozen in liquid nitrogen, and stored at -80ºC until further processing. Experiments were approved by the institutional Committee on Animal Experimentation, according to Dutch legislation.

Isolation of microsomes:

Hepatic microsomes were isolated according to the protocol of Melancon[14], and dissolved in 50mM sodium phosphate buffer of pH7.4.

EROD/PROD:

The activities of CYP1A and CYP2B were measured via 7-ethoxyresorufin O-deethylase(EROD) and 7-ethoxyresorufin O-dependentylase(PROD) activity in triplicates on a microplate reader (Fluoroskan Ascent FL, Labsystems, Sweden) and related to protein content quantified via fluorescamin reaction according to the protocol of Kennedy.[15]

Western Blot:

Vertical SDS gel electrophoresis and semi-dry membrane blot were conducted with 20µg protein per lane as described earlier.[16] For immunoreactions the following antibodies were used. CYP1A1/2: antiRAT CYP1A1/2 (Daiichi Pure Chemicals, Japan); CYP2B1/2: antiRAT CYP2B1/2 polyclonal ab (AB1283, Chemicon, Germany); CYP3A1: antiRAT CYP3A1 polyclonal (AB1253,Chemicon, Germany). The membrane bands were detected with the ECL-Plus detection Kit (Amersham, Germany), Lumi-Imager, and its software LumiAnalyst (Roche, Switzerland).

Isolation of RNA

Hepatic and duodenal total RNA was isolated with TRIzol (Invitrogen, Germany), according to the manufacturer's instructions and dissolved in pure water (LiChrosolv, Merck, Germany). Samples were quantitated by spectrophotometrie with Nanodrop (Nanodrop, USA) and diluted to 100ng/µl. Aliquots of RNA were analyzed by agarose/formaldehyde gel electrophoresis to check RNA integrity.

Development of specific primers for quantitative Real Time PCR. Coding sequences for the genes listed in ***Table 1*** were obtained from GenBank. Specific target regions within the coding sequences, were determined through nucleotide sequence alignment comparisons of targets within multiple member gene families (e.g., MRP-isoenzymes, CYP-isoenzymes). Separation of the isoenzmyes CYP3A1 and CYP3A3 through primer selection proved unsuccessfull, i.e. identical mRNA PCR-products will be generated. Primers spanning introns were designed to the selected target using Beacon Designer 2.1 (PREMIER Biosoft International, USA). All primers were submitted to the National Center for Biotechnological Information for nucleotide comparison using the basic logarithmic alignment search tool (BLASTn) to ensure specificity. Primers were synthesized by MWG Biotech (Germany).

| Gene | GenBank accession no. | 5'- Sense - 3' | 5'- Antisense - 3' |
|---|---|---|---|
| rGAPDH | AF106860 | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG |
| rCYP1A2 | NM_012541 | CGGTGATTGGCAGAGATCGG | GTCCCTCGTTGTGCTGTGG |
| rCYP2B1 | M37134 | ATGGAGAAGGAGAAGTCGAACC | CTTGAGCATCAGCAGGAAACC |
| rCYP3A1/3 | NM_173144 / NM_013105 | CCAGCAGCACACTTTCCTTTG | GGTGGGAGGTGCCTTATTGG |
| rCYP3A9 | NM_147206 | CCGATGTGGAGATTGTGGC | CATTTGTAGCAGGGTATCATAGG |
| rCYP3A18 | NM_145782 | GCCAAGAGTCCCAGAAAGC | GGTGACAGGTGCCTTATTGG |

*Table 1*

RealTime RT-PCR

Each RNA sample was reverse-transcribed before analysis of different gene expression by PCR. Real-time quantitative RT-PCR was performed using an iCycler iQ Real-Time PCR Detection System and iCycler Software version 2.2 (BioRad,Germany).

For RT reaction iScript-Kit (Biorad,Germany) was used according to manufacturer's protocol in a final volume of 20µl, briefly 100ng RNA, 4µl 5 x reaction mix, 1µl of reverse transcriptase, nuclease free water supplied by manufacturer. The iCycler was programmed to 25°C 5min; 42°C 30min; 85°C 5min; 4°C ∞. Samples were then assayed in 25 µl reactions using 12.5µl iQ SYBR Green Supermix (BioRad,Germany) and 6.25 ng of RNA per reaction. Gene-specific primers were used at 5 pmol each per reaction. The iCycler was programmed: 95°C 3min; 40 x (95° 1min, 59°C 1min, 72°C 1min); 95°C 1min; 55°C 1min. A melting curve emerging in a gradient from 55°C to 95°C in increasing steps of 0.5°C verified for the single PCR product.
For standard curves of each primer pair dilution series were generated by diluting one sample 1:1, 1:10, 1:100, 1:1,000, and 1:10,000, as well as including a no template control. The efficiency *E* was calculated from the slope of each standard curve.
GAPDH was used to normalize gene expression in all hepatic samples, since it is a highly expressed gene in rat liver. Fold induction values, ratios of target genes related to the reference genes GAPDH were calculated with the equation of Pfaffl, using one sample in all microtiterplates for comparison.[17] The fold induction, Ratio was related to the mean of the control group, 0 mg/kg bw/d.

