Endocrine Disruptor Susceptibility Genes: Molecular Analysis of Aryl Hydrocarbon Receptors and Dioxin Sensitivity in Wildlife

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Introduction

Some species or populations of wildlife are known to accumulate high levels of chemical contaminants, including persistent organic pollutants. Many of these chemicals have been shown to cause toxicity, including endocrine disruption, in laboratory animals such as rats and mice.

Despite the known effects of these chemicals in experimental animals, it has been difficult to assess the impact of these chemicals in wildlife, especially protected species for which direct toxicity testing is not possible.

Assessing the risk of chemical exposure to wildlife therefore requires extrapolation from what we know in other animals, especially laboratory rodents. But there is uncertainty inherent in the process of extrapolation. In general, we can expect overall similarities in the mechanisms involved in endocrine disruption, and this is the basis for extrapolation. But there may be differences in important mechanistic details that affect our ability to extrapolate accurately from one species to another. For example, there are sometimes large species differences in sensitivity to chemicals, which may be due to differences in capacities for biotransformation or to differences in properties of the cellular targets (e.g. proteins) of the chemical.

One approach to dealing with species differences in sensitivity is to develop "biomarkers of susceptibility" to chemicals. This approach is being used in human health assessment, where protein polymorphisms are being studied as markers of the susceptibility of individual humans to disease. Similarly, species-specific differences in the properties of proteins that are involved in mechanisms of toxicity might be used to predict sensitivity of wildlife. We propose that species-specific cloning and characterization of genes involved in toxicity can contribute to risk assessment by linking mechanistic studies in rodents to epidemiological findings in wildlife.

One group of endocrine-disrupting chemicals that continues to be of concern worldwide are the dioxin-like chemicals, including polychlorinated dibenzo-dioxins, dibenzofurans, and the planar (non-ortho-substituted) polychlorinated biphenyls (PCBs). These chemicals are considered endocrine disruptors because of their effects on reproduction and development and their interference with the functioning of endocrine pathways, including those involving estrogens and androgens.

These chemicals act by a well-known mechanism involving the aryl hydrocarbon receptor (Ah Receptor or AHR), a ligand-activated transcription factor. Dioxins and coplanar PCBs act through the AHR to cause changes in gene expression, which include adaptive responses, such as the induction of cytochrome P450s, as well as changes in the expression of other genes that may lead to toxic alterations in cell growth or function.

We are studying the AHR as a potential susceptibility gene in wildlife because of its demonstrated role as a dioxin susceptibility gene in rodents. Several pieces of evidence support this hypothesis. First, the AHR is necessary for dioxin toxicity in mice, as demonstrated by the insensitivity of AHR-knock-out mice to dioxin effects. Second, studies in mouse strains have shown that properties of the AHR can

determine the sensitivity of those strains to dioxin. Third, the affinity for the AHR is the primary factor determining the structure-activity relationships of dioxin-like chemicals for causing toxicity. Based on this understanding, we propose that the AHR can be studied as a biomarker of susceptibility (or susceptibility gene) in wildlife.

The goal of research in my laboratory is to characterize the Ah receptor signaling pathway in wildlife and its role in determining the sensitivity of wildlife to dioxin-like chemicals. This work involves the cloning and sequencing of AHR cDNAs, subcloning of the cDNAs into expression plasmids, expression of the protein by *in vitro* transcription and translation, and functional characterization of the expressed proteins. Our current focus is on the AHR, but this approach can be applied also to other proteins, including receptors for steroids and other hormones. The work in our laboratory involves a variety of species, including marine mammals, birds, fish, and invertebrates. Today I will present an overview of two sets of studies—one involving marine mammals and the other, birds.

AHR in beluga whale

In one set of studies we are investigating the AHR pathway in the beluga whale. This species is of interest because of an endangered population inhabiting the St. Lawrence estuary in eastern Canada. This population is highly exposed to a variety of chemicals, including dioxin-like chemicals. Studies of this population have shown pathological and reproductive abnormalities that are suspected to be due to the chemical exposure. In addition, biochemical effects have been observed in other populations of beluga and have been linked to chemical exposure. In these experiments, we asked whether beluga express a high-affinity AHR protein.

Brenda Jensen in my laboratory cloned and sequenced a beluga AHR cDNA. This cDNA encodes a 95 kDa (kilodalton) protein, approximately the same size as the human AHR. The beluga AHR shares high amino acid identity with human and mouse AHRs, especially in regions of the protein that are known to be functionally important. These regions include the basic-helix-loop-helix (bHLH) region, which is important for DNA binding, and the PAS domain, which is important for ligand binding.

We used this cloned AHR cDNA to express the beluga AHR protein by *in vitro* transcription and translation, and compared it to the mouse and human AHRs expressed in the same way. When we did this in the presence of ^{35}S-methionine, the labeled proteins could be visualized on denaturing gels, confirming synthesis of the protein. We also showed that the *in vitro*-synthesized beluga AHR was recognized by antibodies against the mouse AHR, confirming its identity as an AHR.

