Fish as Vertebrate Models of Dioxin Toxicity

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Introduction

Historically research on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity and the aryl hydrocarbon receptor (AhR) signaling pathway in fish has lagged by more than a decade behind that in mammals. It was research in laboratory mammals, not fish, which established that TCDD toxicity is AhR-mediated. After TCDD binding, the activated AhR translocates to the nucleus and dimerizes with the AhR nuclear translocator (ARNT) to increase transcription of genes containing dioxin response elements (DREs). AhR and ARNT are members of the PAS family of transcription factors that include SIM, PER, HIF 1α, EPAS-1, AHR-1, AHA-1, CLOCK, NPAS, and TRH (1). Members of this family, found in both vertebrates and invertebrates, may act as molecular and environmental sensors to regulate essential growth and developmental pathways and it is intriguing to speculate similar roles for AhR and ARNT (1).

The relatively recent emergence of zebrafish as a model species for studying molecular genetics and development in vertebrates, along with the finding that fish have a functional AhR signaling pathway and are responsive to TCDD toxicity (2), has increased interest in using fish as vertebrate models for investigating molecular mechanisms of TCDD toxicity and physiological functions of the AhR signaling pathway. Since early life stages of fish are more susceptible to TCDD toxicity than juveniles or adults (2), the focus of the present study is on the developmental toxicity of TCDD and related compounds in fish. We discuss two fish models, one that has greater ecological relevance, the rainbow trout/lake trout model (2,3), and another that is more advantageous experimentally because of the wealth of information on its genetics and development biology, the zebrafish model(4). We compare signs of TCDD developmental toxicity in the trout and zebrafish and discuss cloning of the AhR and AhR nuclear translocator (ARNT) cDNAs in each species and functional characterization of the respective proteins. Lastly, use of fish-specific, relative potencies (*REPs*), determined in this laboratory for trout early life stage mortality (5), is illustrated for

characterizing the risk of mortality in feral Great Lakes lake trout embryos exposed to polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs).

Materials and Methods

References for methods used in conducting the studies are as follows: characterization of TCDD early life stage toxicity (2,3,4), determination of REPs for PCDDs, PCDFs and PCBs based on early life stage mortality (5), assessment of toxic interactions between mixtures of PCDD, PCDF, and PCB congeners (6,7), cloning of the AhR and ARNT cDNAs and functional characterization of the proteins (8,9,10,11), and application of the toxicity equivalency factor (TEF) approach for describing the risk of embryo mortality associated with contamination of Great Lakes lake trout eggs with PCDDs, PCDFs, and PCBs (12,13).

Results and Discussion

Developmental Toxicity of TCDD in Trout and Other Fish Species. Signs of toxicity in lake trout and rainbow trout early life stages, exposed as fertilized eggs to TCDD, were manifested during the sac fry stage as cardiovascular toxicity characterized by reduced perfusion of tissues with blood, yolk sac, pericardial and meningeal edema, hemorrhages, craniofacial malformation, and arrested development of skeletal and soft tissues culminating in mortality (2,3). The TCDD toxicity syndrome is virtually identical to blue sac disease in salmonids which has an unknown etiology. In TCDD-exposed trout sac fry that survive, swim bladder inflation is often impaired. While generally similar signs of TCDD developmental toxicity occur in the 10 freshwater fish species investigated thus far (lake trout, brook trout, rainbow trout, fathead minnow, channel catfish, lake herring, medaka, white sucker, northern pike, and zebrafish) there are wide differences across these species in sensitivity to TCDD-induced early life stage lethality (14). Lake trout is the most sensitive species with egg concentrations of TCDD in the range of 30-100 pg/g causing embryo mortality and zebrafish is the least sensitive species requiring a 40-fold higher egg dose of TCDD than lake trout to cause the same effect (2,3,4). All PCDD, PCDF, and non-ortho PCB AhR agonists, tested as single compounds or as mixtures, produce identical signs of early life stage toxicity in rainbow trout (2,5,7). Potencies of individual PCDD, PCDF and non-ortho PCB congeners, relative to TCDD, in causing rainbow trout early life stage mortality are generally similar to the relative potencies in mammals (2,5). However, a notable exception is the mono-ortho PCBs which are not active in causing early life stage toxicity in trout (2,5). Rainbow trout and lake trout eggs exposed to graded doses of complex mixtures of PCDD, PCDF, and PCB congeners that are found in feral lake trout eggs in the Great Lakes have shown that the congeners interact in an essentially additive fashion to cause early life stage mortality (7).

