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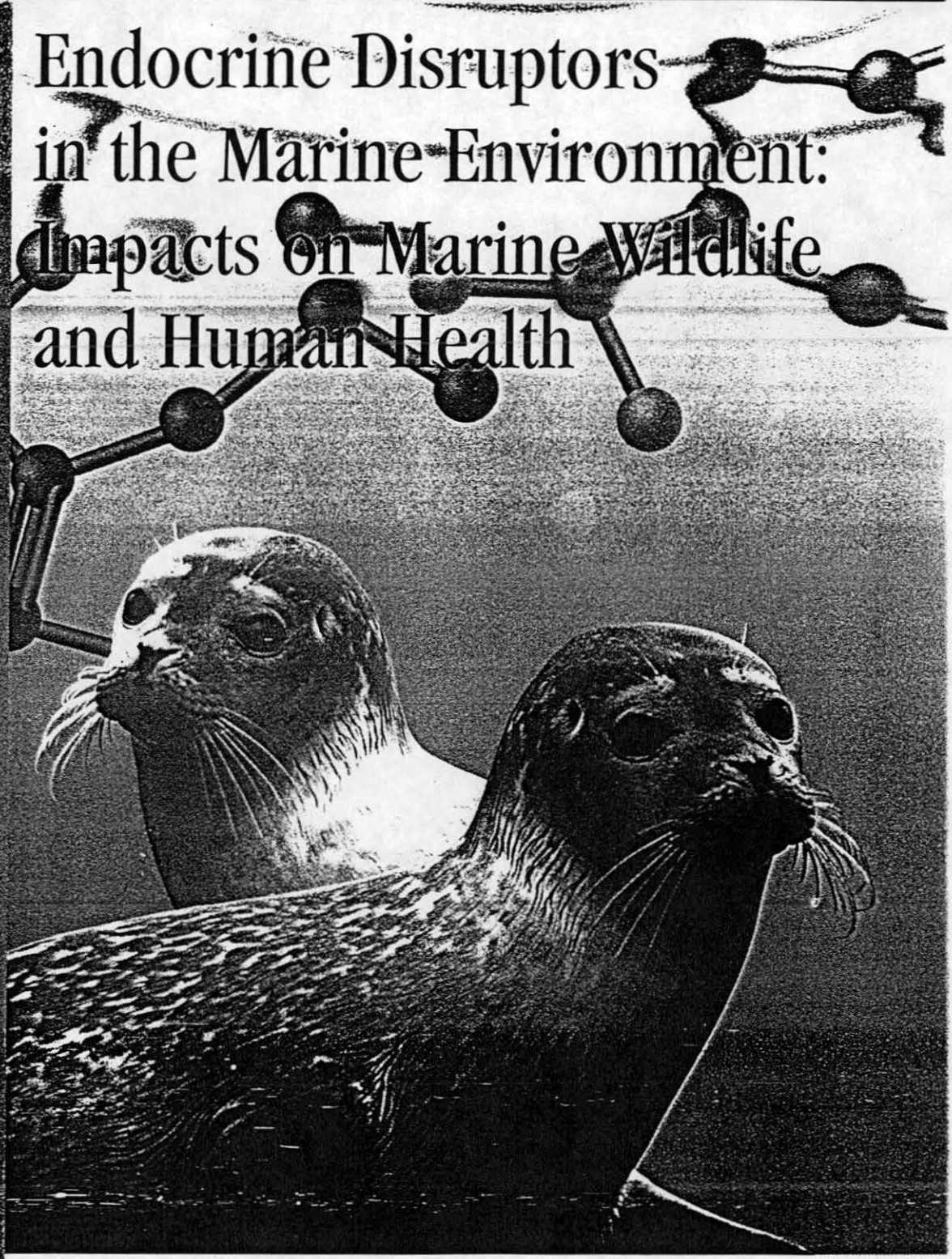
Endocrine Disruptors in the Marine Environment: Impacts on Marine Wildlife and Human Health



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The Ah Receptor: Comparative Biochemistry and Possible Role as a Biomarker of Susceptibility to PHAH

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Introduction

Halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls (PCBs), chlorinated dibenzo-p-dioxins, and related compounds, are ubiquitous contaminants of the global environment. Some HAHs, especially the so-called planar HAH (PHAH) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are highly toxic to many vertebrate species. Some populations of marine mammals are highly exposed to PHAH, and these contaminants have been suggested to contribute to marine mammal mortality and morbidity. The magnitude of the risk that PHAH pose to the health of marine mammals is uncertain, however, because there is little direct information on the sensitivity of these animals to PHAH. Thus, alternative approaches are needed for assessing the susceptibility of these species to effects of these environmental contaminants.

