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SPECIAL CONTRIBUTION

An Aryl Hydrocarbon Receptor Odyssey to the Shores of Toxicology: The Deichmann Lecture, International Congress of Toxicology-XI

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The science of toxicology is devoted, in large part, to understanding mechanisms of toxicity so that we can more accurately assess the risk posed by exposure to xenobiotic agents and, perhaps, intervene in the toxicologic process to mitigate harm. Dioxin-like chemicals continue to be of great concern as environmental toxicants. About 30 years ago the aryl hydrocarbon receptor (AHR) was discovered as a specific binding site for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This giant step led to our current view that essentially all toxic effects of dioxins are AHR-mediated. The AHR serves as the archetype for understanding toxicity mediated by other soluble receptors. The fact that toxicity is receptor-mediated has important implications, especially for dose–response relationships. In laboratory animals genetic differences in *AHR* gene structure lead to profound differences in responsiveness to dioxin-like chemicals. Humans, however, exhibit relatively few *AHR* polymorphisms and these seem to exert only modest effects on downstream events. Dioxin toxicity is fundamentally due to AHR-mediated dysregulation of gene expression. Our current challenging goal is to determine which dysregulated genes underlie specific forms of dioxin toxicity. Mapping AHR-mediated gene expression in a variety of biological systems may help explain why dramatic differences in susceptibility to dioxin toxicity exist among laboratory species and why humans appear to be relatively resistant to adverse effects of dioxins.

Key Words: **aryl hydrocarbon receptor; dioxin; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; gene regulation.**

PRELUDE AND DISCLAIMER

This story is a chronicle of the conception, birth, and growth of aryl hydrocarbon receptor (AHR) research in relation to toxicology with a focus on evolution of AHR investigations in my own laboratory. I ask forbearance from readers as well as from fellow toilers in the AHR field if work from my own laboratory seems overemphasized or overrepresented. This article is intended to give a flavor of various aspects of AHR research rather than a definitive and comprehensive review of each topic and to be prospective as well as retrospective. Due to space constraints it won't be possible to cite the bulk of the important original contributions made by the numerous laboratories who study the AHR. Please see the excellent reviews by other investigators, cited in this paper, which provide additional perspectives on the multitudinous facets of AHR structure and function.

Before we consider recent developments in the AHR arena, it may be useful for newcomers to have an overview of how the AHR field arrived at its current state, illustrated, mainly, by my own research journey. Journeys are more enjoyable when they are taken with affable companions. It's been my good fortune during the AHR voyage to have been accompanied by many talented and congenial trainees and collaborators. This narrative is intended as a tribute to members of my laboratory who contributed so significantly to understanding this intriguing receptor as well as to the international community of AHR scholars with whom I've been privileged to interact over the past 30 years.

Research and research careers do not always proceed in a straightforward, logical, linear fashion. They evolve, just as do biological systems, via the force of natural selection picking from among the range of variants. Along the way, there are serendipitous or fortuitous events (akin to mutations—some good, some not-so-good) that strongly influence the direction and the success of our research (Jensen, 2004; Rothstein, 1986). In my case, two lines of research (which on the surface seemed independent) coalesced, partly by chance, into a 30-year infatuation with the AHR.

Providential Connections: Estrogens and Polycyclic Aromatic Hydrocarbons

My original research interest was induction of mammary cancer by exogenous estrogens. In my Ph.D. thesis research,

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directed by Prof. George Gass, we found that continuous low-dose administration of the potent synthetic estrogen, diethylstilbestrol (DES), induced mammary carcinoma in more than 95% of mice if they carry the mouse mammary tumor virus. If mice lack either the virus or the estrogen stimulus, tumor incidence is very low (Gass *et al.*, 1974; Okey and Gass, 1968).

DES was synthesized in the 1930s from precursors obtained from coal tar (Dodds *et al.*, 1938). Coal tar (not so coincidentally) happens also to be the original source from which another notorious group of carcinogens was isolated, the polycyclic aromatic hydrocarbons (PAHs), typified by benzo[*a*]pyrene (BP).

Although DES can be carcinogenic in animal models (and in humans exposed prenatally to high doses—''DES daughters'') (Herbst *et al.*, 1971), DES also found its way into cancer therapy. Charles Huggins was awarded the Nobel Prize in Physiology or Medicine in 1966 for his discovery that DES and other hormonal therapies have value in some cases of advanced prostate cancer. Huggins also explored hormonal therapy for breast cancer. To this end, his laboratory was a leader in developing a remarkable animal model by showing that PAHs such as 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (3-MC) are superb mammary carcinogens. These PAHs induce adenocarcinomas in young female rats within only a few weeks after a single dose (Dao and Sunderland, 1959; Huggins *et al.*, 1961).

When I took up my first independent research position at the University of Windsor (Ontario, Canada), I pursued my interest in the respective roles of the estrogen and the PAH in the dramatic Huggins model of PAH-induced mammary cancer. It was bemusing to me, as a new investigator, to find that there were two (apparently diametrically opposed) mechanistic explanations being floated about for mammary carcinogenesis. One school held that PAHs were carcinogenic because they mimic the action of estrogens on mammary epithelium. A concurrent and competing view flipped this around to propose that estrogens are carcinogenic because they mimic PAHs—that is, estrogens are bioactivated into mutagenic metabolites. Later it would become established that there is a core of truth in each of these views (see Belous *et al.*, 2007).

