AN INTEGRATED APPROACH TO ASSESS TOXICITY OF BROMINATED FLAME RETARDANTS IN MOUSE MODELS

Josef Rasinger[1], Thomas Carroll[1], Valentina Reffatto[1], Francesca Maranghi[2], Roberta Tassinari[2], Marte Maave[3], Alberto Mantovani[2], Anne-Katrine Lundebye[2] and Christer Hogstrand[1]

[1]King's College London, 150 Stamford Street, Franklin-Wilkins Building, London, UK; [2]Istituto Superiore di Sanità, V.le Regina Elena, Rome, Italy; [3]National Institute of Nutrition and Seafood Research, Postboks 2029 Nordnes, Bergen, Norway

Introduction

Brominated flame retardants are persistent organic pollutants of increasing concern[1]. BDE47 is major component of the commercial flame retardant mixture DE-71 and has been shown to be the most dominant congener present in biota. Exposure to BDE47 has been seen to share many neurological endpoints with non-dioxin PCBs and this is believed to be largely due to their structural similarities[2,3]. BDE47 has been shown to disrupt thyroid hormone homeostasis[4], alter calcium homeostasis and protein kinase C signalling[5] as well increase arachidonic acid release[6]. The brominated flame retardant HBCD is a relatively new persistent organic pollutant when compared to the PCBs and PBDEs. HBCD represents around a third of total brominated flame retardants used in Europe and this accounts for approximately 60% of this brominated flame retardant's world-wide usage[7]. As with BDE47 and all non-dioxin PBDEs and PCBs, HBCD induces Cytochrome P450 enzymes in a CAR/PXR dependent manner[8].

Although both these flame retardants have been shown to have effects on rodents there is a lack of data on the possible modulating effect of the exposure matrix on the observed effects. This may be of importance because fish consumption constitutes a major exposure pathway for brominated flame retardants and fish by itself is a generally a very good source of nutrient and is enriched in nutrients that may influence toxicity outcome. In the present study we therefore compared the effects of BDE47 and HBCD in mice after dietary exposure involving spiked diets based on either salmon or casein.

Materials and methods

Mice were fed a nutritionally balanced diet based on either casein or 15% freeze dried salmon fillet spiked with BDE47 or HBCD for 28 days. Doses were given at two levels, the lower of which represented consumption of salmon from heavily polluted waters and the higher chosen from previous studies to generate mild but definite effects. After completion of the feeding trial, the animals were killed and tissues prepared for analysis of BFRs, histology, serum thyroid hormone levels, and transcriptome and proteome profiles.

Animal trials were complemented with experiments using two mouse neuronal cell lines, N2A and NSC19, to cast light on direct effects on the brain as opposed to those that may have been caused by endocrine disruption.

Genomics assays were carried out on brain tissue and cell cultures only. The Affymetrix platform was used for transcriptomics on brain and an in-house two-colour oligonucleotide array was used to generate transcript profiles in cultured cells. The DIGE system (GE Healthcare) was used for protein profiling followed by protein identification using LC-MS/MS.

Results and discussion:

Histology: The data of the present study indicated spleen, thyroid and liver as target organs for both flame retardants. Interestingly, the casein-based diet alone seemed to increase spleen germinal centres and red cell hypochromacy, compared with the fish-based control.

Thyroid hormone levels: There were changes observed in serum T3 and T4 concentrations, but these were more associated with feed type than BFR exposure. No significant effects on T3 or T3 levels were observed for any toxicant groups, compared to the respective feed-matched controls.

Transcriptomics: BDE47 and HBCD appear to share a common set of effects on gene expression with roughly half of the genes being regulated by one of the toxicants also being regulated by the other. These BFRs display similar phenotypic neurotoxicity and so the high overlap of differentially expressed genes between HBCD and BDE47 is a suitable reflection of these findings. Interestingly, the overlap between HBCD and BDE47 appears much more prevalent for the down regulated genes.

Analysis of biological processes enriched among genes regulated by HBCD revealed many terms associated with brain or neuron development and differentiation. Terms related to cytoskeleton function, inflammatory response, calcium regulation, and androgen signalling were also found to be significant. As with tests of the biological processes ontology, terms relating to cytoskeleton function were found to be enriched including "actin filament binding", "cytoskeletal protein binding", "Rho GTPase binding" and "structural constituent of myelin sheath". Many terms relating to secondary messenger systems such as "protein kinase activity" were found enriched within the HBCD group. The HBCD also caused an over-representation of differentially expressed genes with with molecular function terms suggesting an effect on calcium homeostasis.

The two major classes of biological processes found to be enriched by BDE47 treatment concerned axon generation and regulation as well as the cytokeleton related processes. Both these classes are related to those seen for HBCD but interestingly two further gene terms, "rhythmic process" and "nucleosome positioning", found by PBD47 enrichment testing were shown to have very unrepresented in HBCD results. As with HBCD many functions relating to secondary messenger systems such as "MAP kinase phosphatase activity" and kinase activity were enriched. Terms relating to protein modification and folding were also overrepresented.

