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Altered thyroxine metabolism in rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane (HBCD)

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ABSTRACT

Hexabromocyclododecane (HBCD) is an additive flame retardant used in consumer and industrial applications. Our earlier work suggested that fish exposed to HBCD exhibited an increase in the rate of turnover of thyroxine (T4), the precursor molecule to the active thyroid hormone triiodothyronine (T3). To examine this further, juvenile rainbow trout were held in the laboratory and fed diets containing environmentally relevant concentrations of the individual α, β or γ isomers. Thirty-two days after feeding began, 20 fish from each group were gavaged with gelatin containing 1 μCi [^{125}I]-T4. Uptake of [^{125}I]-T4 from the gut was low and recoveries of the initial [^{125}I]-T4 were <10% after 2 d and <3% and <2% after 6 and 14 d respectively, with no differences among groups. However, measurements of the labeled T4 in tissues 2, 4, 6 and 14 d after gavage showed that tissue disposition and elimination rates of the hormone were altered by HBCD. In particular, on day 14 after gavage feeding, there was significantly lower radioactivity in the thyroid of fish exposed to the HBCD isomers relative to the reference group. This implies that either there was lower iodide uptake by the gland in fish exposed to HBCD or that the rate of thyroid hormone turnover in HBCD treated fish was elevated. There was also a significantly higher type II outer ring deiodinase enzyme activity in livers of fish exposed to the β and γ isomers. Taken together, these results indicate that HBCD can potentially impact the thyroid system of fish.

1. Introduction

Hexabromocyclododecane (HBCD) is the principle fire retardant in polystyrene foams that are used as insulation in the building industry and for upholstering furniture. With the recent inclusion of *penta*- and *octa*-brominated diphenyl ethers (BDEs) in the UNEP Stockholm Convention which now officially bans their use in countries subscribing to the treaty, it is anticipated that HBCD might be a candidate replacement chemical for the BDEs in some applications. However, HBCD itself is now under review by the UNEP to see if it fulfills the necessary screening criteria of persistence, bioaccumulation and toxicity as defined in the Annex D of the Convention (http://chm.pops.int/Convention/POPsReviewCommittee/hrPOPRCMeetings/POPRC5/tabid/588/mctl/ViewDetails/EventModID/871/EventID/69/xmid/2107/language/en-US/Default.aspx).

While it is known that HBCD bioaccumulates in aquatic organisms, there is relatively little data regarding its toxicity (Birnbaum and Staskal, 2004; Law et al., 2005). The limited studies that do exist suggest that HBCD has the potential to impact thyroid function in exposed organisms. For example, rats exposed to 100 mg HBCD kg^{-1} bodyweight per day exhibited significantly lower circulating concentrations of the thyroid hormone, thyroxine (T4) (Chengelis, 1997). We also previously showed that epithelial cell heights of the thyroids were significantly greater in fish exposed to HBCD, indicating thyroid gland hypertrophy (Palace et al., 2008). While mechanisms for these thyroidal effects are not yet clear, an in vitro study using human epithelial cervical cancer cells (HeLa) showed that HBCD at >5 μM augmented TR-mediated gene expression in the presence of T3 (Yamada-Okabe et al., 2005). Germer et al. (2006) also reported that HBCD induces CYP2B1 mRNA and CYP2B1/2B2 protein via the CAR/PXR signalling pathway. Both of these studies suggest that HBCD may alter thyroid status by increasing the clearance rates of thyroid hormones.

The commercial mixture of HBCD consists of three diastereoisomers, α, β and γ. The γ isomer predominates in abiotic compartments such as aquatic sediments but this is in sharp contrast to biota where the α isomer predominates (Janak et al., 2005; Law et al., 2006). Differences in lipid solubility and uptake into biota, varying rates of metabolism among the isomers, and in vivo isomerization of HBCD have all been suggested as potential mechanisms for the contrast between abiotic and biotic isomer profiles (Law et al., 2005, 2006; Zegers et al., 2006). The toxicological relevance of the α-dominated profile in organisms is not known as there is little information on the relative potency of the individual

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diastereoisomers to induce biological effects or specifically to affect the thyroid in exposed organisms (Birnbaum and Staskal, 2004).

To examine potential effects of HBCD diasteroisomers, juvenile rainbow trout (*Oncorhynchus mykiss*) were fed diets containing environmentally relevant concentrations of the individual α, β and γ isomers in the laboratory followed by oral dosing with [^{125}I]-T4 to examine tissue disposition.

2. Materials and methods

2.1. Fish and feeding

Juvenile rainbow trout (approximately 60 g) obtained from the Whiteshell Fish Hatchery (West Hawk Lake, Manitoba) were acclimated in 800 L fiberglass tanks receiving 2 L min^{-1} dechlorinated Winnipeg City tap (12 ± 2 °C) water for >3 months prior to beginning the experimental feeding stages. During acclimation, fish were fed 5 pt Martin Mills trout food (Martin Mills, Elmira ON) at a ration of 1% of bodyweight six times per week.