Steroids and carcinogenic PAHs bear a passing structural similarity (Fig. 1). The first known steroid receptor, the estrogen receptor (ER), was discovered through pioneering work by Elwood Jensen in the late 1960s (see Jensen, 2004). David Keightley, my first Ph.D. student, tested the supremely potent mammary carcinogen, DMBA, to see if it could interact with the ER and found that DMBA did not compete with estradiol-17β for binding to the ER (Keightley and Okey, 1973). DMBA was not a good estrogen but we found that DMBA could interfere with some ER functions *in vivo* (Ianicello and Okey, 1976; Keightley and Okey, 1974). At that time we had not foreseen that a receptor which behaves very much like a steroid receptor might exist for the PAHs themselves. We will revisit the estrogen/PAH/AHR story near the end of this review.

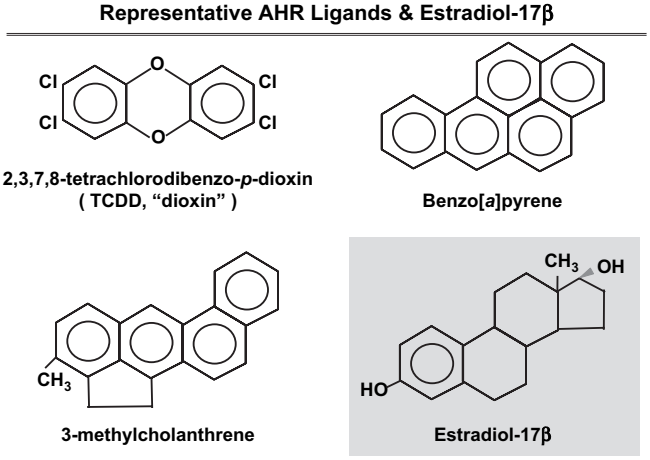

FIG. 1. Representative AHR ligands and estradiol-17β. TCDD is the prototypical AHR ligand among the group of halogenated aromatic hydrocarbons (HAHs). 3-MC and BP are major nonhalogenated PAH ligands, widely studied because of their carcinogenic properties. The structure of estradiol-17β is included because during the embryonic days of AHR research the potential interaction of PAHs with ER was being investigated and because there has been a recent resurgence of interest in estrogens and complex cross-talk between ER pathways and AHR pathways.

Our research on estrogens in relation to mammary carcinogenesis took on a new dimension when the environmental movement was spurred in the late 1960s by the disturbing discovery that pesticides such as dichloro-diphenyl-trichloroethane (DDT) interfere with reproduction in birds (Bitman *et al.*, 1968; Peakall, 1967) and mammals (Bitman *et al.*, 1968). Since we were an ''estrogen lab,'' John Clement, a graduate student, tested DDT for estrogenic activity in standard bioassays and found that the *o,p′*-DDT isomer has estrogenic activity but that the commercial mixture (which predominantly is the *p,p′*-DDT isomer) exerts ''antiestrogenic'' effects and interferes with uterotrophic activity of the natural estrogen, estradiol-17β (Clement and Okey, 1972).

Because DDT exerts both estrogenic and antiestrogenic effects, I wanted to find out how this ubiquitous pesticide would affect development of estrogen-dependent mammary cancer in rats treated with a PAH. Perhaps this ''real-world'' exposure to both a pesticide and a PAH carcinogen would be disastrous to the recipient. In fact, my graduate student, Charles Silinskas, found that brief pretreatment of female rats with DDT, at doses as low as 10 ppm, in the diet confers dramatic protection from DMBA-induced mammary cancer and leukemia (Silinskas and Okey, 1975). This protection appeared to be due to the ability of DDT to enhance metabolism and elimination of DMBA (Okey, 1972).

I became intrigued with the phenomenon of induction of ''drug-metabolizing enzymes.'' I wanted to understand the induction mechanism and how induction might relate to protection from environmental carcinogens. It was serendipitous that I was eligible for a sabbatical leave and I was fortunate that Daniel Nebert was willing to accept this unknown

investigator from a small provincial university into his laboratory, then at the National Institute for Child Health and Human Development, National Institutes of Health (NIH).

Before I continue the tale of my own work during the nascent days of AHR, it's necessary to jump back to events beginning in the 1950s that paved the way for discovery of this captivating receptor.

ARYL HYDROCARBON RECEPTOR: THE EARLY YEARS—FROM CONCEPT TO CLONING

Discovery of "MC-type" Induction Lights the Path

Many important nuclear receptors were discovered by ''reverse endocrinology.'' That is, clones that harbor sequences similar to those of known nuclear receptors were retrieved from complementary DNA (cDNA) libraries. However, the protein products of these novel clones remained ''orphan receptors'' until their ligands and functions eventually were deciphered. This genetic pathway to discovery yielded several nuclear receptors whose acronyms now are widely known in pharmacology and toxicology: RAR, RXR, LXR, PPAR, CAR, PXR, and FXR (reviewed in Evans, 2004; Giguere, 1999; Gustafsson, 1999; Kliewer *et al.*, 1999).

In contrast to the strategy of ''clone first, find function later,'' the AHR's discovery preceded the era of receptor cloning and resulted from efforts to understand the mechanism by which polycyclic hydrocarbons induce their own metabolism. Allan Conney, working in the Millers' laboratory at the University of Wisconsin in the 1950s, discovered that BP and 3-MC induce what then was called ''BP hydroxylase.'' (Later ''BP hydroxylase'' was designated aryl hydrocarbon hydroxylase [AHH] in recognition of the fact that many PAHs in addition to BP are substrates (Nebert and Bausserman, 1970b). Molecular investigations eventually linked AHH activity to CYP1 enzymes.) The phenomenon of ''MC-type'' induction by PAHs was an essential antecedent to discovery of the AHR. See the engaging autobiographical sketch by Conney (2003b) for a full account of the circumstances which led to his discovery of MC-type induction.