Here we present and discuss the hypothesis that the sensitivity of marine mammals to PHAH can be predicted from an understanding of the comparative biochemistry of proteins involved in the molecular mechanisms of PHAH action. PHAHs cause toxicity through activation of the aryl hydrocarbon receptor (AHR)-dependent signal transduction pathway. Studies in laboratory animals have shown that the expression (i.e. tissue concentration) and properties (e.g. dioxin-binding affinity) of the AHR can influence the sensitivity of animals to these compounds. Can the characterization of AHRs in marine mammal species help predict their sensitivity to PHAH? Is the AHR a good "biomarker of susceptibility" in wildlife? We explore these questions in light of recent data on the comparative biochemistry and molecular biology of the AHR and the relationship between AHR properties and differential sensitivity to PHAH. We describe an approach and preliminary results involving AHR cloning, *in vitro* expression, and functional analysis of marine mammal AHRs. The data obtained may provide information of use in assessing the risk of environmental contaminants to protected species, including marine mammals.

PHAH in marine mammals

Numerous species and populations of marine mammals exhibit high levels of PHAH in blubber and other tissues (1, 2). Exposure to PHAH and other organic contaminants has been suggested as a causative factor in cases of marine mammal mortality and morbidity, including reproductive abnormalities, immune dysfunction, and carcinogenesis (1, 2). However, with few exceptions (e.g. 3, 4), direct information concerning the effects of such exposures is lacking (5).

At a recent workshop to assess the impact of persistent organic contaminants on marine mammal health (2), the wealth of data on chemical residues in marine mammal tissues was contrasted with the dearth of biological and toxicological information that would help in interpreting the chemical data. Among the several research needs and recommendations made at the workshop were: (i) the need for a better understanding of processes linking exposure to effects (including subcellular mechanisms), (ii) the need for development and validation of biomarkers, and (iii) the use of model marine mammal species as surrogates for the major marine/aquatic mammal groups.

Mechanism of PHAH action: the Aryl Hydrocarbon Receptor

TCDD and other PHAHs are thought to produce toxicity through changes in the expression of genes involved in the control of cell growth and differentiation. These changes are initiated by the binding to the AHR, a ligand-activated transcription factor (6-8). The AHR and its dimerization partner ARNT (AHR nuclear translocator) belong to the basic-helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of transcriptional regulatory proteins (9, 10). Members of this gene family play important roles in the initiating physiological responses to changing environmental conditions (9, 10).

Multiple lines of evidence indicate that the AHR has an essential role in PHAH toxicity. Initially, the importance of the AHR was inferred from studies showing that differences in PHAH sensitivity among mouse strains were related to the presence of high- or low-affinity AHR alleles. In addition, a strong correlation was found between AHR binding affinity and toxic potency for a series of PHAHs, showing that the AHR is a controlling factor in their toxicity. More recently, molecular and genetic studies of the AHR have revealed important mechanistic details about the AHR-dependent signaling pathway (11) and have shown definitively that the AHR is necessary for TCDD toxicity (12).

Species- and strain-differences in PHAH sensitivity and structure-activity relationships

There are dramatic species and strain differences in susceptibility to effects of TCDD and other PHAHs. The differences among mammalian species in sensitivity to TCDD lethality are well known; a 5000-fold difference in LD50 values separates the sensitive guinea pig from the resistant hamster. These differences in lethality are not necessarily reflected in the range of sensitivities to other effects (13, 14). However, in mice, the 5- to 15-fold difference among strains in the LD50 for TCDD parallels a similar difference in sensitivity to sublethal effects of TCDD (15, 16). Inter-species variability in sensitivity to PHAH effects is also seen in other vertebrate classes, including fish (40-fold), birds (100- to 1000-fold), and amphibians (17-19). In light of these large differences, it is difficult to predict the impact of current PHAH burdens on marine mammal health.

