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August 1992
Workshop Review Draft

Chapter 2. Mechanisms of Toxic Actions

Health Assessment for
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
and Related Compounds

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Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
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CONTENTS

Figures iv

List of Abbreviations v

Authors and Contributors x

2. MECHANISM OF ACTION 2-1

2.1. INTRODUCTION 2-1

2.2. THE "RECEPTOR" CONCEPT 2-1

2.3. THE Ah (DIOXIN) RECEPTOR 2-3

2.4. BIOCHEMICAL PROPERTIES OF THE AH RECEPTOR 2-6

2.5. FUNCTION OF THE Ah RECEPTOR 2-8

2.6. IMPLICATIONS FOR RISK ASSESSMENT 2-14

2.7. FUTURE RESEARCH 2-16

2.8. REFERENCES 2-17

LIST OF FIGURES

2-1 Mechanism of TCDD Action 2-10

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Ah	Aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
ALT	L-alanine aminotransferase
AST	L-asparate aminotransferase
BDD	Brominated dibenzo-p-dioxin
BDF	Brominated dibenzofuran
BCF	Bioconcentration factor
BGG	Bovine gamma globulin
bw	Body weight
cAMP	Cyclic 3,5-adenosine monophosphate
CDD	Chlorinated dibenzo-p-dioxin
cDNA	Complementary DNA
CDF	Chlorinated dibenzofuran
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DCDD	2,7-Dichlorodibenzo-*p*-dioxin
DHT	5α-Dihydrotestosterone
DMBA	Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

LIST OF ABBREVIATIONS (cont.)

DRE	Dioxin-responsive enhancers
DTG	Delayed type hypersensitivity
DTH	Delayed-type hypersensitivity
ED_{50}	Dose effective for 50% of recipients
ECOD	7-Ethoxycoumarin-0-deethylase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EROD	7-Ethoxyresurofin 0-deethylase
EOF	Enzyme altered foci
FSH	Follicle-stimulating hormone
GC-ECD	Gas chromatograph-electron capture detection
GC/MS	Gas chromatograph/mass spectrometer
GGT	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
GST	Glutathione-S-transferase
HVH	Graft versus host
HAH	Halogenated aromatic hydrocarbons
HCDD	Hexachlorodibenzo-p-dioxin
HDL	High density lipoprotein
HxCB	Hexachlorobiphenyl

LIST OF ABBREVIATIONS (cont.)

HpCDD	Heptachlorinated dibenzo-p-dioxin
HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HxCDD	Hexachlorinated dibenzo-p-dioxin
HxCDF	Hexachlorinated dibenzofuran
ID_{50}	
I-TEF	International TCDD-toxic-equivalency
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)
LH	Luteinizing hormone
LDL	Low density liproprotein
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MFO	Mixed function oxidase
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK	Natural killer

LIST OF ABBREVIATIONS (cont.)

NOAEL	No-observable-adverse-effect level
NOEL	No-observed-effect level
OCDD	Octachlorodibenzo-p-dioxin
OCDF	Octachlorodibenzofuran
PAH	Polyaromatic hydrocarbon
PB-Pk	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyl
OVX	Ovariectomized
PBL	Peripheral blood lymphocytes
PCQ	Quaterphenyl
PeCDD	Pentachlorinated dibenzo-p-dioxin
PeCDF	Pentachlorinated dibenzo-p-dioxin
PEPCK	Phosphopenol pyruvate carboxykinase
PGT	Placental glutathione transferase
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
ppm	Parts per million
ppq	
ppt	Parts per trillion
RNA	Ribonucleic acid
SAR	Structure-activity relationships

LIST OF ABBREVIATIONS (cont.)

SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SRBC	Sheep erythrocytes (red blood cells)
$t_{½}$	Half-time
TCAOB	Tetrachloroazoxybenzene
TCB	Tetrachlorobiphenyl
TCDD	Tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factors
TGF	Thyroid growth factor
tPA	Tissue plasminogen activator
TNF	Tumor necrosis factor
TNP-LPS	lipopolysaccharide
TSH	Thyroid stimulating hormone
TTR	Transthyretrin
UDPGT	UDP-glucuronosyltransferases
URO-D	Uroporphyrinogen decarboxylase
VLDL	Very low density lipoprotein
v/v	Volume per volume
w/w	Weight by weight

AUTHORS AND CONTRIBUTORS

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During the preparation of this chapter, EPA staff scientists provided reviews of the drafts as well as coordinating internal and external reviews.