Genetic Models Plant a Seed that Will Yield a Bountiful Harvest

Although Prof. Werner Kalow at the University of Toronto had published his landmark monograph *Pharmacogenetics* in 1962 (Kalow, 1962), the possibility that genetic factors might actually matter in drug metabolism and drug response was still not on the radar screen for most pharmacologists and toxicologists by 1970. Today pharmacogenetics and pharmacogenomics have fully penetrated biomedical research as well as the pharmaceutical industry; their importance may seem obvious to younger investigators but this is a relatively recent enlightenment.

Cell models of AHH induction. Daniel Nebert was an ''early adopter'' who, at the start of the 1970s, had developed a well-honed appreciation for the potential power of a genetic approach to pharmacology and toxicology. Working initially in Harry Gelboin's laboratory at the National Cancer Institute-NIH, Dan Nebert found that induction of BP hydroxylase/AHH is not confined to liver of intact animals. He developed a very informative induction model in hamster fetal cell cultures where many fundamental characteristics of the induction process were worked out (Nebert and Bausserman, 1970a; Nebert and Gelboin, 1968a,b).

A previous *in vivo* survey pointed to substantial differences between mouse strains in AHH inducibility by 3-MC (Nebert and Gelboin, 1969). As a new independent investigator at the National Institute of Child Health and Human Development, Dan demonstrated that AHH activity was much more highly inducible in fetal cells derived from C57BL/6 mice than cells from DBA/2 mice (Nebert and Bausserman, 1970b), establishing the utility of cell models for exploring pharmacogenetic aspects of AHH regulation.

In the late 1970s, Oliver Hankinson (University of California) brought a powerful new tool—somatic cell genetics—to bear on mechanisms regulating induction of AHH. (AHH, by then was becoming associated with CYP1A1.) The Hankinson laboratory exposed Hepa-1 mouse hepatoma cells to BP in culture. BP induces AHH activity in wildtype Hepa-1 cells, thereby causing them to self-destruct because the induced enzyme bioactivates BP into cytotoxic metabolites. The rare mutant cells in the population that are not AHH-inducible survive; these resistant cells then were selected for further study to determine the basis of their nonresponsiveness. The BP-selection process (Hankinson, 1979) and subsequent genetic complementation analyses revealed that, in addition to the *Cyp1a1* gene itself, products of at least two other genes are required to induce CYP1A1. One of these genes encodes the AHR (Legraverend *et al.*, 1982).

The biggest payoff from their somatic cell genetic strategy in the Hankinson laboratory was identification of the other key regulatory gene product. That is, discovery of a novel protein, ARNT (aryl hydrocarbon receptor nuclear translocator), which would turn out to be the essential dimerization partner for the AHR (Hoffman *et al.*, 1991; Reyes *et al.*, 1992) (see below). Discovery of ARNT triggered explosive growth in the AHR field *per se* as well as in areas such as hypoxic signaling where ARNT (also known as HIF-1β) plays a vital role (Fryer and Simon, 2006) and extending into such diverse areas as vascular tumorigenesis (Rankin *et al.*, 2005) and type 2 diabetes (Gunton *et al.*, 2005). Cloning of ARNT and subsequent cloning of the AHR (see below) were instrumental in unveiling an entire family of regulatory proteins containing bHLH/PAS domains (Gu *et al.*, 2000).

James Whitlock Jr's laboratory (Stanford University) used a fluorescence-activated cell sorter to select cells that are unresponsive to induction by BP and derived AHR-deficient mutant cell lines akin to those produced by the Hankinson laboratory (Miller and Whitlock, 1981; Miller *et al.*, 1983). The AHR-deficient mutant Hepa-1 cells produced by the Hankinson

laboratory and the Whitlock laboratory have been invaluable to other investigators who use them to determine if a particular response requires the AHR.

In vivo model—the mouse Ah locus. The original Nebert discovery of a strain difference in AHH induction laid the foundation for a classic genetic approach to inheritance of AHH regulation *in vivo*. Breeding studies in the Nebert laboratory showed that inheritance of inducibility essentially is an autosomal dominant trait. The genetic locus controlling induction was defined as *Ah* for aromatic hydrocarbon responsiveness (reviewed in Nebert, 1988, 1989; Nebert *et al.*, 1981). C57BL/6 mice (Ahr^{b1} allele in current nomenclature) constitute the prototype ''responsive'' strain and DBA/2 mice (Ahr^{d} allele) the prototype ''nonresponsive'' strain. The Nebert laboratory and many other laboratories would go on to show that genetic differences at the *Ah* locus (now termed the *Ahr* locus) influence sensitivity of mice to a very broad range of responses to xenobiotic chemicals including mutagenesis, carcinogenesis, teratogenesis, and dioxin toxicity (summarized in Nebert, 1989; Okey *et al.*, 2005b).

The Induction-Receptor Hypothesis Arises

Marshall McLuhan, media guru at the University of Toronto, inverted the dictum ''seeing is believing'' to: ''If I hadn't believed it, I wouldn't have seen it.'' In other words, for some discoveries, having the conviction that a particular phenomenon exists is the precondition that permits us to recognize evidence which supports that phenomenon. For the AHR, the ''belief'' that there was an induction receptor was based on a combination of genetic findings along with the precedent of receptors for other small hydrophobic molecules (i.e., steroids) which was burgeoning in the 1970s.

The first hint that AHH induction might be mediated by a receptor goes back to the statement by Nebert and Bausserman (1970a) who proposed that: ''the process of hydroxylase induction involves a rate-limiting step, which may be the saturation of 'inducer-binding' sites in the cell.''

2,3,7,8-Tetrachlorodibenzo-p-dioxin, a Super-potent AHH Inducer, Becomes the Ideal Radioligand for the Receptor Search

The plausibility of the ''induction-receptor'' hypothesis took a great leap forward because of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). ''Nonresponsive'' mouse strains are so called because they do not exhibit AHH induction when treated with nonhalogenated PAHs such as 3-MC, even at very high doses. TCDD shifted the focus of investigation away from the enzyme and toward pathways that regulate AHH expression. TCDD would soon establish that the ''*Ah* locus'' is a regulatory locus that encodes the AHR.