Four genes that were consistently regulated by either HBCD or BDE47 in two studies were selected to test by qPCR the effect of diet base (casein vs. salmon) on gene expression in brain of mice with the hypothesis that the fish-based diet may ameliorate the effects of brominated flame retardants. Of the four selected biomarker genes (Bdnf, Hsp5, Plag, and Pak7), one showed a difference in brominated flame retardant response that could be related to diet. Whilst Pak7 was significantly upregulated by both HBCD and BDE47 in the casein based diet, there was no effect in the diet that was based on salmon.

Proteome profiles: Changes in abundance of proteins in response to HBCD or BDE47 in casein and salmon based diets were also investigated. In terms of numbers of proteins being regulated, there were more proteins showing significant differential expression by HBCD in the salmon based diet than there was in that based on casein. In either diet background there were changes in specific proteins being regulated by HBCD at concentrations that are environmentally realistic and representative of highly contaminated fish. Indeed, more proteins appeared to be regulated at the lower dose than at the higher. There were considerable overlaps between treatments in terms of the identities of regulated proteins. This confirms the results from microarrays.

Hierarchial clustering and principal component analysis showed that protein expression in the brain was more influenced by the basis of the diet (casein vs. salmon) than by the exposure to toxicants. This demonstrates a pronounced and somewhat surprising influence that fish consumption had on the metabolism in the brain.

In terms of functions of proteins affected by BDE47 and HBCD there was again an overlap between the toxicants and also with the results from the gene expression analysis. Regulated proteins were predominantly associated with pathways involved in neurological disease, genetic disorder, phychological disorder, cancer, reproductive system disease, cardiovascular disease, cell cycle, and vitamin and mineral metabolism.

Experiments on neuronal cell lines: Using the MTT assay, it was established that N2A or NSC19 cells incubated with concentrations between 2 and 6 μM BDE47 or HBCD have decreased cell viability between 20 and 30% relative to the DMSO-treated control. Caspase-3 activity was significantly increased in cells treated with either of the two BFR at concentrations as low as 1μM, indicating cell death through apoptosis. HBCD was also found to cause LDH leakage which was reduced by pre-treatment of cells with the omega-3 fatty acid, docosahexaenoic acid (DHA), which is abundant in salmon.

Microarray analysis of cells incubated with 1 or 2µM of BDE47 revealed three main enriched functional groups: (1) genes involved in cell development and differentiation, (2) membrane-bound and (3) genes implicated in cellular metabolic processes. In HBCD treated cells (1 or 2µM), the list of regulated genes were enriched in genes involved in (1) cell cycle and cell division and (2) development and in metabolic process, along with several other functions. DHA was shown to regulate genes that were also regulated by HBCD. Furthermore, the potency of HBCD in modulating DHA-induced gene expression was dependent of the level of DHA treatment. These findings are in accordance with the reduced HBCD cytotoxicity in DHA treated cells would support the idea of a protective role of DHA against HBCD toxicity.

Conclusions: Mice fed diets spiked with HBCD or BDE47 for 28 days at levels that might be expected in salmonids only from heavily polluted areas showed histological aberrations of spleen, liver and kidney, and changes in gene transcript and protein profiles in brain. Functional analysis of the genomics data obtained provided evidence for a causal link between dietary BFR exposure and genes linked to nervous system function and neurodegenerative diseases. The data obtained also showed that the diet has a large influence on gene and protein expression in the brain and indicated that the diet did indeed modulate the BFR-induced stress.

Acknowledgements:
The authors wish to thank Drs Mathew Arno and Estibaliz Aldecoa-Otalora Astarloa at the King's College Genomics Centre for technical support with microarray analyses and Dr Manuel Mayr, King's College London for help with mass spectroscopy. This project was financially supported by EU AQUAMAX project, contract no. 016249-2.

References:
1. Birnbaum, LS, Staskal DF (2004); *Environmental Health Perspectives* 112: 9-17.
2. Mariussen, E, Fonnum, F (2003); *Neurochemistry International* 43: 533-542.
3. Schecter, A, Päpke, O, Tung, KC, Staskal, D, Birnbaum, L (2004) *Environmental Science & Technology* 38: 5306-5311.
4. Hallgren, S, Sinjari, T, Håkansson, H, Darnerud, PO (2001); *Archives of Toxicology* 75:200-208.
5. Kodavanti, PR, Ward, TR, Ludewig, G, Robertson, LW, Birnbaum, LS (2005); *Toxicological Sciences* 88:181-192.
6. Kodavanti, PR, Derr-Yellin, EC. (2002); *Toxicological Sciences* 68: 451-7.
7. Covaci, A, Gerecke, AC, Law, RJ, Voorspoels, S, Kohler, M, Heeb, NV, Leslie, H, Allchin, CR, De Boer, J. (2006) *Environmental Science & Technology* 40: 3679-3688.
8. Germer, S, Piersma, AH, van der Ven, L, Kamyschnikow, A, Fery, Y, Schmitz, HJ, Schrenk, D. (2006) *Toxicology* 218:229-236.