AUTHORS

James Whitlock, Jr.
Department of Pharmacology
Stanford University School of Medicine
Stanford, CA

EPA CHAPTER MANAGER

William H. Farland
Office of Research and Development
Office of Health and Environmental Assessment
Washington, DC

2. MECHANISM OF ACTION

2.1. INTRODUCTION

The environmental contaminant 2,3,7,8-TCDD has generated worldwide concern because of its wide-spread distribution, its persistence, its accumulation within the food chain and its toxic potency in experimental animals. Epidemiological studies have not produced a well-defined estimate of the risk that the dioxin poses to human health (Bailar, 1991). Knowledge of the mechanism of TCDD action may facilitate the risk assessment process by imposing constraints upon the assumptions used to estimate an acceptable exposure to the dioxin. Here, we review our current knowledge of dioxin action, with emphasis on the contribution of the Ah receptor to the mechanism. Other reviews provide additional background on the subject (Couture et al., 1990; Landers and Bunce, 1991; Nebert, 1989; Poland and Knutson, 1982; Safe, 1986; Silbergeld and Gasiewicz, 1989; Skene et al., 1989; Whitlock, 1990).

2.2. THE "RECEPTOR" CONCEPT

The idea that a drug, hormone, neurotransmitter or other chemical produces a physiologic response by interacting with a specific cellular target molecule (i.e., a "receptor") evolved from several observations. First, many chemicals elicit responses that are restricted to specific tissues. For example, ACTH stimulates the secretion of cortisol by the adrenal cortex, but has no effect on other tissues. This type of observation implied that the responsive tissue (i.e., the adrenal cortex) contained a "receptive" component, whose presence was required for the physiological effect (i.e., cortisol secretion). Second, many chemicals are quite potent. For example, nanomolar concentrations of numerous hormones and growth factors elicit biological effects. This type of observation suggested that the target cell contained a site(s) to which the chemical could bind with high affinity. Third, stereoisomers of some chemicals (e.g., catecholamines, opioids) differ by orders of magnitude in potency. This type of observation indicated that the molecular shape of the chemical strongly influenced its biological activity; this, in turn, implied that the binding site on or in the target cell also had a specific geometric configuration. Together, these three types of observation predicted that the biological responses to some

chemicals involve stereospecific, high affinity binding of the chemical to particular receptor sites, located on or in the target cell.

The availability of compounds of high specific radioactivity permitted the quantitative analysis of their binding to tissue components *in vitro*. To qualify as a potential "receptor," a binding site for a given chemical must satisfy several criteria. (1) The binding should be saturable, (i.e., there should be a limited number of binding sites per cell). (2) The binding should be reversible. (3) The binding affinity measured *in vitro* should be consistent with the potency of the chemical observed *in vivo*. (4) If the biological response exhibits stereospecificity, so should the *in vitro* binding. (5) For a series of structurally-related chemicals, the rank order for binding affinity should correlate with the rank order for biological potency. (6) Tissues that respond to the chemical should contain binding sites with the appropriate properties.

The binding of a ligand to its cognate receptor is assumed to obey the law of mass action; therefore, a rectangular hyperbola represents the relationship between the concentrations of ligand and ligand-receptor complex. Conceptually, ligand-receptor binding is analogous to substrate-enzyme binding, and similar analytical approaches are often applied to both types of interaction. For example, the Michaelis-Menton model, developed to account for the kinetic characteristics of some enzymatic reactions, may also account in some instances for the kinetics of ligand-receptor binding. According to this model, at very low concentrations of ligand, the formation of the ligand-receptor complex is directly proportional to the ligand concentration; at very high concentrations of ligand, the formation of the ligand-receptor complex is independent of the ligand concentration. However, it is not clear that all receptors obey Michaelis-Menton kinetics, just as some enzymes (such as those that exhibit allosteric effects) do not.

Ligand binding constitutes only one aspect of the receptor concept. By definition, a receptor transduces a chemical signal into a biological effect. Thus, ligand binding must lead to a response, and the functional consequences of ligand-receptor binding represent an essential component of the receptor concept. Receptor theory attempts to quantitatively relate ligand binding to the biological response. The classical "occupancy" model postulates (1) that ligand-

receptor binding is a reversible, bimolecular reaction, (2) that the magnitude of the biological response is directly proportional to the number of occupied receptors, and (3) that the response is maximal when all receptors are occupied. The expected rectangular hyperbolic "dose-response" relationships predicted by the occupancy model have been observed for numerous drugs and other chemicals (Clark, 1933; Limbird, 1986; Parascandola, 1980; Stephenson, 1956). One empirical aspect of this type of relationship is that, when receptor occupancy is sufficiently low, a small increase in ligand concentration may elicit no detectable biological response. Thus, a drug or other chemical may produce no observable effect, even though finite concentrations are attained at its receptor.

