MOLECULAR AND CELLULAR APPROACHES TO UNDERSTANDING THE SENSITIVITY OF MARINE MAMMALS TO PERSISTENT ORGANIC POLLUTANTS

Mark E. Hahn,[1] Brenda A. Jensen,[1] Eun-Young Kim,[1,2]
Sibel I. Karchner[1], Diana G. Franks[1], Joy M. Lapseritis[1],
Kristen E. Whalen[1], and Michael J. Carvan[3]

[1]Woods Hole Oceanographic Institution, Woods Hole, MA, U.S.A.
[2]Ehime University, Matsuyama, Japan
[3]University of Wisconsin-Milwaukee, Milwaukee, WI, USA

Introduction

Numerous persistent organic pollutants (POPs) are distributed globally, accumulate in animal tissues, and have the potential to disrupt the reproduction and development of wildlife. Some of these compounds, including certain planar halogenated aromatic hydrocarbons (PHAHs), have been suggested as causative factors in recent episodes of mortality and morbidity in aquatic mammals and birds.[1, 2] However, the magnitude of the risk that PHAHs pose to the health of aquatic vertebrates is controversial and has not yet been established for most species.

Assessing the risk of chemical exposure to wildlife is complicated by a number of factors. It is well known that there are dramatic differences in PHAH sensitivity among vertebrate species.[3-5] However, for most aquatic mammals and birds there is little or no direct information on their sensitivity to PHAHs, because legal and ethical concerns preclude the direct testing of toxic chemicals on protected animals. In addition, logistical challenges hinder systematic sampling for detailed epidemiological analyses. Thus, alternative approaches for assessing the susceptibility of these species to effects of PHAHs and other POPs are needed.

Many POPs act by interfering with specific receptors for hormones, growth factors, and other signaling molecules. We propose that the species-specific, molecular/biochemical characterization of proteins and pathways involved in mechanisms of POP toxicity can contribute to risk assessment by linking mechanistic studies in rodents to observational findings in wildlife. This approach involves the cDNA cloning, *in vitro* expression, and functional characterization of receptors and enzymes involved in toxicity, complemented by experiments performed using primary cell cultures or cell lines derived from target species. We illustrate this approach using the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor through which PHAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) cause altered gene expression and toxicity.

cDNA cloning and in vitro characterization of the AHR in wildlife.

Some marine mammals accumulate extremely high concentrations of PHAHs in their tissues, but the sensitivity of marine mammals to these chemicals is not well known and cannot be determined directly. To infer the sensitivity of marine mammals from biochemical data, we have cloned AHRs from an odontocete, the beluga *Delphinapterus leucas,* and a pinniped, the

harbor seal *Phoca vitulina*. The beluga AHR cDNA encodes an 845 amino acid protein that shares 85% identity with the human AHR and 75% identity with the mouse AHR Ah^{b-1} allele.[6] Beluga AHR protein synthesized *in vitro* bound [^{3}H]TCDD with an affinity that was at least as high as that of the mouse AHR and significantly greater than that of the human AHR. Comparing the beluga AHR affinity with concentrations of AHR ligands (TCDD-EQs) in beluga tissues suggests that levels of receptor occupancy are sufficient for effects to occur. Measurement of competitive AHR binding affinities for a series of PHAHs has provided the first assessment of beluga-specific relative potencies (RPs) and revealed close agreement between beluga-specific RPs and RPs determined using the mouse AHR (Jensen & Hahn, manuscript in preparation). The harbor seal AHR contains 843 amino acids and shares 82% and 79% identity with beluga and human AHRs, respectively[7]. Like the beluga AHR, the seal AHR bound [^{3}H]TCDD with high affinity, consistent with experimental studies showing that seals may be sensitive to PHAH effects[8]. An AHR cDNA has also been cloned from Baikal seal[9].

