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Calibration and Modulation of Cytochrome P4501A Induction in Dioxin Exposed Atlantic Tomcod

Submitted by:

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The following final report is designed to highlight the major findings of our study. More details on these results can be obtained from the six papers or manuscripts that accompany this report. In addition, one more manuscript is being prepared with Dr. Michael Ikonomou (Department of Fisheries and Oceans, Canada) on the spatial distribution of hepatic levels of dioxin, furan, and PCB congeners in young-of-the-year tomcod from the Newark Bay complex and multiple sites in the main stem of the Hudson River. The major objective of that paper will be a determination of sources of these contaminants in the Hudson River based on individual congener pattern profiles.

1. Tomcod from the Hudson River population are less responsive to dioxins and co-planar PCB congeners than tomcod populations from cleaner Atlantic coast estuaries (Courtenay et al. 1999).

Dose-response curves were developed for hepatic cytochrome P4501A1 (CYP1A1) mRNA expression in adult tomcod from the Hudson River and Miramichi River, New Brunswick, which were i.p. injected with graded doses of 2,3,7,8- TCDD and three co-planar PCB congeners (PCB 77, PCB 126, and PCB 169). For all four aromatic hydrocarbons, levels of contaminants needed to induce initial significant or maximum hepatic CYP1A1 mRNA expression were significantly higher in tomcod of Hudson River ancestry than in tomcod from the Miramichi River. However, CYP1A1 inducibility with benzo[a]pyrene(B[a]P) or beta-naphthoflavone did not differ significantly between tomcod from the Hudson River and Miramichi River. These results suggest that mechanisms of CYP1A1 transcriptional activation in tomcod may differ for halogenated (dioxins, furans, and PCBs) compared to non-halogenated (PAHs) aromatic hydrocarbons. Additionally, chronic exposure to Hudson River mixtures of environmental contaminants probably impaired subsequent CYP1A1 inducibility in tomcod either through heritable genetic change or through physiological acclimation.

2. Levels of hepatic dioxin, furan, and PCB congeners (and total TCDD TEQs) are substantially higher in adult tomcod from the Hudson River than in tomcod from two other Atlantic coast estuaries. Within the estuary, levels of these contaminants were higher in adult tomcod from the Hackensack River than in tomcod from Garrison, NY (River Mile 51) (Courtenay et al. 1999).

High-resolution gas chromatography/high-resolution mass spectrometry was used to quantify levels of all individual dioxin, furan, and PCB congeners in pools of livers (n=6-15) from male and female tomcod, respectively, from Garrison, NY, the Hackensack River, Miramichi River, and Margaree River, Nova Scotia. Hepatic levels of 2,3,7,8-TCDD in tomcod from the Hackensack River are among the highest ever reported in a fish population. Wet weight levels of hepatic 2,3,7,8-TCDD were 554 and 208 ng/kg in male and female tomcod, respectively, from the Hackensack River compared to 1 ng/kg in Margaree and 38 ng/kg in Miramichi River females, respectively. In the Hackensack River sample, more than 80% of total TCDD TEQs from dioxins and furans was contributed by 2,3,7,8-TCDD, whereas 2,3,7,8-TCDD contributed 35-47% of total TCDD TEQs in tomcod from the main stem of the Hudson River at Garrison.

Additionally, the ratio of total dioxins to furans differed between tomcod from the Hackensack River and Garrison. Dioxins predominated in the Hackensack River sample and furans in the main stem of the Hudson River. This indicates that the sources of dioxins and furans to the Hackensack River and the main stem of the Hudson River are different and that migration of tomcod between the Newark Bay complex and the main stem Hudson River is minimal.

As expected levels of total PCBs and co-planar PCB congeners were higher in tomcod from the Hudson River estuary than in conspecifics from the two Canadian rivers. For example, wet weight levels of hepatic PCB 77, PCB 126, and PCB169 were 18 fold and 40 fold higher in female tomcod from Garrison than in fish from the Miramichi and Margaree rivers, respectively. Surprisingly, levels of total co-planar PCBs were almost identical between adult tomcod from Garrison and the Hackensack River. This indicates the presence of significant local sources of bioavailable co-planar PCBs in the Newark Bay complex.