Developmental Toxicity of TCDD in Zebrafish. As with trout, research on zebrafish embryos exposed to waterborne TCDD for 1 hour, 3-5 hours post fertilization (hpf), has shown that the cardiovascular system is a primary site of toxicity (4). Reduced perfusion in vascular beds of the head, trunk and gills was observed 72 hpf, and at a higher egg dose of TCDD, as early as 48 hpf in the caudal vein. Yolk sac and pericardial edema, craniofacial malformations, and failure of the swim bladder to inflate occur later. Mortality in the zebrafish embryos is typically delayed until 68-240 hpf (7-10 days). This is considerably less time than the 46-56 days after egg fertilization

needed for TCDD-exposed rainbow trout to exhibit mortality or the 180 days needed for lake trout. For investigating molecular mechanisms of TCDD development toxicity, zebrafish have several advantages over trout and other vertebrates: they are easily cultured, develop rapidly, produce large numbers of synchronously developing embryos daily, are transparent permitting easy observation of developmental events, their development has been extensively studied, hundreds of mutants from systematic saturation mutagenesis screens are available, and genetic screens based on phenotype are possible. In the latter regard, generation of a pool of mutant zebrafish, each one differentially insensitive to a specific sign of TCDD developmental toxicity, should be useful in elucidating molecular pathways that lead to these developmental defects.

Cloning of the AhR and ARNT from Rainbow Trout and Zebrafish. To determine in fish the molecular basis for the species difference in sensitivity to TCDD, lack of toxicity of the mono-ortho PCBs, and to understand the various potential roles of the AhR and ARNT in normal development, it is essential to characterize the AhR signaling pathway. We have cloned AhR and ARNT from both zebrafish and rainbow trout (8,9,10,11). There are several differences between the fish and mammalian AhR and ARNT proteins. We have found multiple forms of both AhR and ARNT in fish. We have cloned three rainbow trout and one zebrafish AhR. Comparison of the amino acid sequences reveal that the C-terminal domain of fish AhRs are approximately 200 amino acids longer and do not contain a Q-rich transactivation domain found in mammalian AhRs. The trout and zebrafish AhRs are expressed and functional in the early embryo suggesting a potentially important role for AhR in normal development. Interestingly, unlike mammals, TCDD exposure increases AhR mRNA in zebrafish and rainbow trout (10,11). We have also identified multiple forms of ARNT in zebrafish and rainbow trout. In rainbow trout, two alternately spliced ARNTs with different C-terminal domains (ARNTa and ARNTb) have been partially characterized (8). Both ARNTa and ARNTb interact with the AhR and DREs, but only AhR/ARNTb dimers have transactivation activity. Furthermore, ARNTa acts as a dominant negative transcription factor (8). Recently we have also begun to characterize three forms of ARNT from zebrafish (Tanguay, unpublished results). Comparison of the amino acid sequence reveals that two of the clones are most similar to ARNT1 and ARNT2 from rat, and the third clone is a truncated form of ARNT2 ($ARNT2_t$). $ARNT2_t$ mRNA is highly expressed and may have impaired function since the protein terminates within the PAS B region and lacks the entire C-terminal transactivation domain.

The TEF Approach and Early Life Stage Mortality Risk Characterization in Lake Trout. The TEF method has been proposed for characterizing the risk of mortality to fish early life stages caused by exposure to PCDDs, PCDFs and PCBs (12). The TEF method describes the potency of individual congeners relative to TCDD and calculates a TCDD equivalents (TEQ) concentration for the mixture based on concentrations of congeners in eggs. The TEF method for trout early life stage mortality risk assessment was validated by determining early life stage mortality-specific REPs for PCDD, PCDF and PCB congeners in rainbow trout (5), showing that congeners interact additively to produce the response (6,7), and finding that REPs determined in rainbow and lake trout are similar (12). The TEF method was used in a retrospective risk assessment of early life stage mortality in Lake Ontario lake trout (13). In the 1940s populations of lake trout in Lake Ontario declined and by 1960 were virtually extinct. Subsequent stocking with yearling trout resulted in a large adult population after the 1970s, but survival of sac fry to the yearling stage (recruitment) was not detected until recently. The finding that lake trout early life stage mortality

occurs at relatively low parts per trillion concentrations of TCDD in lake trout eggs (2,3), led to an investigation of a TCDD etiology for Lake Ontario's lake trout problems (13). Concentrations of TCDD and related compounds determined in archived lake trout collected since 1978 indicated that exposures of embryos to TCDD in the 1970s should have caused extensive sac fry mortality. To estimate lake trout egg concentrations of TCDD and related compounds farther back in time, radionuclide-dated 1 cm segments of sediment cores from Lake Ontario were analyzed. Biota-sediment accumulation factors (BSAFs) were measured for lake trout eggs and used with the sediment core data to predict retrospectively lake trout egg concentrations of TCDD and related chemicals from 1920 to 1987 (13). The additive TEF approach was then used to calculate TEQ concentrations in lake trout eggs over time. From results of laboratory toxicity studies on lake trout, early life stage mortality was predicted to increase from 0-100% as TEQ concentrations in eggs increase from 30-100 pg/g (2-3). 100% mortality was predicted from the 1940s into the 1970s. However, based on the recent improvement in lake trout early life stage survival and expected decreases in egg exposures, any residual impairment of lake trout natural reproduction by TCDD and related compounds in Lake Ontario may be completely eliminated in 5-10 years.

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