There are dramatic differences in sensitivity to PHAHs among species of birds[5]. For example, common terns are ~80-fold less sensitive than chickens to effects of PHAHs[5, 10]. To investigate the molecular mechanism of differential PHAH sensitivity, we have cloned and sequenced AHR cDNAs from white leghorn chicken (*Gallus gallus*) and common tern (*Sterna hirundo*) [11]. The chicken AHR cDNA encodes a protein of 858 amino acids (96.2 kDa); the tern AHR is 859 amino acids (96.3 kDa). Chicken and tern AHRs share 93% amino acid identity overall, and 98% in the ligand binding domain. Chicken and tern AHRs synthesized by *in vitro* transcription and translation exhibited specific binding of [^{3}H]TCDD. However, saturation binding analysis (0 - 10 nM [^{3}H]TCDD) showed that the binding affinity of the tern AHR was approximately 7-fold lower than that of the chicken AHR. Similarly, the tern AHR displayed reduced ability to activate transcription of a dioxin-responsive luciferase reporter construct in a transient transfection assay. Domain-swapping experiments and site-directed mutagenesis showed that two amino acids in the ligand-binding domain were responsible for the difference in affinity between chicken and tern AHRs (Karchner *et al*., manuscript in preparation). We conclude that differences in the TCDD-binding affinity and other properties of the common tern AHR contribute to the reduced sensitivity of this species to PHAH effects. Together, these results obtained for marine mammals and marine birds show that the use of *in vitro*-expressed proteins is a promising approach for understanding and predicting the molecular basis of PHAH toxicity in wildlife.

Molecular and biochemical data from skin biopsies

Some aquatic mammals are highly endangered or extremely rare. For such species, obtaining tissue samples that can be used for the *in vitro* studies like those described above can be difficult. For example, the western North Atlantic right whale (*Eubalaena glacialis*) is highly endangered, with a known population of approximately 300 individuals and a low rate of reproductive success[12]. Right whale tissues are seldom available for study. However, skin/blubber biopsies are sometimes taken for genetic studies[13, 14]; right whale skin biopsies[15] and those of other cetaceans[16] have also been used to measure expression of cytochrome P450-dependent monooxygenases as a marker of chemical exposure. We tested the feasibility of using right whale skin/blubber biopsies as a source of RNA for use in identifying, isolating, and characterizing contaminant susceptibility genes and other biomarker genes related to contaminant effects and physiological condition. RNA was purified from right whale biopsies and a reverse transcription-polymerase chain reaction (RT-PCR) approach was used to clone a complementary DNA (cDNA) encoding the right whale aryl

hydrocarbon receptor (AHR) protein (Lapseritis & Hahn, manuscript in preparation). These results show that RNA of sufficient quality to clone and sequence contaminant susceptibility genes can be obtained from whale skin biopsies. Our goal is to use this information to better understand the possible role of contaminants in the decline of the North Atlantic right whale. Future studies could apply this approach to examine other biomarker genes related to contaminant effects and physiological condition, and the relationship of gene expression and function to cetacean health.

Marine mammal cells in culture

Studies using primary cell cultures or cell lines have been instrumental in providing a mechanistic, species-specific understanding of PHAH effects in rodents and humans[17, 18]. The availability of cultured cells from marine mammal species would greatly facilitate studies to understand species-specific aspects of POP toxicity in these animals. However, currently there is a dearth of marine mammal cell lines that can be used for this purpose.

We have evaluated the possibility of using cells obtained from stranded animals to study molecular responses to PHAH exposure. Using tissues dissected from stranded, euthanized animals (or in the case of beluga, animals harvested in subsistence hunts), primary cultures of kidney cells were established from false killer whale (*Pseudorca*), striped dolphin (*Stenella*), white-sided dolphin (*Lagenorhynchus*), common dolphin (*Delphinus*), and beluga (*Delphinapterus*). Attempts to culture cells from other tissues (e.g. liver) have not yet been successful. In addition to the primary cell cultures, a kidney cell line (CDK) derived from the bottlenose dolphin (*Tursiops truncatus*) [19] is also being utilized. These cells express an AHR[19] that exhibits saturable, high-affinity binding to [^{3}H]TCDD (Whalen, Hestermann, Jensen, and Hahn, unpublished data). In several experiments in which these cells were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other AHR ligands, no cytochrome P450 1A1 (CYP1A) induction response could be detected by measurement of CYP1A catalytic activity (ethoxyresorufin O-deethylase activity) or immunodetectable CYP1A protein. Incubation with the demethylating agent 5-aza-2'-deoxycitidine, which restores CYP1A1 inducibility in other cells,[20] was not effective in CDK cells. Currently, we are evaluating other treatments that might restore the CYP1A1 inducibility of these cells, as well as other approaches whereby cetacean cells can be used to obtain a better understanding of the responsiveness and sensitivity of these animals to PHAHs.

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