Alan Poland's laboratory (then at the University of Rochester) was devoted to determining mechanisms of toxicity of halogenated aromatic compounds. They found that TCDD, which they previously showed to be a potent inducer of δ-aminolevulinic acid synthase (Poland and Glover, 1973a), also was a potent AHH inducer in chick embryo liver (Poland and Glover, 1973b). Moving to mammals, TCDD proved to be 30,000 times more potent than 3-MC at inducing AHH in rat liver (Poland and Glover, 1974). Even more revealing was the ability of TCDD to overcome the ''nonresponsive'' phenotype in mice. In collaboration with Dan Nebert's group, the Poland–Nebert team found that TCDD was able to induce hepatic AHH activity in five mouse strains that are nonresponsive to 3-MC (Poland *et al.*, 1974). The fact that TCDD induced AHH in ''nonresponsive'' mice led to the conclusion that the P450 gene encoding the enzyme was normal and to the hypothesis that nonresponsiveness was due to a mutation which leads to production of an ''inducer-binding receptor'' with a reduced affinity for nonhalogenated aromatic hydrocarbons. Breeding studies in mice supported this hypothesis (Nebert *et al.*, 1975; Poland and Glover, 1975), setting the stage for use of radiolabeled TCDD in the search for the induction receptor.

Alan Poland's chemistry collaborator at the University of Rochester, Andrew Kende, prepared [^{3}H]TCDD as the quintessential bait for the receptor fishing expedition. In addition to its great potency as an AHH inducer, TCDD has the virtue of being chemically stable and highly resistant to metabolism in most biological systems *in vitro* or *in vivo*. Armed with [^{3}H]TCDD, Poland, Glover, and Kende brought forth the eagerly sought first experimental evidence for an induction receptor in their landmark *JBC* paper in 1976 (Poland *et al.*, 1976b). The hypothetical receptor had become real.

The Okey Lab Enters the Induction-Receptor Arena

Adventures with PAH radioligands. In 1976, prior to my sabbatical leave in the Nebert lab at NIH, [^{3}H]TCDD was not generally available. My laboratory, therefore, made our initial foray into the induction-receptor field with tritiated versions of the nonhalogenated AHH inducers, BP and 3-MC. We attempted to identify a [^{3}H]BP-binding component in C57BL/6 hepatic cytosol that could be saturated at reasonable radioligand concentrations and that would show specificity when nonradioactive AHH inducers were introduced as competitors. Binding profiles, after separating radiolabeled cytosol by velocity sedimentation on sucrose gradients, revealed a very large radiolabeled peak with a sedimentation coefficient of about 4S. There also was a small peak at about 9S but we could not get ''clean'' competition by other AHH inducers for the 9S component and did not attempt to publish these data.

In retrospect, the binding component that sedimented at 9S does, in fact, represent binding of the PAH radioligands to AHR as we (Okey and Vella, 1982, 1984; Okey *et al.*, 1984) and Poellinger *et al.* (1983) would demonstrate in the 1980s by direct binding studies with [^{3}H]BP, [^{3}H]3-MC and [^{3}H]dibenzo[*a*,*h*]anthracene. The identity and function of the 4S component that becomes labeled by [^{3}H]BP and [^{3}H]3-MC remains

mysterious. The abundant 4S component was tentatively identified as glycine N-methyltransferase and it was proposed that the 4S binder mediates CYP1A1 induction (Bhat and Bresnick, 1997; Raha *et al.*, 1995). However, other studies do not support a role for the 4S binding component in P450 regulation (Harris *et al.*, 1988; Kamps and Safe, 1987). Most recently it was reported that β-naphthoflavone, a well-known CYP1A1 inducer, binds a 4S component but the functional significance of this binding for regulating gene expression remains unclear (Brauze, 2004; Brauze and Malejka-Giganti, 2000).

[^{3}H]TCDD at the NIH. Gregory Bondy, an exceptionally talented graduate student from my laboratory at the University of Windsor, joined me at the Nebert NIH lab for a few months in the summer of 1978 prior to his entry into medical school. We applied techniques we previously had used to study ER (Okey and Bondy, 1977, 1978a,b) to the study of [^{3}H]TCDD binding in the Nebert *Ah*-locus mouse model. It may be difficult for current students to envision this Paleozoic era in receptor research when the only method available to identify and characterize soluble receptors was by reversibly tagging them with a dissociable radioligand. Nevertheless, rapid progress was made, riding on the back of the splendid radioligand, [^{3}H]TCDD.

In addition to the important receptor properties of *K*d, B_{max} and specificity that were revealed in Alan Poland's milestone 1976 paper, we wanted to understand macromolecular structure of the binding protein. To that end we employed velocity sedimentation on sucrose density gradients (SDG) along with ion exchange chromatography. The chromatography experiments were greatly facilitated by the collaboration of the late Howard Eisen who was a glucocorticoid receptor specialist and part of the Nebert laboratory. Both the SDG technique and column chromatography were widely used in the 1970s in studies of steroid hormone receptors; our use of these methods reflected our presumption that the dioxin-binding protein was a type of steroid receptor.

With data gathered primarily by SDGs, we confirmed Alan Poland's findings. Namely, that hepatic cytosol from C57BL/6 mice contains a saturable, high-affinity [^{3}H]TCDD binding site that is selective for compounds known to be AHH inducers and that this specific binding component was not detectable in cytosol from genetically nonresponsive DBA/2 mice. It was Dan Nebert's wisdom and logic to designate this binding component the ''AH receptor,'' since it is the product of the *Ah* locus; we introduced the ''AH receptor'' terminology in our first paper (Okey *et al.*, 1979).