2.3. THE Ah (DIOXIN) RECEPTOR

The remarkable potency of TCDD in eliciting its toxic effects suggested the possible existence of a receptor for the dioxin. Poland and co-workers using radiolabeled TCDD as a ligand, demonstrated that C57BL/6 mouse liver contained a soluble, intracellular protein that bound the dioxin saturably (i.e., ~10^5 binding sites per cell) and with high affinity (i.e., in the nanomolar range, consistent with TCDD's biological potency (*in vivo*). Furthermore, competition binding studies with congeners of TCDD revealed that ligands with the highest binding affinity were planar and contained halogen atoms in at least three of the four lateral positions; thus, ligand binding exhibited stereospecificity. Together, these findings indicated that the intracellular, TCDD-binding protein had the ligand-binding properties expected for a "dioxin receptor." In addition, the binding of TCDD to the protein *in vitro* resembled a rectangular hyperbola and, therefore, appeared to obey the law of mass action (Poland and Knutson, 1982).

Biochemical and genetic evidence implicates the TCDD-binding protein in the biological response to dioxin. For example, studies of structure-activity relationships among congeners of TCDD reveal a correlation between a ligand's binding affinity and its potency in eliciting a biological response, such as enzyme induction. Furthermore, inbred mouse strains in which TCDD binds with lower affinity to the receptor exhibit decreased sensitivity to dioxin's

biological effects. Thus, from both a ligand-binding standpoint and a functional standpoint, the intracellular TCDD-binding protein satisfies the criteria required for a receptor. The protein is designated as the "Ah receptor," because it binds and mediates the response to other *a*ryl *h*ydrocarbons, in addition to TCDD (Poland and Knutson, 1982).

The Ah receptor evolved prior to the introduction of halogenated aromatic hydrocarbons into the environment (Czuczwa et al., 1984). Therefore, some other compound(s) must represent the "natural" ligand(s) for the receptor. Other, naturally-occurring high affinity ligands for the receptor are present in the environment (Gillner et al., 1985, 1989; Rannung et al., 1987; Bjeldanes et al., 1991). Presumably, the structure of TCDD is similar enough to such compounds that it mimics their binding to the receptor.

Inbred mouse strains differ quantitatively in their sensitivities to aromatic hydrocarbons, such as TCDD. This polymorphism is genetic in origin, and in crossbreeding studies, the sensitive phenotype segregates as an autosomal dominant trait. Numerous responses to aromatic hydrocarbons (e.g., enzyme induction, thymic involution, cleft palate formation, hepatic porphyria) exhibit a similar segregation pattern. For this reason, the genetic locus responsible for the polymorphism is envisioned as regulatory in nature. It is designated as the "Ah locus", and is thought to encode a regulatory proteins(s). Crossbreeding studies also reveal that the segregation patterns for TCDD binding and aromatic hydrocarbon responsiveness are identical. These observations imply that the Ah locus encodes the Ah receptor (Nebert, 1989; Poland and Knutson, 1982).

Electrophoretic studies reveal the existence of several forms of the TCDD-binding protein in inbred mouse stains. These observations imply the existence of multiple alleles at the Ah locus in mice (Perdew and Hollenbeck, 1990; Poland and Glover, 1990). The biochemical properties of the different forms of the Ah receptor remain to be described. In particular, the extent to which the different receptor forms affect the sensitivity of the host to TCDD is not known. An analogous receptor polymorphism might exist for humans. If differences in the properties of the Ah receptor do exist among humans, they might produce interindividual differences in sensitivity to TCDD. However, this possibility remains to be tested.

Human cells contain an intracellular protein whose properties resemble those of the Ah receptor in animals. Ligand-binding studies and hydrodynamic analyses have identified an Ah receptor-like protein(s) in a variety of human tissues (Cook and Greenlee, 1989; Harris et al., 1989; Lorenzen and Okey, 1991; Roberts et al., 1990; Waithe et al., 1991). The human receptor has not been studied extensively, and it is not yet clear if the properties of the human protein differ substantially (in a functional sense) from those of the Ah receptor in animals. However, by analogy with the existence of multiple receptor forms in mice, we anticipate that the human population will also be polymorphic with respect to Ah receptor structure and function. Therefore, we would expect humans to differ from one another in their susceptibilities to TCDD.

