SUBCHRONIC EFFECTS OF HEXABROMOCYCLODODECANE (HBCD) ON HEPATIC GENE EXPRESSION PROFILES IN RATS

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Abstract

Hexabromoyclododecane (HBCD), used as flame retardant (FR) mainly in textile industry and in polystyrene foam manufacture, has been identified as a contaminant at levels comparable to other brominated FRs, as TBBPA or PBDEs. The commercial HBCD formulation contains 3 isomers (α,β,γ), which is dominated by the γ isomer. As a result of its widespread use and their physical and chemical properties, HBCD is now an ubiquitous contaminant in the environment and humans. HBCD levels in biota are increasing slowly and seem to reflect the local market demand. One important observation is the shift from the high percentage of the γ-HBCD stereoisomer in the technical products to the α-HBCD stereoisomer in biological samples. The toxicological database of HBCD is too limited in order to perform a solid integrated risk assessment, combining data from exposure and effect studies. In order to complete some of these gaps, a 28-day HBCD repeated dose study (OECD407) was done in Wistar rats, within the framework of the European project FIRE as described earlier by van der Ven *et al.*, 2006. Liver tissues from these animals used for gen expression profile analysis. Our results show that cholesterol biosynthesis and estrogen metabolism were affected pathways by the daily oral exposure of 30 and 100 mg/kg HBCD to rats.

Introduction

Hexabromocyclododecane (HBCD) is a brominated aliphatic cyclic hydrocarbon used as flame retardant in extruded or expanded polystyrene foam and in textile back coating. The commercial HBCD formulation contains 3 isomers (α,β,γ) and is dominated by the γ isomer. Several investigations indicated a sharp increase in HBCD concentrations from 2001 onwards [1-4], which can possibly be explained by the withdrawal of the penta- and octabrominated diphenylether (PBDE) mixtures from the market and subsequent increased use of other brominated flame retardants (BFRs) such as HBCD and tetrabromobishpenol A (TBBPA). The world-wide demand for HBCD was reported to be over 16000 ton per year in 2001 [5]. In the environment, concentrations of HBCD from several sediment or air samples range from <0.1 – 514 ng/g (dw) [6, 7]. In wildlife, the highest total HBCD levels so far were measured in harbour porpoises on Scottish coast with median concentrations of 51 mg/g lipid weight [4]. HBCD has also been detected in different fish marine mammals and bird species, such as falcons, glaucous gulls or owls [8, 9]. In the few human studies conducted to date, low HBCD concentrations have been reported with median values varying between 0.35 and 1.1 ng/g lipid weight [1]. From a toxicological point of view, HBCD exerts *in vivo* as well as *in vitro* effects on different endpoints. *In vitro*, neurotoxic potential effects were described when rat cerebellar granule cells were exposed to HBCD at low µM concentrations [10]. Furthermore, HBCD exposure of transfected HeLaTR human cervical carcinoma cells, expressing the thyroid hormone responsive element and luciferase as reporter gen, showed enhanced gene activation [11]. *In vivo*, subchronic effects of HBCD on hepatic cytochrome P450 levels with induction of drug-metabolism enzymes have also been described, probably via the CAR and/or PXR signalling pathway [12]. In the present study, we examined gene expression profiles in male and female rat livers after 28days of daily HBCD treatment according to OECD-407 guidelines [3].

Material and Methods

Animal experiment. Male and female rats of the WU (CPB) strain were exposed subchronically to vehicle, 30 mg/kg bw or 100 mg/kg bw HBCD daily for 28 days as described earlier [3]. The animal experiments were performed

according to OECD407 guideline [13]. Livers tissues were removed and snap-frozen in liquid nitrogen. Subsequently they were stored at -80˚C until further processing.

RNA isolation. Livers were taken from -80˚C and total RNA was isolated using RNA Instapure according to supplier's instructions (RNA Instapure, Eurogentec, Maastricht, The Netherlands). Isolated RNA was resuspended in 100 µl of sterile RNAse-free water and after purification, using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), purity and concentration were assessed spectrophotometrically at wavelengths of 230, 260 and 280 nm. 230/260 ratios were higher than 1.8 for most samples and 260/280 ratios were all higher than 1.9. Samples were stored at –80ºC prior to mRNA analysis.

Preparation of labeled cRNA and microarray hybridization. From each treatment group, 5 livers were pooled. Labeling of the pooled samples was performed using a fluorescent direct label method (G2555-66002, Agilent Technologies, Amstelveen, The Netherlands). The common reference sample, i.e. a pool of all control RNA samples, was labeled with Cy3 (PerkinElmer, Wellesley, MA, USA) and the pooled RNA samples of each treatment group and control group were labeled with Cy5 (PerkinElmer, Wellesley, MA, USA). Subsequently, the labeled RNA was purified (QIAquick spin columns, QIAGEN, Venlo, The Netherlands) and hybridized to 22K rat oligo microarrays (G4130A, Agilent Technologies, Amstelveen, The Netherlands) according to manufacturer's instructions (In situ hybridization kit plus, 5184-3568, Agilent, Amstelveen, The Netherlands). After a 17h incubation period, slides were washed and fluorescent images were obtained with a scanner at 543 for Cy3 and 633 for Cy5 (Agilent Technologies, Amstelveen, The Netherlands) using supplier's software (Feature Extraction 8, Agilent Technologies, Amstelveen, The Netherlands).