In the following sections I will concentrate on findings made by my laboratory but attempt to do justice to the many other AHR investigators by placing our discoveries in the context of overall developments in the field.

Nuclear Translocation—Behaving Like a Steroid Receptor

Cloning in the 1990s would reveal that the AHR's primary structure does not qualify it for bona fide membership in the formal nuclear receptor superfamily, notably because the AHR lacks the zinc-finger domain that typifies steroid receptors. However, despite belonging to a different gene family, the AHR behaves very much like a steroid receptor. Because we had a preconception in the 1970s (based on the steroid receptor precedent) that liganded AHR should translocate from cytoplasm into nucleus, we prepared both cytosol and nuclear extract from livers of mice injected with [^{3}H]TCDD. We were rewarded with beautifully symmetrical [^{3}H]TCDD-binding peaks in both cell fractions (Figs. 2 and 3). Clearly, however, the cytosolic and nuclear forms of AHR had different sedimentation velocities (Fig. 3). Further experiments would be required to find out why.

Although nuclear uptake of the [^{3}H]TCDD • AHR complex could be demonstrated *in vivo*, (as shown by our experiments and by William Greenlee's studies in Alan Poland's laboratory at about the same time; Greenlee and Poland, 1979), *in vivo* studies are cumbersome. Thus, after I completed my sabbatical leave in the Nebert laboratory, we collaborated with Michael Dufresne, a cell biologist at the University of Windsor, to refine our understanding of the cytosol-to-nucleus translocation process in a more tractable system, Hepa-1 cells in culture (Okey *et al.*, 1980). Cell culture experiments confirmed that sedimentation properties are quite different between cytosolic and nuclear forms of the AHR. Moreover, we found that translocation into the nucleus is a temperature-dependent process. The nuclear compartment is devoid of AHR until cells are

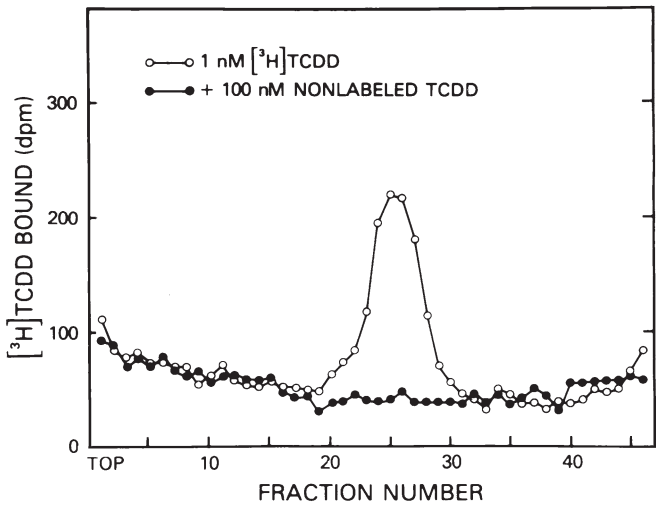

FIG. 2. Macromolecular snapshot that provides clues to AHR structure. Our first SDG velocity sedimentation profile of [^{3}H]TCDD binding, performed by Gregory Bondy in 1978. Hepatic cytosol from a C57BL/6N mouse was incubated with a concentration of 1nM [^{3}H]TCDD in the presence or absence of a 100-fold excess of nonradioactive TCDD. When [^{3}H]TCDD is present alone, there is a clear peak of radioactivity near the middle of the gradient. Radioactivity in this region is extinguished by excess ''cold'' TCDD or by other known CYP1A1 inducers (not shown). The 9S binding component proved to be the specific TCDD binding site that we designated the AHR (reproduced from: Okey *et al.* 1979).

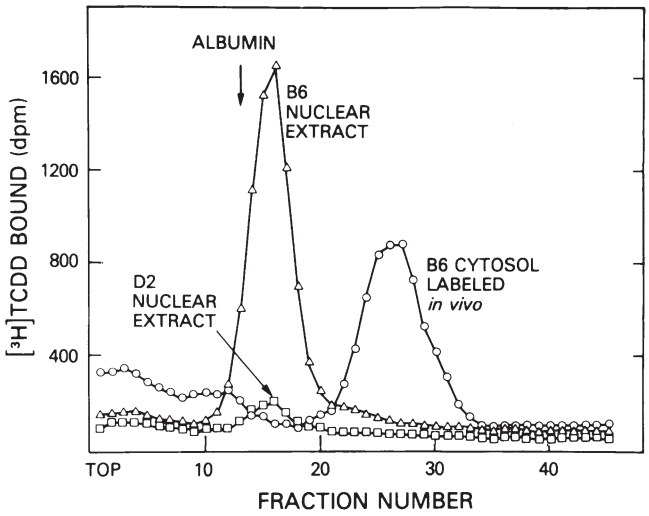

FIG. 3. Cytosolic and nuclear forms of AHR have different macromolecular properties. We injected a mouse intraperitoneally with [^{3}H]TCDD, then removed the liver 2 h later and prepared cytosol and nuclear extracts. Sedimentation of the binding component from nuclear extract (near fraction 15) is much slower than sedimentation of the cytosolic binding component (near fraction 26). This was the first evidence that the nuclear form of the AHR is a substantially smaller macromolecular complex than the receptor that initially binds TCDD in cytosol. See the text for details of this ligand-induced transformation. (Figure from Okey *et al.*, 1979, with permission of the publisher.)

exposed to a ligand at physiologic temperature. Recent work by Kawajiri and colleagues shows that the AHR protein contains a motif for nuclear localization as well as a motif for nuclear export and that phosphorylation of the nuclear-localization motif inhibits nuclear uptake of the AHR (Ikuta *et al.*, 1998, 2004a,b). However, the exact nature of the temperature-dependent step which is required for nuclear uptake still is not resolved.