We then studied the function of the *in vitro*-synthesized beluga AHR. We focused on dioxin specific binding, the initial step in toxicity. The best method for measuring binding of dioxin to the AHR is velocity sedimentation on sucrose gradients. This method involves incubation of the *in vitro*-synthesized beluga AHR with radiolabeled 2,3,7,8-tetrachlorodibenzo-para-dioxin ([^{3}H]TCDD). One incubation contains [^{3}H]TCDD alone and measures "total binding". A second incubation contains [^{3}H]TCDD plus an excess of unlabeled tetrachlorodibenzofuran (TCDF) and measures "nonspecific binding". The difference between these two tubes is "specific binding". Binding is measured by scintillation counting after fractionation of the incubation mixtures on sucrose gradients.

In these experiments, [^{3}H]TCDD bound to the *in vitro*-synthesized beluga AHR in a peak of total binding that was displaced by cold ligand, and therefore represents specific binding. A similar result was obtained with the mouse AHR expressed *in vitro*. It is interesting to compare the peaks obtained with the *in vitro*-expressed proteins to those obtained using liver cytosol. Even the freshest beluga liver samples give only small peaks of specific binding. This shows that cloning and *in vitro*-synthesis provide a good way to generate large amounts of beluga AHR for study, and reduces the need for collection of field samples.

One of the main questions we had about AHRs from marine mammals is how their affinities for TCDD compare to the affinities of AHRs from laboratory rodents. To measure TCDD-binding affinities, we performed saturation binding analysis of *in vitro*-synthesized beluga, mouse, and human AHRs. This involves determining specific binding as the difference between total binding and non-specific binding, over a range of [^{3}H]TCDD concentrations. From the specific binding curves, we calculated the equilibrium dissociation constants (Kd), which are measures of the binding affinities. (A low Kd indicates a high affinity.) These experiments showed that the beluga AHR has a significantly lower Kd (or higher affinity) than the human AHR. Thus, the beluga AHR is a high-affinity AHR, similar to that of a dioxin-sensitive strain of mouse.

How does the affinity of the beluga AHR compare to the concentrations of dioxins found in their tissues? We can make some comparisons using data in the literature, keeping in mind that *in vitro* versus *in vivo* comparisons are complicated by the many differences between in vitro and in vivo systems. From a study of PCBs and dioxins in beluga by Derek Muir in Canada, we calculated that the amount of dioxin equivalents (TCDD-Eqs) in liver of adult males from the St. Lawrence estuary is 0.13 nM (nanomolar). With a Kd value of 0.43 nM, this concentration of dioxin equivalents would result in 23% of the receptors being occupied by a ligand. This percent occupancy is one at which effects might be expected to occur, based on experiments in rodents and other systems. The magnitude of those effects would depend on the concentration of receptors and other factors. However, this calculation suggests that the concentrations of chemicals to which some beluga are exposed may be sufficient to be causing effects, given the presence of a high-affinity AHR.

Another objective of our research on beluga is to begin to assess whether there are differences in relative potencies of PCB congeners between marine mammals and rodents. Relative potency values (also called Toxic Equivalency Factors or TEFs) are used to calculate the relative contributions of dioxin-like PCB congeners to the overall dioxin-equivalents of environmental samples. However, it is known that there can be substantial species differences in these relative potencies (TEFs) and that this can affect risk assessment calculations. TEFs have not yet been determined for any marine mammal.

To estimate relative potencies of PCBs in beluga, we used the *in vitro*-expressed beluga AHR to determine the ability of PCB congeners to inhibit binding of TCDD, as a measure of their relative binding affinity for the AHR. Competitive binding curves obtained with a series of PCB congeners, including non-ortho and mono-ortho-substituted PCBs, were used to determine inhibition constants (K_I values) for these compounds with the mouse and beluga AHRs, from which we could calculate relative binding affinities. The beluga K_I values for this limited set of PCBs match almost perfectly with those of mouse, suggesting that relative potencies determined in rodents may be appropriate for use in beluga. However, it is still important to determine whether equal binding affinities translate into equal relative potencies.

Harbor seal AHR

The same kind of studies that I just described for beluga have also been done using harbor seal. This is the work of Eun-Young Kim of Ehime University, when she was a visiting fellow in my laboratory. She cloned and sequenced a harbor seal AHR cDNA and showed that the harbor seal AHR shares high amino acid identity with other mammalian AHRs.

Like the beluga AHR, the harbor seal AHR can be expressed *in vitro* and used in binding assays to determine its binding affinity for TCDD. Sucrose gradient analysis showed that [^{3}H]TCDD bound to the *in vitro*-expressed harbor seal AHR. Saturation analysis showed that the TCDD-binding affinity of the seal AHR is similar to that of the high-affinity mouse AHR. Thus, the seal, like the beluga, also possesses a high-affinity AHR.

AHR in birds

In another set of studies, we investigated the molecular basis for differences in sensitivity to dioxins that occur among species of birds. This is work done by Sibel Karchner and Diana Franks. Unlike beluga and seal, there were data from *in vivo* and *in vitro* studies of birds that provide a direct comparison of their sensitivity to dioxin-like chemicals. These studies show that common terns are 80-times less sensitive than chickens to effects of dioxins. We asked whether that difference might be due in part to differences in the properties of Ah receptors from these two species.

