United States Environmental Protection Agency
Research and Development
Health Effects Research Laboratory
Research Triangle Park NC 27711
EPA/600/S1-88/006 Jan. 1989

Project Summary

Characterization of the Ah Receptor

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The rat liver cytosolic receptor protein containing the Ah-receptor protein was purified and studied using a photochemical assembly of 2,3,7,8-TCDD. The receptor protein was purified using various chromatographic procedures. The unbound receptor protein rapidly lost its capacity to bind 2,3,7,8-TCDD. However, the 2,3,7,8-TCDD bound Ah receptor did not readily dissociate, probably reflecting the high potency and persistence of the toxicity of 2,3,7,8-TCDD.

Results are based on a new one-step methodology which allows activation parameters to be calculated directly from raw experimental measurements which allows the uncertainty in the activation enthalpy, expressed as a 95% confidence interval, to be obtained unambiguously.

The enthalpies of activation for both the formation and the interaction of the receptor-ligand complex are the same within the statistical uncertainty. This led to a kinetic model in which the receptor was activated to an intermediate followed by competitive degradation of the unoccupied receptor and formation of the receptor-ligand complex, both of these latter steps being fast compared with the first. The conclusion is that ligand binding and receptor degradation both involve the protein in a conformational reorganization.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Complex halogenated compounds such as dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), chlorinated biphenyls (PCBs) and brominated biphenyls (PBBs) are industrial compounds or by-products with a number of common biologic and toxic effects. The activities of these toxic halogenated aryl hydrocarbons (HAH) are structure dependent. It has been proposed that the effects of the toxic HAH are dependent on the initial interaction of these compounds with a cytosolic receptor protein (the Ah receptor) in the target tissues.

Procedure

Evidence for receptor-mediated mechanism:

1. *Saturable Binding Criteria.*

The synthesis of radiolabeled 2,3,7,8-TCDD with a high specific activity triggered several important mechanistic studies; it was apparent that in the soluble fraction of hepatic and extra-hepatic tissues from several species there was a protein which exhibited saturable binding with the radioligand. Moreover, several reports have shown that 3-methylchloanthrene, benzo[a] pyrene and dibenz[a,h]anthracene also exhibit saturable binding with this cytosolic receptor protein.

2. *Tissue or Cellular Specificity.*

Endogenous receptor ligands such as steroid hormones and neurotransmitters interact with receptors which are

located within specific tissues or cells. Tissue specificity has also been demonstrated with 2,3,7,8-TCDD receptor in rats and mice; C57B1/6J mice and Sprague-Dawley rats which are highly responsive to 2,3,7,8-TCDD exhibit tissue-dependent concentrations of the receptor which vary from 0-54 fmol/mg cytosolic protein. In contrast, non-detectable levels of the receptor protein are observed in cytosol from DBA/2J which are relatively nonresponsive to the effects of 2,3,7,8-TCDD and related toxic HAHs.

3. High Affinity Ligand-Receptor Binding.

2,3,7,8-TCDD, 3-MC and several other toxic HAHs bind with high affinity to the cytosolic receptor protein with Kd values in the range of 0.1 to 10 nM which approximate Kd values for steroids binding to their cytosolic receptor proteins.

4. Correlation Between Structure-Dependent Binding and Their Biologic and Toxic Responses.

Several studies with polychlorinated dibenzodioxins and polychlorinated dibenzofurans congeners clearly demonstrate the effects of structure on their binding affinities, AHH induction potencies and toxicities. The most active compounds contain 4 lateral (2,3,7 and 8) Cl substituents and the removal of these groups or the addition of 2 or more non-lateral Cl substituents gives cogeners with overall diminished activities.

Results and Discussion

1. The interaction of several photolabile chemicals with the receptor protein will be demonstrated using a photochemical assembly which has been set up for this study. The first phase utilizes hydrocarbons which exhibit highbinding affinities for the Ah receptor and which are available as radiolabled [3-H] compounds with a high specific activity. These compounds include 2,3,7,8-TCDD, 3-methylcholanthrene and benzo[a]pyrene. A second series of photolabile compounds, including azido derivatives of radiolabeled 2,3,7,8-TCDD and benzo[a]pyrene used in these photoaffinity studies. The competitive binding of several ligands to the covalently modified receptors were investigated to probe the possible differences in ligand binding site(s) on the receptor protein(s) and the 3-H-photoaffinity labeled protein adducts isolated and used as markers for the purification studies.

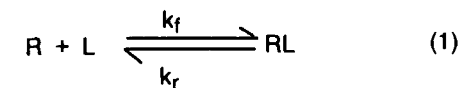
2. The receptor protein was purified using a series of chromatographic procedures including ion exchange column chromatography, hydroxyapatite column chromatography affinity chromatography and gel permeation high pressure liquid chromatography. The key step in this approach is the preparation of several affinity column supports which have been functionalized with synthetic substituted chlorinated dibenzo-p-dioxins. These functionalized column supports are utilized to preferentially adsorb the receptor protein from the cytosol and therefore facilitate purification. Since it had been reported that ligand binding activity of the Ah receptor is labile, the purification scheme used the covalently modified radiolabeled ligand-receptor complex as a marker protein.

3. The third objective focused on the preparation of monochlonal antibodies to the purified Ah receptor. This approach was to facilitate the detection and quantitation of the receptor in animal and human tissue which would serve as a probe for the determination of individual susceptibilities to the toxic HAHs.

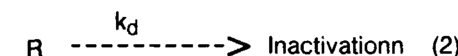
Progress

1. Ligand Binding Studies

The initial on understanding receptor-ligand interactions resulted in the development of two possible kinetic models. The receptor-ligand interactions resulted in the development of two possible kinetic models.