Physicochemical Characterization: Appreciating the AHR as a Macromolecule

Kinetics of ligand binding (Kd and B_{max}) can be determined without knowing anything about the structure of the binding protein. However, in order to truly understand receptor function it is imperative to know the receptor's macromolecular properties.

When I returned to Canada after completion of my sabbatical year in the Nebert laboratory I was offered a position in the Division of Clinical Pharmacology in the Research Institute at The Hospital for Sick Children, Toronto, where we continued to investigate multiple facets of the AHR. One limitation in our research was that the SDG assay we initially used required overnight centrifugation in a swinging-bucket rotor that held only six samples. In Toronto, to increase our analytical capacity, my postdoctoral fellow, Hing Wo Tsui, developed a 2-h vertical-tube-rotor SDG assay (Tsui and Okey, 1981) that became a mainstay in our research program for several years and was widely adopted by other laboratories.

Devotees of the author George Orwell might have anticipated the dawn of the year 1984 with trepidation. But, in fact, 1984 was a very good year for my laboratory because it saw the arrival of two exceptionally productive postdoctoral fellows, Michael Denison and Patricia Harper, and an excellent Ph.D. student, Rebecca Prokipcak. Mike Denison and Becky Prokipcak immersed themselves in physicochemical characterization of the AHR while Patricia Harper (a cell biologist by training) spearheaded our transition toward a molecular approach to the AHR.

Mike Denison's hydrodynamic experiments showed that cytosolic AHRs from Sprague–Dawley rat liver and C57BL/6 mouse liver exist as macromolecular complexes of 250–280 kDa that can be dissociated, under conditions of high ionic strength, into smaller ligand-binding subunits of about 120 kDa for rat and 105 kDa for C57BL/6 mouse (Denison *et al.*, 1986c). These experiments provided the first evidence that the AHRs from rats and mice are similar but not identical molecular species, a finding that later would be confirmed and extended in the eras of immunoblotting and cloning.

As described above, our initial experiments *in vivo* and in cell culture showed that cytosolic and nuclear forms of AHR have different sedimentation properties (Okey *et al.*, 1979, 1980). One possibility was that the nuclear receptor is simply a monomeric ligand-binding subunit contained within a multimeric cytosolic AHR complex and that the ligand-binding subunit generated when we exposed labeled cytosol to high salt is the same macromolecule as the nuclear receptor. Further hydrodynamic analysis by Becky Prokipcak showed that this simple scenario is not true. Exposure of cytosol to high salt yields a [^{3}H]TCDD-binding component with a mass of 105 kDa (as seen in the Denison experiments), whereas the form of receptor recovered by high-salt extraction of nuclei from cells treated with [^{3}H]TCDD has a mass of about 176 kDa (Prokipcak and Okey, 1988). Clearly, the nuclear AHR was not simply the monomeric ligand-binding subunit. Becky went on to show, by photoaffinity labeling with [^{3}H]TCDD and electrophoresis under denaturing conditions, that the size of the ligand-binding component in nuclear AHR is the same as the ligand-binding component in cytosol (Prokipcak and Okey, 1990). Some additional component would need to be identified to account for the extra mass of the nuclear form of AHR detected in our laboratory and others (Elferink *et al.*, 1990; Gasiewicz *et al.*, 1991). The ''missing piece'' of the nuclear complex turned out to be the ARNT protein, later identified by Oliver Hankinson's laboratory (Hoffman *et al.*, 1991; Reyes *et al.*, 1992). The identities of the multiple constituents of the cytosolic AHR complex would eventually be identified by several laboratories (see below).

Of course my laboratory was not the only group involved in this early euphoric phase of AHR characterization. Jan-Åke Gustafsson's laboratory at the Karolinska Institute, Stockholm, had great expertise and experience with the glucocorticoid receptor and turned some attention to the ''TCDD receptor'' or

"dioxin receptor" (aka, AH receptor). They first used isoelectric focusing of [^{3}H]TCDD-labeled rat liver cytosol to identify a specific binding component that had high affinity and selectivity for CYP1A inducers (Carlstedt-Duke *et al.*, 1978). However, partial proteolysis with trypsin was required in order to focus the specific band; thus it was not possible to determine physicochemical properties of the native receptor protein. Lorenz Poellinger (initially in the Gustafsson laboratory) and Thomas Gasiewicz (University of Rochester) also performed extensive physicochemical analysis of the AHR and found that its overall properties were reminiscent of steroid receptors but with some distinct differences (Gasiewicz and Bauman, 1987; Gasiewicz and Rucci, 1984; Nemoto *et al.*, 1990; Poellinger *et al.*, 1982, 1983).

Since the AHR has many physicochemical properties in common with steroid receptors, both Lorenz Poellinger and Tom Gasiewicz adapted a steroid receptor technique based on adsorption to hydroxylapatite to measure [^{3}H]TCDD binding to AHR (Gasiewicz and Neal, 1982; Poellinger *et al.*, 1985). The "HAP" method became widely used by many laboratories as a rapid assay in AHR binding studies.

The ultimate physicochemical characterization for a protein is to derive a 3D crystallographic structure that will reveal how the protein functions. The AHR has not yet been crystallized. However, some insight into AHR 3D structure has been obtained by recent homology modeling based on similarities in primary structure between the AHR and related proteins (Pandini *et al.*, 2007). Modeling and site-directed mutagenesis reveal several structural features that are important to the ligand-binding function.