Analyses of mouse hepatoma cells in culture reveal additional genetic and biochemical aspects of the Ah receptor. It is possible to use physical (fluorescence-activated cell sorting) and/or chemical (resistance to benzo(a)-pyrene) techniques to select cells that are defective in the Ah receptor (Hankinson, 1979; Miller and Whitlock, 1981). One class of cells exhibits a defect in its ability to bind TCDD; however, the liganded receptors that do form are able to accumulate normally within the cell nucleus. These cells respond poorly to TCDD (as measured by aryl hydrocarbon hydroxylase induction). In another class of variants, ligand binding is normal; however, the liganded receptors are unable to bind to DNA and fail to accumulate in the nucleus. These cells do not respond to TCDD. Genetic studies of these variant cells using cell fusion reveal that the receptor-defective phenotype is recessive and that the variants fall into different complementation groups. The latter observation implies that more than one gene contributes to receptor function (Hankinson, 1983; Miller et al., 1983; Whitlock, 1990). It is possible, for example, that the Ah receptor has both a ligand-binding component and a separate, DNA-binding component. Furthermore, there could be multiple alleles for each component. In principle, the fact that multiple genes influence receptor function increases the potential for polymorphisms in receptor structure and function among both animals and humans.

Hankinson and co-workers also have described mouse cells which exhibit alterations in both ligand binding and nuclear accumulation; these variants

comprise a third genetic complementation group (Karenlampi et al., 1988). Furthermore, analyses of dominant nonresponsive variants suggests the existence of a repressor protein that may block Ah receptor function (Watson and Hankinson, 1988). Together, these observations imply that multiple genetic loci influence Ah receptor function in mice. Presumably, the situation is similar for other animals and humans; if so, we anticipate that humans will differ in their responses to TCDD because of genetic differences in the Ah receptor.

2.4. BIOCHEMICAL PROPERTIES OF THE AH RECEPTOR

The Ah receptor has been difficult to purify in substantial quantity, and insufficient knowledge of its structural and functional properties represents a major impasse in our understanding of dioxin action (Bradfield et al., 1991). The receptor is a soluble (as opposed to a membrane-bound) intracellular protein, which, upon binding TCDD, acquires a high affinity for DNA. The ligand-binding properties of the receptor have been studied in some detail, in order to define the ligand characteristics important for binding and for eliciting a biological response. The analysis of such structure-binding and structure-response relationships is useful for evaluating the role of the Ah receptor in systems where receptor-defective variants are not available. For example, studies with congeners of TCDD reveal a good correlation between their binding affinities for the Ah receptor and their potencies in inducing aryl hydrocarbon hydroxylase activity (Poland et al., 1979). This finding suggests that the receptor participates in the induction response. The results of analogous structure-activity studies implicate the Ah receptor in a broad spectrum of biochemical, morphological, immunologic, neoplastic, and reproductive effects produced by TCDD (Poland and Knutson, 1982; Safe, 1986). For this reason, we envision that the receptor participates, directly or indirectly, in every biological response that the dioxin elicits.

The ligand-binding and hydrodynamic properties of the Ah receptor differ relatively little across species and tissues; it is difficult to account for the diversity of TCDD's biological effects by these criteria (Henry et al., 1989). Relatively little is known about the human Ah receptor. In particular, the variability among humans in the receptor's binding affinity for TCDD remains to be determined; therefore, the extent to which this factor influences human

sensitivity to TCDD is unknown. The hydrodynamic properties of the human receptor are similar to those of the rodent protein. It is currently unknown whether the rather small structural variations in the receptor observed among species or tissues are associated with differences in receptor function.

Ligand binding increases the affinity of the Ah receptor for DNA *in vitro* and is associated with the accumulation of the receptor in the cell nucleus *in vivo*. Several investigators have used a gel retardation technique to show that ligand binding converts the Ah receptor to a DNA-binding form, which is found predominantly in nuclear extracts from TCDD-treated cells (Cuthill et al., 1991; Denison et al., 1989; Denison and Yao, 1991; Hapgood et al., 1989; Nemoto et al., 1990; Saatcioglu et al., 1990a,b). Thus, the binding of TCDD to the receptor "transforms" the protein to a DNA-binding species. The process of transformation is poorly understood. Biochemical studies indicate that transformation is associated with changes in thermostability, surface charge, and sensitivity to sulfhydryl reagents, implying that a conformational alteration (i.e., a change in the shape of the receptor protein) occurs (Denison et al., 1987; Gasiewicz and Bauman, 1987; Kester and Gasiewicz, 1987). Hydrodynamic studies suggest that ligand binding leads to the dissociation from the receptor of another factor (possibly, a heat-shock protein), a process which could expose the receptor's DNA-binding domain (Perdew, 1988; Wilhelmsson et al., 1990). Other analyses reveal that the transformed receptor undergoes an increase in its sedimentation coefficient, implying that the ligand-binding component of the receptor associates with a second protein(s) during the transformation process (Henry et al., 1989). Thus, the transformation process appears to involve multiple events and interactions between the Ah receptor and other proteins. These hydrodynamic results are consistent with biochemical evidence that the DNA-binding form of the receptor is composed of at least two different proteins. For example, the results of both protein-DNA crosslinking experiments and protein-protein crosslinking studies imply that the liganded Ah receptor binds to DNA as a heteromer (Elferink et al., 1990; Gasiewicz et al., 1991). Both the biochemical findings and the hydrodynamic results are consistent with the genetic evidence that multiple genes and proteins contribute to receptor function.