In addition to the species differences in absolute sensitivity, there exist also species differences in the relative potencies of selected PHAH congeners as compared to TCDD. This is true especially for the mono-*ortho*-substituted PCBs (18, 20, 21). These congeners exhibit substantial dioxin-like activity in some species, but not in others. Such differences are important for risk assessment, as these congeners often contribute a significant fraction of the total dioxin equivalents in marine mammal

tissues (as calculated using toxic equivalency factors (TEFs) derived from rat experiments).

Role of the AHR in differential dioxin sensitivity

There is little dispute that most of the acute effects of TCDD and related PHAH occur through the AHR (7). Recently, targeted disruption of the AHR gene—producing "AHR knock-out" mice—has confirmed that PHAH toxicity requires a functional AHR. Mice bearing the disrupted AHR gene are much less sensitive to the biochemical, lethal, and teratogenic effects of TCDD (12, 22-24). This essential role of the AHR suggests that the presence, expression, or properties of AHRs (or other components of the AHR-dependent signal transduction pathway) may control the sensitivity of animal species to the effects of PHAHs. Consistent with this idea, there are several examples in which differences in AHR expression or characteristics appear to be the primary determinant of differential dioxin sensitivity.

Mouse strains classified as "responsive" and "nonresponsive" (to PHAH and PAH) express distinct AHR alleles (25, 26) and the 5- to 15-fold difference in TCDD sensitivity of the two strains can be explained by the ~9-fold difference in TCDD-binding affinities of their respective AHR proteins (27). A single amino acid change appears to be responsible for the altered ligand-binding affinity (28).

In TCDD-sensitive and TCDD-resistant rat strains, an AHR polymorphism is linked to physicochemical differences in the protein products of two AHR alleles (29, 30). Genetic analysis showed that of several factors contributing to the resistant phenotype, the AHR locus was the most important (31).

The sensitivity of humans to PHAH has been an important and controversial topic for many years. Although this issue is not yet resolved, some human cell lines appear to be approximately 10-fold less sensitive than rodent cells to PHAH, and this difference is associated with a similar difference in TCDD-binding affinity of the human AHR (reviewed in 32).

AHR ligand-binding affinities have also been measured in birds (33). The 15-fold higher affinity for TCDD of the chicken AHR as compared to AHRs from great blue heron and double-crested cormorant is similar to the difference in sensitivity to the *in ovo* CYP1A-inducing potency of TCDD in these species. The results of this and other studies in birds (34) are consistent with the hypothesis that AHR expression and/or ligand-binding affinity is an important determinant of PHAH susceptibility.

Of course, the AHR is not the only factor that can influence the susceptibility to PHAH effects. Some variations in TCDD sensitivity among mammalian species are not explained solely by differences in biochemical characteristics of their respective AHRs (35). Altered expression or function of other components of the AHR-dependent signal transduction pathway can also influence responsiveness (reviewed in 36). In addition, other species-specific characteristics (e.g. biotransformation activities, pharmacokinetic differences) may also be important, especially with respect to PHAH structure-activity relationships (37, 38). Nevertheless, it is clear that the AHR plays an important—and possibly primary—role in determining susceptibility to PHAH toxicity.

The AHR as a Biomarker of Susceptibility

Biomarkers are biochemical, physiological, or other types of biological changes that indicate the presence or effects of xenobiotic compounds (39-41). In addition to the commonly used biomarkers of exposure and effect, which are especially useful in biomonitoring, some biological characteristics can be used as biomarkers of susceptibility (42, 43). In light of the evidence discussed above, we propose that the AHR might be useful as a biomarker of susceptibility to PHAH toxicity in marine mammals. What do we know about the AHR in marine mammals and other wildlife species?