Lonely No More: Multiple AHR Partner-Proteins are Identified

As shown in Figure 4, the AHR resides in cytoplasm until binding of ligand triggers transformation of the receptor and its translocation into the cell nucleus. Our early physicochemical characterization indicated that both the cytosolic and the nuclear forms of AHR are oligomeric complexes composed of the AHR protein in association with other macromolecules. Identities of AHR-interacting proteins were resolved, beginning in the late 1980s, through the efforts of many laboratories, particularly those of Gary Perdew, Christopher Bradfield, and Lorenz Poellinger.

The heat-shock protein, hsp90, is a major constituent of the cytosolic complex; its presence was sought in the AHR complex because hsp90 already was well-known to be a vital chaperone for steroid receptors. In addition to hsp90 the cytosolic AHR complex contains at least two other proteins, ARA9 (also known as AIP or XAP2) which assists in stabilizing the AHR and retaining it in the cytoplasmic compartment and p23 which appears to aid release of hsp90 from the AHR after a ligand binds (reviewed in Harper *et al.*, 2006; Petrulis and Perdew, 2002).

At first glance things seem simpler in the nucleus since the nuclear AHR complex contains only the AHR itself tightly

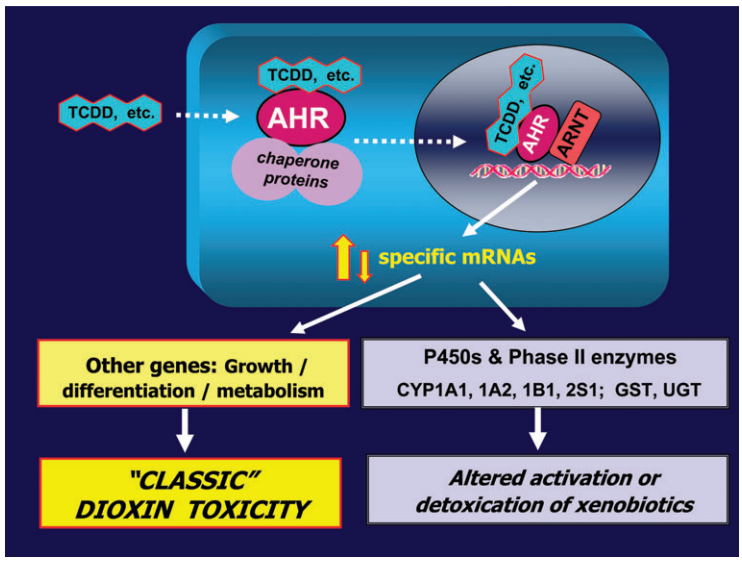

FIG. 4. Simplified diagram of the AHR's mode of action. Prior to ligand binding the AHR resides in cytoplasm where it is bound to hsp90 and additional chaperone proteins. Ligand binding causes translocation into the nucleus, dissociation of chaperone proteins, and dimerization of AHR with the ARNT protein. The ligand • AHR • ARNT complex binds to AHREs located in the 5′-flanking region of numerous genes. When occupied by the liganded AHR complex, AHREs act as transcriptional enhancers to increase the rate of synthesis of specific mRNAs. Note that the liganded AHR also may suppress (downregulate) transcription of some genes (Tijet *et al.*, 2006) through mechanisms that are not yet clear (Riddick *et al.*, 2004). Induction of Phase I and Phase II enzymes is important because it strongly influences the balance of bioactivation/detoxication for a wide range of xenobiotic chemicals. However, "classic" forms of dioxin toxicity appear to be due primarily to dysregulation of genes that encode products other than Phase I/Phase II enzymes. See the text for details. (Diagram modified from Okey *et al.*, 2005b.)

bound to its dimerization partner, ARNT. However, the nuclear ligand • AHR • ARNT complex undergoes a host of protein–protein interactions with coactivators, coreppressors, chromatin remodeling proteins, and basal transcription factors (reviewed in Hankinson, 2005; Kewley *et al.*, 2004; Rowlands *et al.*, 1996; Swanson, 2002).

The formal family of nuclear receptors (including steroid receptors) and the bHLH/PAS family (to which the AHR belongs) are structurally unalike, even though both gene families encode ligand-dependent transcription factors. It should not come as a surprise that many of the chaperones, coactivators, and corepressor proteins which interact with the AHR also interact with other receptors. The evolutionary tool-kit contains component parts that frequently are shared by multiple cellular pathways.

Identification of Response Elements: the AHR Finds its Home on DNA

Successful biological regulation requires a degree of specific recognition at multiple levels in signaling pathways such as ''specificity'' of a receptor for its ligands. Within the nucleus, the ligand • AHR • ARNT complex also must be recognized by specific sites in order to regulate gene expression in an orderly fashion. The specific nucleotide sequence to which the nuclear AHR complex binds was first identified by Mike Denison who, after a very productive stint as a post-doc in my laboratory, joined Jim Whitlock's laboratory at Stanford where they located and sequenced a ''dioxin-responsive element'' in the 5′-flanking sequence of the highly inducible mouse *Cyp1a1* gene (Denison *et al.*, 1988a,b). This subsequently was corroborated by Yoshiaki Fujii-Kuriyama's laboratory (then at Tohoku University, Japan) who termed the enhancer element ''xenobiotic-responsive element'' (XRE) (Fujisawa-Sehara *et al.*, 1988). (In keeping with terminology for the receptor itself, we prefer the term ''AHRE'' [AH response element].) The Whitlock laboratory went on to extensively describe how the AHR affects the *CYP1A1* promoter and chromatin structure to alter gene expression (reviewed in Swanson, 2002; Whitlock, 1999).