Hankinson and coworkers have used molecular genetic approaches to identify a human cDNA that partially corrects the defect in variant mouse hepatoma cells in which the liganded Ah receptor fails to bind DNA (Hoffman et al., 1991). The cDNA encodes a protein (termed "Arnt" by Hankinson's group) whose function remains to be determined. The protein apparently does not bind TCDD. It contains a basic helix-loop-helix domain analogous to those present in other proteins that bind DNA-binding regulatory proteins. Therefore, the Arnt protein may be a DNA-binding component of the Ah receptor. It is also possible that the Arnt protein is a translocase that transports the receptor from cytoplasm to nucleus (Hoffman et al., 1991). Ema et al. (1992) and Bradfield et al. (1991) have identified a mouse cDNA that appears to encode the ligand-binding component of the Ah receptor (Burbach et al., 1992). Like the Arnt protein, the ligand-binding component also contains a basic helix-loop-helix motif, which presumably contributes both to DNA binding and to protein-protein interactions. It is notable that neither component of the Ah receptor contains the zinc finger DNA-binding motif that is characteristic of the steroid/thyroid/retinoid family of receptors. Therefore, the Ah receptor presumably belongs to a different class of regulatory factors proteins.

Electrophoretic studies reveal that multiple alleles exist for the ligand-binding component of the Ah receptor (Poland and Glover, 1990). By analogy, it is reasonable to expect that the DNA-binding component of the receptor will also exhibit polymorphisms and exist in multiple forms. In principle, such a situation raises the possibility that different functional forms of the receptor can exist, created by the association of receptor subunits in different combinations. Such combinatorial diversity could contribute to the variety of biological responses produced by TCDD.

2.5. FUNCTION OF THE Ah RECEPTOR

TCDD induces microsomal AHH activity in many tissue types, as well as in cells in culture. Hydroxylase activity is measured using a simple and sensitive fluorescence technique. Therefore, the induction of AHH activity represents a convenient TCDD-dependent response to study.

Much of our current understanding of the mechanism of dioxin action stems from analyses of AHH induction in mouse hepatoma cells in culture (Whitlock,

1990). Figure 2-1 outlines some of the molecular events that are thought or known to contribute to the mechanism of TCDD action. Nuclear transcription experiments reveal that TCDD induces hydroxylase activity by stimulating the transcription of the corresponding CYP1A1 gene. The response to TCDD occurs within a few minutes and is direct, in that it does not require ongoing protein synthesis. Thus, the regulatory components required for the activation of CYP1A1 transcription are present constitutively within the cell. TCDD fails to activate CYP1A1 transcription in Ah receptor-defective cells; therefore, the response is receptor-dependent.

The observations that TCDD activates transcription and that the liganded Ah receptor is a DNA-binding protein, led to the discovery of a regulatory DNA domain, upstream of the CYP1A1 gene, which responds to TCDD in a receptor-dependent manner. Recombinant DNA methods were used to construct chimeric genes, in which potential regulatory DNA domains from the CYP1A1 gene were ligated to a heterologous "reporter" gene. After transfection of the recombinants into mouse hepatoma cells, TCDD was observed to activate the expression of the reporter gene. This type of experiment revealed that the dioxin-responsive DNA had the properties of a transcriptional enhancer (Jones et al., 1986; Neuhold et al., 1986; Fujisawa-Sehara et al., 1987; Fisher et al., 1990). In addition, the DNA upstream of the CYP1A1 contains a second control element (a transcriptional promoter), which functions to ensure that transcription is initiated at the correct site. Neither the enhancer nor the promoter functions in the absence of the other, and the response of the CYP1A1 gene to TCDD requires that both control elements function properly in combination (Jones and Whitlock, 1990). Analyses of stably-transfected mouse hepatoma cells reveals that the dioxin-responsive enhancer can function in a chromosomal location distinct from that of the CYP1A1 gene (Fisher et al., 1989). These observations imply that analogous enhancer and promoter elements, possibly in combination with additional regulatory components, can mediate the transcriptional response of other genes to TCDD. This hypothesis remains to be tested. However, the combinatorial model is attractive in that it provides a plausible biological mechanism for generating the diversity of effects that TCDD elicits (Whitlock, 1990).