To answer this question, we cloned AHRs from these two species, expressed the proteins *in vitro*, and measured the binding of [^{3}H]TCDD by sucrose gradient analysis as I described earlier. We found that the tern AHR was less able to bind TCDD as compared to the chicken and mouse AHRs. To measure the binding affinities of the chicken and tern AHRs, we performed saturation binding analysis as I described earlier. This experiment showed that the tern AHR had an affinity for binding to TCDD that was approximately 7-times lower than that of the chicken AHR. This result shows that differences in the properties of AHRs between terns and chickens account for at least part (though not all) of the difference in sensitivity to dioxins.

Implications

How will this information be used? As I noted earlier, one of the challenges of understanding the impact of contaminants is extrapolating from results of rodent studies, to effects observed in wildlife. Some have proposed a "weight of evidence" approach, which considers a variety of data, including epidemiological studies in wildlife plus mechanistic data from rodent studies.

We suggest that comparative biochemical and molecular information, such as the type presented here, will help to "bridge the gap" between mechanistic studies in rodents and epidemiological or observational studies in wildlife. Studies of susceptibility genes in wildlife could facilitate extrapolation by reducing the uncertainty that is inherent in this process. Such a comparative molecular approach will complement the other approaches that are being used to assess the impact of endocrine disrupting chemicals on wildlife. This approach (study of susceptibility genes) is already being used in human health risk assessment.

In conclusion, we have shown that:

- Whales and seals express high-affinity AHRs that are highly conserved as compared to AHRs of terrestrial mammals.
- PCB structure-binding relationships are similar in beluga and mouse.
- The presence of a high-affinity AHRs suggests that cetaceans and pinnipeds could be among the more sensitive mammalian species to effects of dioxin-like chemicals.
- Chickens and terns express AHRs that differ by 7-fold in affinity for TCDD. This difference explains part, but not all, of the difference in sensitivity to dioxin effects.

In general, these results suggest that:

- Comparative studies of proteins involved in mechanisms of toxicity can contribute to extrapolation as one component of a "weight of evidence" approach.
- The AHR and other receptors may be useful as biomarkers of species susceptibility to "dioxins" and other endocrine-disrupting chemicals.

Finally, I would like to acknowledge the US National Institutes of Health and National Sea Grant program for funding this research.

Q&A

Iguchi: Thank you very much.

As for dioxin receptors, it seems the way dioxin is received differs substantially from city to city. If you have a question, please step up to the microphone.

Daston: George Daston, Procter & Gamble. Mark, that is very nice work and I think that you are very much on the right track in terms of how to quantitate the extrapolation from lab animal studies to wildlife species. One question, maybe the central question is: what is the range of variability likely to be, in this case, in AH receptor ligand affinity, such that we can set appropriate uncertainty factors in the extrapolation?

Hahn: Are you talking about the range among individuals of those particular species?

Daston: Well, that would be one question, but I guess what I was thinking about would be the range among species in an order, a family or a class, whatever we wanted to do a risk assessment for.

Hahn: There are several answers to that. Within mammals, the range of the mammals that have been looked at so far, the range of affinities is probably only an order of a magnitude or so. The same may be true generally within the vertebrates. But again, there are a lot of groups that have not yet been looked at, like the amphibians, which may be somewhat less sensitive.

But when you get beyond the vertebrates you begin to see some major differences. We have cloned AH receptors from mollusks, and as far as we can tell they have no ability to bind dioxin-like compounds, which is very interesting both from a risk assessment perspective as well as from a mechanistic and evolutionary perspective. So I think the answer to your question is that the range could be huge depending on the phylogenetic distance that you are dealing with.

Iguchi: Any other questions. Go ahead, Dr. Sekizawa.

Sekizawa: Sekizawa, from the National Institute of Health Sciences. Since marine mammals are exposed more to PCBs than dioxins, I think. If you compare complete contribution from exposure to PCBs and dioxins and their affinity difference, it might explain the susceptibility of those animals.

Hahn: I am not sure I understood the question.

Sekizawa: I think that marine mammals, especially in the polar regions, are exposed more to PCBs than to the dioxins. So if you compare the affinity or dissociation constant of PCBs and dioxin, you might elucidate their relative contributions of PCBs and dioxins.

Hahn: Yes, and our hope was to see whether there were some differences perhaps in the relative binding affinities of PCBs as compared to what had been seen in other mammalian species, so that we could get a more accurate calculation of the total dioxin equivalents in marine mammals, which, as you suggest, may be dominated by the PCBs.

Iguchi: Anything else? Dr, Hahn finished a little bit early, so we still have plenty time for questions. Any questions?

I have a question: have you checked the binding affinity of coplanar PCBs?

Hahn: I am sorry, of what?

Iguchi: The binding affinity of the coplanar PCBs.

Hahn: Oh, the coplanar PCBs. Just the limited set that I showed you: PCB 77 and 126 and 169, but they are very similar to what we see with mice.

Iguchi: Any other questions? OK, thank you very much.