Analysis of gene expression data. Spot intensities were quantified using Feature Extraction software. Subsequently, quality control was performed with R, using the limmaGUI interface, and data were normalized and corrected for random and systematic error in Genemaths XT (Applied Maths, Sint-Martens-Latem, Belgium). A principal component analysis (PCA) was performed to visualize clustering of the treatment groups. In order to identify differentially regulated genes, fold changes were calculated compared to the control groups. Subsequently, genes that were regulated more than 2 in at least one treatment group were selected for further analysis. Affected pathways were analyzed with MetaCore version 3.2.0 (GeneGo, Inc., St. Joseph, MI, USA).

Results and Discussion

In the present study, liver mRNA expression profiles of subchronic HBCD exposed rats were analyzed.

Based on the principal component analysis (PCA), there was a clear gender-specific difference (see Figure 1). Expression profiles in female rat liver samples differed from the males and several specific pathways were found te be affected by HBCD exposure (Table 1). Fatty acid and cholesterol biosynthesis pathways were clearly down-regulated by HBCD in female rat livers at both dose levels. From literature, it is known that a reduction of the biosynthesis of cholesterol and plasma triglycerides (TG) can be caused by hypothyroidism [14]. In addition, thyroxin stimulates hepatic *de novo* cholesterol synthesis by inducing the enzyme hydroxymethyl-glutaryl-CoA-reductase (HMG-CoA-reductase). This enzyme catalyses the conversion of HMG CoA to mevalonate, which is a precursor of cholesterol. Furthermore, elevated total thyroxin (TT4) levels exert a negative feedback loop towards the thyroid. Therefore, decreased TT4 levels might stimulate the thyroid to grow in order to compensate for the low levels of TT4. Remarkably a decrease of TT4 serum levels with an increased thyroid gland and pituitary weight was observed suggesting hypothyroidism [3]. Also, reduced thyroxin levels caused by hypothyroidism can consequently lead to decreased cholesterol synthesis [14]. A possible suggestion for a mechanism of action of HBCD in relation to the thyroid is presented in Figure 2. In addition, the expression profiles of, glutathione metabolism showed to be up-regulated several folds that was primarily seen in the male rats. Furthermore, important genes in the conjugation process were shown to be down-regulated (see Table.1). Here it should be noted that hepatic HBCD concentrations were higher in females than in males across the entire dose range [3]. These combined observations suggest a better elimination process of HBCD in males compared to the females. In males, HBCD also regulates phase I and II detoxifying enzymes, such as CYP3A5 and glutathione-S-transferase, which were 12-fold up regulated, in the highest dose group compared to the controls. Clearly, further individual Q-PCR analysis of individual genes is

needed to confirm the results obtained from these microarray analyses, but our results indicate a more complicate mechanism of action of HBCD than was previously known.

Figures and Tables

Table 1. Summary of affected pathways by HBCD after being analyzed with MetaCore.

Pathway	Genes	Fold change (males)	Fold change (females)
Cholesterol biosynthesis	HMCS	-1.1	-1.7
	HMDH	1.0	-3.2
Phase I metabolism	CYP51	1.1	-2.9
	CYP3A5	12.0	1.4
Phase II metabolism	GSTM1	2.5	1.3
	GSTA3	2.2	-1.4
	GSTA5	2.8	-1.4
	GSTP1	11.9	1.2

Figure 1 Principal component analysis (PCA) of analyzed samples to visualize clustering of the treatment groups.

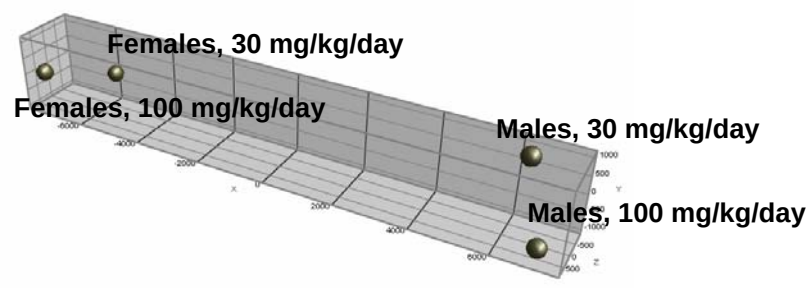
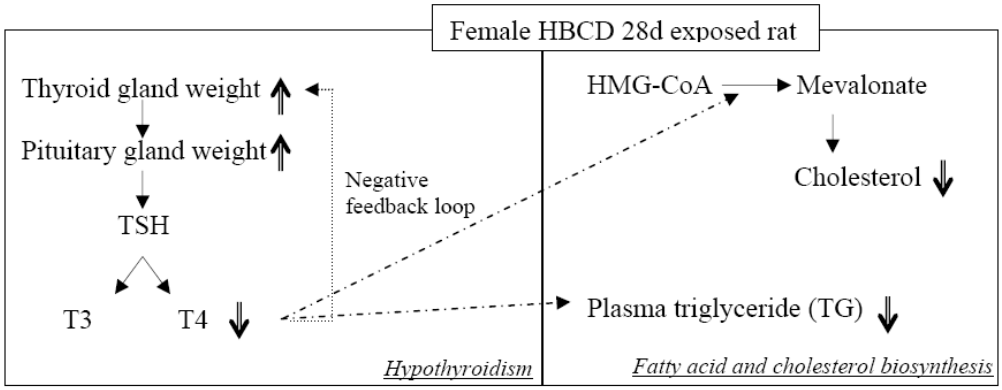


Figure 2 Proposed hypothesis of mechanism of action of HBCD in relation to lipid synthesis disorders and hypothyroidism in subchronically exposed female rats.



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