- Diffusion into cell
- Binding to ligand-binding component of Ah receptor
- Transformation of receptor to DNA-binding form
    - Dissociation from heat-shock protein(s)?
    - Phosphorylation?
    - Association with DNA-binding component of receptor?
    - Translocation from cytoplasm to nucleus?
- Binding of liganded receptor to enhancer DNA
- Enhancer activation
    - DNA bending?
    - Histone modification?
    - Recruitment of additional proteins?
- Nucleosome disruption
- Increased accessibility of transcriptional promoter
- Binding of transcription factors to promoter
- Increased mRNA and protein synthesis
- Primary biological response
- Cascade of compensatory changes
- Secondary biological effects

Figure 2-1

Mechanism of TCDD Action (see text for details)

Transcriptional enhancement by TCDD requires a functional Ah receptor; furthermore, the liganded receptor is a DNA-binding protein. Together, these observations suggest that the induction of CYP1A1 gene transcription by TCDD involves the binding of the liganded receptor to the dioxin-responsive enhancer. Electrophoretic (gel retardation) studies, using enhancer DNA and nuclear extract from uninduced and TCDD-induced cells, reveal the existence of an inducible, receptor-dependent protein-DNA interaction *in vitro*, whose characteristics are those expected for the binding of the liganded receptor to DNA (Denison et al., 1989; Hapgood et al., 1989; Saatcioglu et al., 1990a,b). The liganded receptor recognizes a specific nucleotide sequence

```
5'T-GCGTG 3'
            , which is present in multiple copies within
3'A-CGCAC 5'
```

the enhancer. Studies with an [^{125}I]-labeled dioxin indicate that the liganded receptor binds in a 1:1 ratio to the recognition sequence (Denison et al., 1989). Methylation protection and interference experiments *in vitro* reveal that the liganded receptor lies within the major DNA groove and contacts the four guanines of the recognition sequence (Shen and Whitlock, 1989; Neuhold et al., 1989; Saatcioglu et al., 1990a,b). These properties of the liganded Ah receptor are consistent with those described for other transcriptional regulatory proteins.

Experiments involving the use of phosphatases and/or protein kinase inhibitors suggest that phosphorylation of the Ah receptor, possibly by protein kinase C, plays a role in the biological response to TCDD (Pongrantz et al., 1991; Carrier et al., 1992; Okino et al., 1992). The nature of the phosphorylation(s) and its precise functional role remain to be determined.

Use of a methylation protection technique to analyze receptor-enhancer binding within the intact cell reveals evidence for TCDD-inducible, Ah receptor-dependent protein-DNA interactions, which are similar to those observed *in vitro* (Wu and Whitlock, 1992). These findings strongly imply that the studies of receptor-enhancer interactions *in vitro* do, in fact, reflect biologically

relevant effects of TCDD and provide accurate insights into the mechanism of dioxin action *in vivo*.

The DNA recognition sequence for the liganded Ah receptor contains two CpG dinucleotides. Studies in other systems reveal that cytosine methylation at CpG is associated with decreased gene expression, often in tissue-specific fashion. Cytosine methylation within the Ah receptor's recognition sequence diminishes both the binding of the receptor to the enhancer (as measured by gel retardation) and the function of the enhancer (as measured by transfection). These observations suggest that DNA methylation may contribute to the observed differences among tissues in their biological responses to TCDD (Shen and Whitlock, 1989).

Gel retardation analyses reveal that the binding of the receptor to its recognition sequence bends the DNA *in vitro* (Elferink and Whitlock, 1990). Thus, the receptor-enhancer interaction has the potential to alter the configuration of the DNA. Analyses of the CYP1A1 gene in intact nuclei reveal that the enhancer/promoter regulatory region undergoes a dioxin-inducible, Ah receptor-dependent increase in nuclease susceptibility within minutes exposure of the cell to TCDD. This observation suggests that the receptor-enhancer interaction leads to an alteration in chromosomal structure, such that the regulatory DNA region becomes more accessible to the protein factors that are required for transcription of the CYP1A1 gene (Durrin and Whitlock, 1989). Methylation protection studies of the promoter region *in vivo* reveal that TCDD does, in fact, induce a receptor-dependent increase in protein binding, which probably reflects the increased accessibility of the promoter region in the intact cell (Wu and Whitlock, 1992).