After the acclimation period, fish (approximately 210 g) were randomly distributed so that each 800 L fiberglass tank held 20 fish. Each group was fed 1% bodyweight six times per week of either the reference diet or diets enriched with α, β or γ-HBCD. The diets were formulated as described by Law et al. (2006). Briefly, 1.8 kg of powdered Martin Mills Silver Cup trout starter food was combined with 20 mL corn oil (Sigma Chemical Co., St. Louis, MO) for the reference diet or 20 mL corn oil containing α, β or γ-HBCD to attain a concentration of 5 ng g^{-1} of the appropriate isomer in the final diet. The powdered starter food and corn oil were thoroughly mixed using a Hobart food Mixer for 20 min after which 1.5 L of deionized distilled water warmed to 37 °C containing 40 g of 60 bloom gelatin (Sigma Chemical, Co.) was added. The entire mixture was allowed to mix for a further 20 min, followed by a drying period of 40–60 min. After drying, the food paste was extruded through a 5 mm die and the noodles were allowed to air dry in the dark at 25 °C for a period of 48 h. Noodles were manually broken into pellets and the dry food was stored at −20 °C. Weighed portions of food were removed from storage daily, and allowed to warm to room temperature (15 min) before being fed to the fish.

2.2. [^{125}I]-T4 gavage dosing

After 32 d of feeding the experimentally fortified diets, fish from each group were individually anesthetized in MS222 (0.1 g L^{-1}, pH neutralized with NaOH), weighed and measured. Each fish then received an oral gavage of 200 μL of gelatin warmed to 37 °C containing 1 μCi of [^{125}I]-T4 (specific activity 1200 mCi mg^{-1}). The gelatin solution was injected into the stomach (n = 20/treatment) using a 1 mL syringe with 1.57 mm ID polyethylene tubing that was heat flared at the insertion end to avoid tissue damage. After gavage, fish were allowed to recover in anesthesia-free and aerated water until they regained equilibrium (<2 min) after which they were returned to their original tanks until the appropriate sample period. The procedure was repeated until 20 fish from each dietary group had been dosed with [^{125}I]-T4.

2.3. Tissues and analysis

Five fish from each of the initial dietary groups were randomly selected 2, 4, 6 and 14 d after the gavage treatments. Fish were individually euthanized in MS222 (0.4 g L^{-1}, pH neutralized with NaOH) (<3 min), after which a 2–4 g section of muscle, as well as the gallbladder containing bile, thyroid gland (sampled as the entire lower jaw region), intestine (from stomach to vent) viscera (included stomach, adipose, spleen, gonad, pancreas), liver and whole blood were dissected from each carcass. Whole blood was centrifuged at 6000*g* for 10 min to separate plasma from red blood cells and the liver was weighed and then sectioned into two parts, one of which was frozen at −90 °C until analysis for deiodinase enzyme activity was to be completed. The other portion of liver and all the other tissues were placed in pre-weighed borosilicate glass culture tubes. After re-weighing the tubes to determine tissue weights, the [^{125}I] radioactivity in each tissue was determined using liquid scintillation counting. Total [^{125}I] activities in blood and muscle tissue compartments were calculated based on each fish containing 4.09 mL of blood per 100 g of fish (Gingerich et al., 1987) and an estimate that 52% of the total bodyweight was muscle for each fish (Webb, 1978).

Deiodinase type I and II activities were determined in individual liver tissues from five fish in each diet treatment group that were sampled 2 d post gavage. Assays were performed using the techniques of Eales and Brown (1993) but with modifications taken from Van der Geyten and Darras (2005). Briefly, microsomes containing 0.5–0.8 mg protein were incubated at room temperature for 15 min in 0.1 M KH_2PO_4/$NaHPO_4$ buffer (pH = 7.2) containing 10 mM Dithiothreitol (DTT) and 1 mM Ethyldiaminetetraacetic acid (EDTA) and either 1 nM (for deiodinase type I) or 100 nM (for deiodinase type II) unlabeled thyroxine. At time zero, [^{125}I]-T4 was added to each tube and the reaction was allowed to continue at room temperature for 90 min. The reaction was terminated by adding 40 μL of 2 M potassium iodide (KI). An aliquot of the reaction solution was pipetted onto gel chromatography columns containing 0.25 g of LH-Sephadex 20 that had been equilibrated in 0.1 N hydrogen chloride (HCl). The [^{125}I] liberated by deiodination of the [^{125}I]-T4 was eluted from each column using 3 mL DDW, which was counted using LSC. Each assay was carried out in triplicate.

2.4. Statistical analysis

Group means were analyzed using ANOVA followed by Tukey's test to determine specific group differences. Statistical significance was accepted at $\alpha = 0.05$.

3. Results and discussion

There were no significant differences in fish weight, length or condition among the HBCD dietary groups or the reference fish prior to, or after gavage treatment. There were also no mortalities in any of the treatment groups throughout the experiment. Not surprisingly, a relatively small proportion of the initial oral gavage dose of [^{125}I]-T4 was recovered in all sampled tissues combined (muscle, bile, thyroid gland, intestine, viscera, liver and whole blood from each fish; Fig. 1). Uptake and assimilation of T4 from the gut is known to be relatively low in fish (Sweeting and Eales, 1992; Brown et al., 2004). Whitaker and Eales (1993) showed that only 5 to 6% was assimilated in rainbow trout receiving a single dose of [^{125}I] labeled T4 delivered directly to the duodenum. Furthermore, a large portion of the radioactivity that was taken up was in the form of [^{125}I] liberated from the hormone rather than the intact labeled hormone containing [^{125}I].

In the current experiment, gel chromatography was used to separate radiolabelled iodothyronines, that were retained on the LH-Sephadex columns, from free [^{125}I] that elutes through the columns (Eales et al., 1999). Using this technique, >85% of the recoverable radioactivity from plasma and liver was determined to be in the form of iodothyronines and <15% could be attributed to liberated [^{125}I] (data not shown). While the specific iodothyronines present in the plasma were not determined, it is likely that more [^{125}I]-T3 than [^{125}I]-T4 was absorbed owing to the inefficient