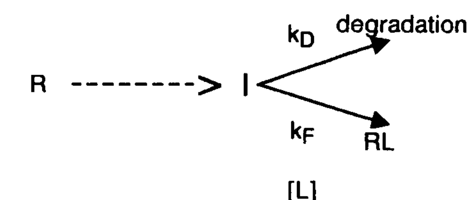
scheme 1.



As shown in scheme 1, the unbound receptor rapidly loses its capacity to bind TCDD. Therefore, the concentrations of R, L and RL at saturation will not represent equilibrium concentrations and can lead to inaccurate estimation of Kass and the initial concentration (Ro) of receptor binding sites. The values for Kf and Kd were determined and the value for Kr and Ro were estimated b matching the experimental results witl the computer simulated curve. Kr was to small to measure experimentally. Sinc Kr was a maximum estimate for th dissociation constant, the Kass values ar minimum estimates. The Kass values (x 10.10 to 3 x 10.11 M^{-1}) were at leas two orders of magnitude greater tha those corresponding to the publishe values of Kd. Consistent with this highe estimate for Kass is the argument that i Kf and Kd had the values determine experimentally in this study and if Kr ha a magnitude consistent with the literatur values of Kd, the complex should readil dissociate with time. This effect was nc observed experimentally.

The enthalpies of activation for botl the formation and inactivation of th receptor-ligand complex are the sam both graphically and computationaly within the statistical uncertainty. This le to the consideration of a second kineti (Scheme 2) in which the receptor i activated to an intermediate, I, followe by competitive degradation of th unoccupied receptor and formation of th RL complex with both of these latte steps being fast compared with the first.

scheme 2



Scheme 2 provides a ready explanatio of why the enthalpies of activation fo complex formation and recepto degradation should be the same, sinc both depend on the temperatur coefficient of Ko only. The entropies c activation are different due to th multiplying term [Ro] in the case c complex formation. The conclusion i that ligand binding and recepto degradation both involve the protein in conformational manner. The recepto degradation occurs competitively witl binding over the whole range o temperatures from 4 to 37°C; there is a yet no direct evidence for comple dissociation, and as a result, the bindin of the ligand stabilizes the receptor to a extent much greater than is found witl steroid hormones.

Photoaffinity Labeling Studies

Attempts to photoaffinity label (PAL) the Ah receptor(s) have been complicated by the low concentration of this protein in most tissues (i.e., hepatic < 100 fmol/mg) which is a problem not encountered with many other receptors. The photolysis of 2,3,7,8-TCDD in H_2O was carried out and the results indicate that 2,3,7,8-TCDD is rapidly photolyzed to unknown product(s) when irradiated with ultraviolet (UV-A, 250-400 nm) light. The observed loss of 70% of the starting material within the first 15 minutes of photolysis indicates that this ligand is significantly photolabile within the time frame of stability of the liganded Ah receptor in the cytosolic preparations. Precipitation of the photolyzed cytosolic protein with acetone (which solvates and removes excess unbound ligand) provides direct evidence for photocovalent attachment of the radioligand. In the absence of photolysis (time 0), acetone treatment removes all radioactivity from the precipitated protein pellet whereas, with increasing photolysis time, an increase in unextractable radioactivity was observed in the protein pellet.

With evidence that the radioligand [^{3}H]-2,3,7,8-TCDD was covalently adducted to cytosolic protein, the proteins were separated with sodium dodecyclsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was impregnated with a fluorographic enhancer, dried and loaded on ultrasensitive x-ray film and stored at -70°C for exposure. A detectable pattern was observed only after film exposure of the gel for a minimum of 24 weeks; however, very little significant information could be obtained. This problem was circumvented by utilizing a method involving slicing the acrylamide gel lanes into 2-3mm slices and determining the radioactivity content of each slice. This was accomplished using an oxidizer. The radioactivity contained in the gel slices was determined by liquid scintillation counting of the recovered tritiated water (>95% recovery).

The typical gel profile had specific labeling of a 95, 90 and 71 kDa protein subunit, which was in good agreement with some published results.

Purification of the Ah Receptor

Attempts to purify this receptor in its unbound form have been carried out with a variety of biochemical techniques including column chromatography and sucrose density centrifugation. It appeared that biochemical manipulation of the unliganded receptor resulted in a rapid loss of specific ligand binding. However, significant stabilization of the receptor occurs when ligand, i.e., 2,3,7,8-TCDD, was bound. When ^{3}H-TCDD liganded receptor from Long-Evans rat hepatic cytosol was separated on an equilibrated Sephacryl-300 column, there were two specifically bound radioactive peaks and this procedure resulted in up to a 10-fold purification of the receptor protein. A comparable 10-fold purification was obtained by centrifugation on a 5-25% sucrose density gradient. Current studies have been initiated to utilize a series of column chromatographic and velocity sedimentation procedures to further the Ah receptor.

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***K. Diane Courtney** is the EPA Project Officer (see below).*
The complete report entitled, "Characterization of the Ah Receptor," (Order No. PB 89-118 657/AS; Cost: $13.95, cost subject to change) will be available only from:
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5285 Port Royal Road
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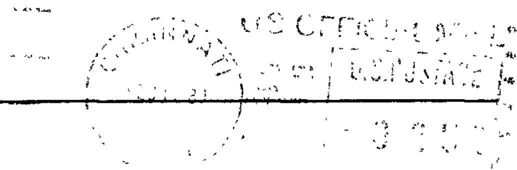

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