The evidence to date implies that the Ah receptor participates in every biological response to TCDD (Poland and Knutson, 1982; Safe, 1986; Whitlock, 1990). This hypothesis predicts that TCDD will be found to activate the transcription of other genes via a receptor- and enhancer-dependent mechanism analogous to that described for the CYP1A1 gene. Preliminary data suggest that this is the case. For example, TCDD induces the expression of a cytochrome P4501A2 gene, a glutathione S-transferase Ya subunit gene, an aldehyde dehydrogenase gene, and a quinone reductase gene; in some cases, induction is

known to occur at the transcriptional level, to be Ah receptor-dependent, and to involve a genomic regulatory element(s) analogous to that found upstream of the CYP1A1 gene (Quattrochi and Tukey, 1989; Telakowski-Hopkins et al., 1988; Dunn et al., 1988; Jaiswal et al., 1988; Favreau and Pickett, 1991). In addition, recent observations suggest that, in human keratinocytes, TCDD activates the transcription of plasminogen activator inhibitor-2 and interleukin-1β, as well as other genes which remain to be identified (Sutter et al., 1991). The mechanism by which dioxin activates the expression of these genes is currently unknown. For dioxin-responsive genes other than CYP1A1, and especially for those genes that respond in tissue-specific fashion, the presence of the receptor/enhancer system may not be sufficient for dioxin action, and other, tissue-specific regulatory components may play a dominant role in governing the response to TCDD. Thus, future research may reveal the existence of additional positive or negative gene regulatory components that can influence the response of the cell to TCDD.

Compensatory changes, which occur in response to TCDD's primary effects, can complicate the analysis of dioxin action in intact animals. For example, TCDD can produce changes in the levels of steroid hormones, peptide growth factors and/or their cognate cellular receptors (Choi et al., 1991; Harris et al., 1990; Lui et al., 1991; Ryan et al., 1989; Sunahara et al., 1989; Umbreit and Gallo, 1988). In turn, such alterations have the potential to produce a series of subsequent biological effects, which are not directly mediated by the Ah receptor. Furthermore, the hormonal status of an animal appears to influence its susceptibility to the hepatocarcinogenic effects of TCDD (Lucier et al., 1991). Likewise, exposure to other chemicals can alter the teratogenic response to TCDD (Couture et al., 1990). Therefore, in some cases, TCDD may act in combination with other chemicals to produce its biological effects. Such phenomena increase the difficulty of analyzing dioxin action in intact animals and increase the complexity of risk assessment, given that humans are routinely exposed to a wide variety of chemicals.

The fact that TCDD may induce a cascade of biochemical changes in the intact animal raises the possibility that the dioxin might produce a response such as cancer by mechanisms that differ among tissues. For example, in one case, TCDD

might activate a gene(s) that is directly involved in tissue proliferation. In a second case, TCDD-induced changes in hormone metabolism may lead to tissue proliferation secondary to increased secretion of a trophic hormone. In a third case, TCDD-induced changes in hormone receptors for growth factors or hormones may alter the sensitivity of a tissue to proliferative stimuli. In a fourth case, TCDD-induced toxicity may lead to tissue death, followed by regenerative proliferation. At present, it is unknown whether any of these hypothetical mechanisms actually occurs, whether they would exhibit similar sensitivities to TCDD, or whether they would occur in all animal species exposed to the dioxin.

Under some circumstances, exposure to TCDD elicits beneficial effects. For example, TCDD protects against the carcinogenic effects of polycyclic aromatic hydrocarbons in mouse skin; this may reflect the induction of detoxifying enzymes by the dioxin (Cohen et al., 1979; DiGiovanni et al., 1980). In other situations, TCDD-induced changes in hormone metabolism may alter the growth of hormone-dependent tumor cells, producing a potential anti-carcinogenic effect (Spink et al., 1990). These (and perhaps other) potentially beneficial effects of TCDD complicate the risk assessment process for dioxin.