3. CYP1A1 mRNA was inducible by PCB 77 in tomcod larvae that were the offspring of laboratory matings of Hudson River parents, but not to the same magnitude as in the offspring of Miramichi River parents. This suggests that inhibition of CYP1A1 mRNA induction in adult tomcod from the Hudson River, at least in part, results from a physiological acclimation response (Roy et al. 2001; Roy et al. submitted).

Controlled laboratory matings were conducted with tomcod broodstock from the Hudson River and the Miramichi River and pure Hudson, pure Miramichi, and hybrid offspring were produced. Embryos and yolk-sac larvae from each group were exposed to waterborne PCB 77 (10 ppm), B[a]P (10 ppm), and vehicle and CYP1A1 mRNA expression was quantified in individual yolk-sac larvae using a newly developed competitive reverse transcriptase PCR (RT-PCR) technique. CYP1A1 mRNA was significantly inducible with PCB 77 in pure Hudson River larvae (9 fold), but not to the same extent as in pure Miramichi River (22 fold) or hybrid larvae. B[a]P highly induced CYP1A1 in larval tomcod from both rivers (47 and 52 fold), but once again gene expression was highest in tomcod of Miramichi descent. These results indicate that a physiological acclimation response probably inhibits CYP1A1 induction in adult Hudson River tomcod, although a multi-generational genetic adaptation may also have occurred in the Hudson River population.

This competitive RT-PCR was also used to measure CYP1A1 mRNA expression in individual, environmentally-exposed, post-yolk sac larvae from multiple sites in the main stem Hudson River. No difference in CYP1A1 expression was observed among larvae from multiple sites in the main stem Hudson River and surprisingly levels of gene expression in these Hudson River exposed larvae were not induced over those detected in hatchery-reared (unexposed) larvae. This suggests that environmental exposures of juvenile tomcod to aromatic hydrocarbons and bioaccumulation must occur when independent feeding begins.

4. Wet weight levels of dioxin, furan, and PCB congeners were lower in the eggs of

tomcod from the Hudson River and Miramichi River than in their livers; however, levels of these contaminants were still much higher in the eggs of Hudson River compared to Miramichi River tomcod (Roy et al. 2001).

The same technology as described above was used to quantify levels of individual dioxin, furan, and PCB congeners in the eggs of Hudson River and Miramichi River females and in their matched livers. Wet weight concentrations of all aromatic hydrocarbons was higher in the livers of fish from both rivers than in their eggs. For example, mean wet weight concentrations of total TCDD TEQs was five and three-fold higher in the livers than eggs of Hudson River and Miramichi River female tomcod, respectively. Similarly, wet weight levels of total co-planar PCBs were 5-6 fold higher in livers than in eggs of tomcod from both rivers. The higher wet weight levels of aromatic hydrocarbons in livers than in eggs suggests that some threshold level of contaminants must be reached before CYP1A1 transcription is impaired.

5. The tomcod CYP1A1 promoter region was cloned, sequenced, and its dioxin response elements (DREs) characterized (Roy et al. 1996).

The tomcod CYP1A1 promoter was the first characterized in a fish taxon and its structure proved very similar to that observed in mammals. Most importantly, tomcod CYP1A1 contained four DREs whose sequence (5'-TNGCGT-3') is identical to that observed in mammals. This suggested that the mechanisms of CYP1A1 transcriptional induction in fish are similar to those occurring in mammals. The binding of inducible proteins to the tomcod DREs was tested in gel mobility shift assays using protein extracts prepared from PCB77, B[a]P, and environmentally exposed tomcod from the Hudson River and the Miramichi River. Patterns of protein binding to the DREs were consistent with the CYP1A1 expression profiles in tomcod from the two populations. Inducible protein binding was observed in tomcod from the Miramichi River that were treated with PCB77 and B[a]P. Tomcod from the Hudson River only exhibited inducible protein binding when treated with B[a]P, not PCB77, suggesting that differences in CYP1A1 inducibility may be due to inter-population variation in functioning of the aryl hydrocarbon receptor (AhR) pathway. Interestingly, environmentally exposed Hudson River tomcod exhibited multiple DRE-binding proteins suggesting that other proteins in addition to AhR may participate in activating CYP1A1 expression in environmental situations.