$$Ratio = \frac{E_{tar}^{(Ct_{Control} - Ct_{treated})}}{E_{ref}^{(Ct_{Control} - Ct_{treated})}}$$

For statistical analysis outlayers were extracted by means of Grubbs test at 95% confidence.
Proceeding analysis was performed by the parametric one way analysis of variance between groups (ANOVA) at $p \leq 0.05$ if Bartlett's test suggested the differences among the standard deviations to be not significant. Comparison between the groups were made using Tuckey's test as a post ANOVA test.

## Results and Discussion

We found that the EROD and PROD activities corresponding to CYP1A and CYP2B activities vary noticeably, independent of dose groups. The amount of the CYPs under consideration does not seem to be affected by treatment in male animals for CYP1A1/2, 2B1/2, 3A1 as detected in Western blot. In females the CYP3A1 amount shows a nonsignificant trend to be upregulated with dose increase. This trend could not be verified on the transriptional level with CYP3A1/3. The trend may be due to differences between individuals as well as differences in feed consumption appended with TBBPA exposure of each rat. None of the other CYPs seems to be influenced by oral exposure to TBBPA up to 300mg/kg body weight per day, neither at protein nor at RNA level. A further possible cause for the minor inducing effect of TBBPA is its low oral bioavailibility and a rapid excretion of TBBPA.[9,10]

Related to the low levels of TBBPA in human detected so far[18] it is not expected to reveal inducing effects of TBBPA in human liver cytochromes. However, low dose long term effects may play an important role in changing the body's homeostasis of hormones, or causing diseases.

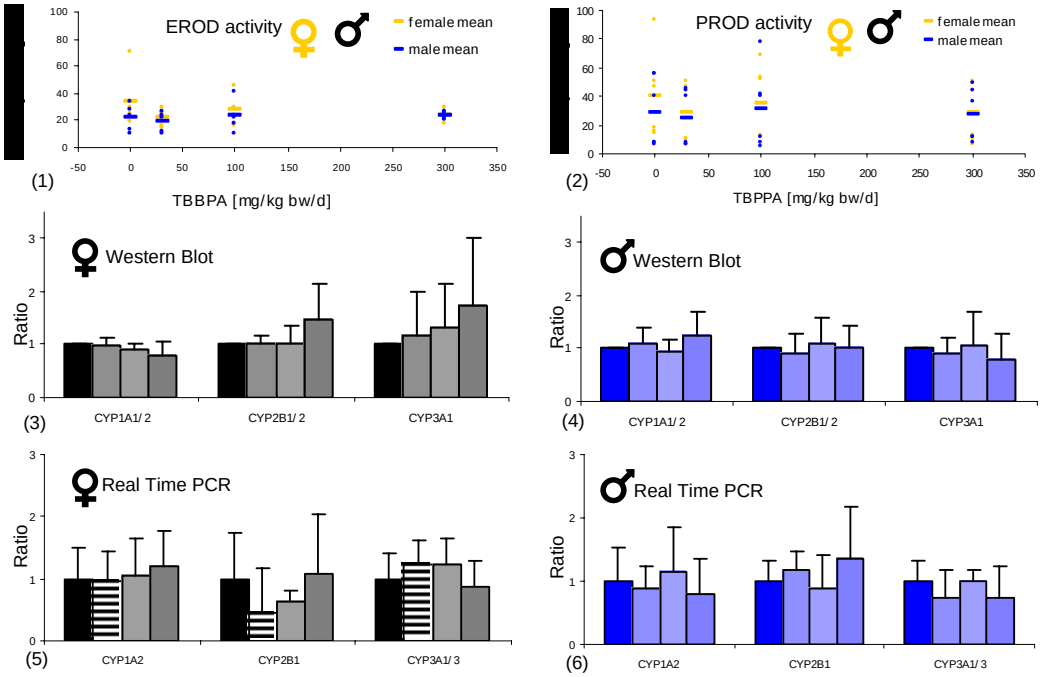


*Figure:* (1) EROD activities of hepatic microsomes(2) PROD activities of hepatic microsomes
(3) protein amount of CYPs detected via Western blot of hepatic microsomes of female animals
(4) protein amount of CYPs detected via Western blot of hepatic microsomes of male animals
(5) RNA amount of CYPs in female livers detected via Real Time PCR
(6) RNA amount of CYPs in male livers detected via Real Time PCR

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