2.6. IMPLICATIONS FOR RISK ASSESSMENT

A substantial body of biochemical and genetic evidence indicates that the Ah receptor mediates the biological effects of TCDD. This concept implies that a response to dioxin requires the formation of ligand-receptor complexes. TCDD-receptor binding appears to obey the law of mass action and, therefore, depends upon (1) the concentration of ligand in the target cell; (2) the concentration of receptor in the target cell; and (3) the binding affinity of the ligand for the receptor. In principle, some TCDD-receptor complexes will form even at very low levels of dioxin exposure. However, in practice, at some finite concentration of TCDD, the formation of TCDD-receptor complexes will be insufficient to elicit detectable effects. Furthermore, biological events subsequent to TCDD-receptor binding may not necessarily exhibit a linear response to dioxin. For example, the ability of the liganded Ah receptor to activate transcription does not correlate with its binding affinity for DNA *in vitro* (Shen and Whitlock, 1992). In addition, in some systems, the induction of gene transcription appears

to require a threshold concentration of transcription factor(s) (Fiering et al., 1990). An analogous situation may exist for the liganded Ah receptor.

The concentration of TCDD required to produce a biological effect may vary among individuals. For example, inbred mice vary in their sensitivity to TCDD according to the receptor's ligand-binding affinity (Poland and Knutson, 1982). Analogous phenomena commonly occur in humans, who differ in their responses to numerous xenobiotics (Nebert and Weber, 1990). Thus, a concentration of TCDD that is sufficient to induce a response in one individual may be insufficient in another. For example, studies of humans exposed to dioxin at Seveso fail to reveal a direct relationship between blood TCDD levels and the development of chloracne (Mocarelli et al., 1991).

The challenge for risk assessment is to use the known properties of the Ah receptor, together with the known dose-response relationships and pharmacokinetic data for TCDD, to estimate an intracellular concentration of TCDD below which no detectable effects occur. This estimate will, in principle, make it possible to set limits on acceptable human exposure.

Other issues also influence the risk assessment process. Given TCDD's widespread distribution, its persistence, and its accumulation within the food chain, it is likely that most individuals (at least in industrialized societies) are exposed to the dioxin. Therefore, the population at potential risk is extended and heterogeneous. This fact implies that individuals are likely to differ in their susceptibility to dioxin, either because of genetic differences or because of exposure to other chemicals.

Complex TCDD-induced effects (such as cancer) probably require multiple steps and are likely to involve several genetic and/or environmental factors. For example, a homozygous recessive mutation at the hr (hairless) locus appears necessary for TCDD's action as a tumor promoter in mouse skin (Poland et al., 1982). Furthermore, tumor induction requires exposure to a second chemical, which acts as a tumor initiator. Presumably, a similar situation exists for humans. If so, only certain individuals may be at risk from exposure to TCDD, because of their particular genetic makeup and/or their exposure to other chemicals. Continued analyses of the mechanism of dioxin action in the future

may lead to methods for identifying individuals who are especially at risk from exposure to TCDD.

2.7. FUTURE RESEARCH

The paucity of information about the structure and function of the Ah receptor represents the major impasse to a better understanding of the mechanism of dioxin action. The probable heteromeric composition of the receptor, implied by both genetic and biochemical observations, imposes important constraints upon approaches which are likely to be successful for the purification of the receptor proteins themselves or the cloning of the corresponding genes. Recently-developed biochemical methods, such as photoaffinity labeling (Poland et al., 1986; Landers et al., 1989) or DNA affinity chromatography (Kadonaga and Tjian, 1986), as well as genetic techniques (Hoffman et al., 1991) constitute new experimental approaches, which appear likely to generate novel insights into receptor structure and function in the near future. Antibodies generated against a receptor-related synthetic peptide also provide new information about the biological properties of the Ah receptor (Poland et al., 1991). *In vitro* transcription provides a new method for analyzing the function of the purified Ah receptor (Wen et al., 1990). Together, these experimental approaches may help to reveal the extent to which receptor heterogeneity contributes to inter-individual differences in susceptibility to TCDD.

Studies of other tissues (e.g., skin, thymus) are likely to reveal additional TCDD-responsive genes, which exhibit tissue-specific expression (Sutter et al., 1991). Analyses of the mechanism of dioxin action in such systems appear likely to reveal additional factors that influence the susceptibility of a particular tissue to TCDD. In addition, studies of other TCDD-inducible genes, such as glutathione S-transferase, quinone reductase, and aldehyde dehydrogenase, may reveal whether differences in enhancer structure, receptor-enhancer interactions, or promoter structure affect the responsiveness of the target gene to TCDD (Whitlock, 1990).

Analyses of dioxin action may provide some insight into the mechanisms by which TCDD and related compounds produce birth defects or cancer, effects which are of particular public health concern. A major challenge for the future will

be the establishment of experimental systems in which such complex biological phenomena are amenable to study at the molecular level.

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