Comparative Biology of the Ah Receptor

The AHR has been extensively characterized in laboratory mammals (6), but much less is known about this protein in wildlife species, including marine mammals, non-mammalian vertebrates and invertebrates (9). AHR cDNAs have been cloned from birds (44), fish (45-50), and an amphibian (44). Fish are particularly interesting because they express two AHR genes, in contrast to the single AHR identified in other vertebrate groups (45, 46). Invertebrate AHR homologs are known to exist (46, 51, 52), but their ligand-binding properties appear to differ substantially from those of vertebrate AHRs (51, 53).

Little is known about the presence or properties of AHRs in aquatic mammals. One might expect that cetaceans (whales), pinnipeds (seals, sea lions, walruses), sirenians (manatees, dugongs), mustelids (mink and otter), and ursids (polar bears) would express AHR proteins closely related structurally and functionally to those in other mammalian taxa. However, these five groups represent distinct evolutionary lineages within class mammalia (54). Given the heterogeneity that is known to exist among other mammalian AHRs (e.g. 35, 55), it is important to establish the features of AHR signaling in each of these aquatic mammal groups.

Indirect evidence of AHR function in aquatic mammals has come from studies of cytochrome P4501A (CYP1A) expression in relation to PHAH burdens. For example, White *et al.* (56) found a strong correlation between the content and activity of CYP1A in beluga (*Delphinapterus leucas*) liver and the concentration of selected PCBs in blubber of these animals, suggesting activation of the AHR. Similar results were obtained for polar bear (*Ursus maritimus*) (57).

Two laboratories have more directly identified an AHR in cetaceans by measuring specific binding of radioligands *in vitro*. Specific binding of [^{3}H]TCDD was observed in a kidney cell line from the bottlenose dolphin (*Tursiops truncatus*) (58) and the AHR photoaffinity ligand [^{125}I]N$_3$Br$_2$DD was used to identify an AHR in beluga (59).

In vitro expression and functional analysis of cloned AHRs: A new approach in comparative toxicology

AHR cloning and expression in marine species

The use of reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers is a powerful approach for isolating homologous cDNA sequences from new species (60). We have used

this method to clone AHR homologs from mammals, fish, birds, an amphibian, and several invertebrates (9, 45-47, 61). To evaluate AHR function and make comparisons among species, we have established systems for the *in vitro* expression and functional analysis of cloned AHRs. These systems utilize *in vitro* transcription and translation reactions together with ligand-binding assays. One advantage of this approach is that the AHRs from several species (including laboratory rodents, humans, and marine mammals) are all expressed under identical conditions, facilitating comparisons. Using this approach, we have expressed fish and mammalian AHRs and studied their dioxin binding activity *in vitro* (45, 49).

Cetacean AHRs

The RT-PCR approach described above was used to obtain the full-length AHR sequence from beluga and a partial AHR sequence from the white-sided dolphin (*Lagenorhynchus acutus*) (61). The beluga AHR—the first AHR cloned from any marine mammal—is 845 amino acids and shares 75% and 85% amino acid identity with the mouse and human AHRs, respectively.

Beluga AHR protein synthesized by *in vitro* transcription and translation demonstrated specific, high affinity [^{3}H]-TCDD binding. In a charcoal-dextran binding assay with varying concentrations of [^{3}H]-TCDD, the binding affinity of the beluga AHR was compared with that of an AHR from a dioxin sensitive mouse strain and with the human AHR. In this comparison, the beluga AHR bound ligand with an affinity that was at least as high as that of the mouse AHR (high-affinity allele), and substantially greater than that of the human AHR (61).

Harbor Seal AHR

Using the RT-PCR approach described above, we also have obtained a full-length AHR sequence from the harbor seal (E.-Y. Kim and M.E. Hahn, unpublished data). The seal AHR shares a high degree of amino acid identity with the human and beluga AHRs. Although binding affinities have not yet been measured, the seal AHR exhibits strong, specific binding of [^{3}H]-TCDD.

The results of these initial studies demonstrate that there is a high degree of conservation of AHR structure between terrestrial and some marine mammals, and suggest that the mechanism of dioxin toxicity may be similarly conserved in these two groups. Our results further suggest that beluga, and perhaps cetaceans generally, may be among the more sensitive mammalian species to effects of PHAHs. Further understanding the comparative biochemistry of the AHR in marine mammals may provide insight to the potential sensitivity of these animals to these ubiquitous environmental contaminants.

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