6. The aryl hydrocarbon receptor gene was cloned and sequenced in a tomcod from the Hudson River (Roy and Wirgin 1997).

A single *AhR* gene was cloned and characterized from our tomcod genomic and cDNA libraries. At the time, it was among the first full length fish *AhR* genes reported in the literature. Tomcod *AhR* exhibited structure that was very similar to mammalian *AhR* including number of exons, overall deduced protein size, and moderate sequence conservation of functional domains including basic helix-loop-helix, PAS A, and PAS B. However, no similarity was observed between tomcod and mammalian *AhR* in the 3' transactivation domains. In total, these results suggested that the mechanisms of

CYP1A1 induction and mediation of toxic responses to dioxin exposure in fish and mammals are probably quite similar.

We also compared levels of *AhR* mRNA among tissues in tomcod from the Hudson River and Miramichi River that were untreatead or treated with dioxin or co-planar PCB congeners. That was done to determine if there are inter-population differences in *AhR* levels that might confer resistance to these toxicants and to initially evaluate if chronic exposure to high levels of pollutants in the Hudson River could down-regulate *AhR* levels and thereby alter sensitivities to these compounds. We found no significant difference in *AhR* mRNA levels among populations, tissues, or treatment groups suggesting that variation in *AhR* mRNA levels is not responsible for differences in sensitivities to dioxin-like compounds. Because, it is also possible that variation in AhR protein expression may occur among groups in the absence of variation in *AhR* mRNA expression, we developed an antibody against recombinant tomcod AhR protein. However, we found low levels of *AhR* expression in all tissues investigated suggesting that fish express lower levels of AhR protein than mammals or that the antibody was insufficiently sensitive to detect differences in AhR expression levels.

Using Southern blot analysis with a full-length tomcod *AhR* cDNA probe, we observed considerable variation in 3'*AhR* genomic DNA between tomcod from the Hudson River and elsewhere. As a result, exon specific tomcod*AhR* probes were developed which localized this variation to exons 10 and 11. For example, all tomcod from the Hudson River exhibited a 3.5 kb DNA fragment in *AhR* exon 11 which was absent in tomcod from the St. Lawrence, Miramichi, and Margaree rivers. Complete molecular characterization of these variants has continued and will be followed by functional analysis of variant alleles in *in vitro* transfection assays.

7. Hepatic CYP1A1 mRNA levels differed significantly among collections of young-of-the-year tomcod from multiple sites in the lower Hudson River estuary but did not correlate with total hepatic TCDD TEQs in the same fish (Yuan et al. 2001).

Young-of-the-year tomcod were collected from the Hackensack River, the apex of Newark Bay, and multiple Hudson River sites extending from river mile 0 to river mile 85. Levels of hepatic CYP1A1 mRNA were quantified in individual juvenile fish from 42 sites and hepatic concentrations of all dioxin, furan, and PCB congeners were quantified in a subset of these collections (n=11). Significant heterogeneity in CYP1A1 mRNA expression was observed among sites with highest levels observed in fish from the three sites in the Newark Bay complex. Lowest levels of gene expression were generally detected in fish from the most upriver sites in the main stem of the Hudson River. In some comparisons, levels of gene expression differed by 23-34 fold between tomcod from different sites in the estuary.

Surprisingly, hepatic levels of TCDD TEQS were higher in y-o-y tomcod (5-6 month old) from the Newark Bay complex than in adults collected from the same sites. This suggests that exposure and bioaccumulation occurs very early in life. Although levels of dioxins, furans, and PCBs expressed as TCDD TEQs and CYP1A1 mRNA were

highest in tomcod from the Newark Bay complex, there was no relationship between hepatic halogenated aromatic hydrocarbon levels and hepatic CYP1A1 mRNA expression in tomcod from sites in the main stem Hudson River. This indicates that levels of CYP1A1 mRNA expression in fish from sites highly polluted with mixtures of halogenated aromatic hydrocarbon and other contaminants may not always be reflective of bioavailable xenobiotics. These results also suggest that induced CYP1A1 mRNA expression in tomcod from the Hudson River may be due primarily to other contaminants not measured in this study, such as PAHs.