In collaboration with Mike Denison, Patricia Harper and I showed that human AHR can be activated by ligand to bind to the same nucleotide sequence that comprises the mouse AHRE (Harper *et al.*, 1992). The fundamental mechanism of gene regulation by the AHR is well-conserved across mammalian species. The core pentanucleotide AHRE sequence (GCGTG) occurs frequently within mammalian genomes (Lee *et al.*, 2006; Sun *et al.*, 2004; Tijet *et al.*, 2006). Recently Oliver Hankinson's laboratory reported that the mouse *Cyp2s1* gene contains three overlapping AHRE sequences upstream of the promoter and three overlapping hypoxia response elements (HREs) embedded within the region containing the AHREs (Rivera *et al.*, 2007). The potential complexity of gene regulation by the AHR is illustrated by the fact that not only does the AHR • ARNT dimer bind to this regulatory region, the region also binds the HIF-1α • ARNT dimer which is a powerful regulator of genes that respond to hypoxia. As we will see later, the architecture of receptors and their response elements provide ample opportunity for cross-talk in a potentially very complex combinatorial fashion.

The AHRE sequence originally identified in the mouse *Cyp1a1* gene probably is the response element for the majority of AHR-regulated genes. However, induction of CYP1A2 has perennially been a more complex problem than induction of CYP1A1. Sogawa *et al.* (2004) identified a novel enhancer element in the rat *CYP1A2* gene which seems to be the site of action of the TCDD • AHR • ARNT complex and termed this response element ''XRE-II.'' As a twist on the ''standard model'' of AHR signaling, the TCDD • AHR • ARNT complex does not bind directly to the XRE-II response element; rather, the complex appears to couple to XRE-II through binding to an unidentified adapter protein which itself binds XRE-II. My laboratory wondered whether the XRE-II motif (which we term AHRE-II) was unique to the rat *CYP1A2* gene or whether this element might be involved in other AHR-mediated gene responses. Paul Boutros, an insightful bioinformaticist in my laboratory, used phylogenetic footprinting to show that the AHRE-II motif is conserved in at least 36 genes across the genomes of mouse, rat and human. By gene expression array analyses we found that about 15 genes which contain conserved AHRE-II motifs respond to TCDD. Rather surprisingly, many of these genes that appear to respond through the AHRE-II element encode ion-channel proteins and transporters rather than enzymes related to metabolism of xenobiotic chemicals (Boutros *et al.*, 2004).

AHR Downregulation by its Ligands

Our initial AHR studies in cell culture hinted that treatment with TCDD causes the total cellular AHR content to decrease rapidly (Okey *et al.*, 1980). My Ph.D. student, Becky Prokipcak, performed a thorough accounting of cytosolic and nuclear forms of AHR in Hepa-1 cells and established, for the first time, the phenomenon of ligand-induced downregulation of AHR (Prokipcak and Okey, 1991).

A skilled technical assistant in my laboratory, John Giannone, then showed that if protein synthesis is blocked with actinomycin D or cycloheximide, nuclear AHR levels do not decrease after cells are exposed to TCDD. These data were the first evidence that ligand-dependent downregulation of the AHR likely results from protein degradation involving a short-lived protease (Giannone *et al.*, 1995). Subsequently, we confirmed that downregulation of cellular AHR content is not due to a decrease in AHR messenger RNA (mRNA) but, rather, via loss of AHR protein while sparing its dimerization partner, ARNT (Giannone *et al.*, 1998). The laboratories of Richard Pollenz, Murray Whitelaw, and Qiang Ma then independently discovered that the mechanism of ligand-induced AHR downregulation in cell culture is predominately through the

ubiquitin-proteasome pathway (Ma and Baldwin, 2000; Pollenz, 2002; Roberts and Whitelaw, 1999).

As described above, downregulation was first discovered in cell culture. Downregulation also can occur in TCDD-exposed tissues *in vivo* (Pollenz *et al.*, 1998; Sommer *et al.*, 1999). However, a Ph.D. student in my laboratory, Monique Franc, found that downregulation in rodent liver is transient following a single TCDD dose *in vivo* and that after a few days TCDD actually causes a slight upregulation in AHR mRNA and AHR protein (Franc *et al.*, 2001a). She also mimicked real-world environmental/dietary exposure to TCDD and found that AHR levels remain relatively constant in the face persistent, low-dose TCDD intake (Franc *et al.*, 2001b).

AHR downregulation presumably represents a cell's method of desensitizing itself and preventing excessive stimulation from potent agonists. Downregulation is dramatic in cell culture but its transient nature *in vivo* suggests that most tissues will not be desensitized following persistent, low-dose TCDD challenge. So far as we know, resistance to TCDD toxicity cannot be attributed to sustained downregulation of AHR levels in any animal species. AHR levels *in vivo* and in cell culture are affected by a bewildering variety of factors, including the receptor's own ligands; for a recent summary please see Harper *et al.* (2006). Our understanding of the ''what regulates the regulator?'' remains rudimentary. New factors continually are being discovered such as the recent reports that Erk kinase participates in AHR degradation (Chen *et al.*, 2005) and that NS1BP, a protein which contains a ''kelch'' domain, may regulate functional levels of AHR in cells both by tethering AHR to the cytoskeleton and by influencing proteasomal degradation (Dunham *et al.*, 2006).

Ubiquitous Expression of the AHR

The original discovery of a specific [^{3}H]TCDD binding site in mouse liver (Poland *et al.*, 1976b) naturally led to the question of how widely this new receptor is distributed across animal species and tissues.

Ontogeny and tissue distribution. As described above, fetal cells were a valuable early model system to study induction mechanisms for CYP1 enzymes (Nebert and Gelboin, 1968b). In addition, TCDD is one of the most potent teratogens known in rodents. With these motivations, multiple laboratories mapped AHR expression during development.

The developing mouse kidney is exceptionally sensitive to teratogenesis by TCDD which induces hydronephrosis in an AHR-dependent fashion (Lin *et al.*, 2001; Mimura *et al.*, 1997; Peters *et al.*, 1999). Sharon Choi, a Ph.D. student working with my colleague Patricia Harper in Toronto, found that AHR mRNA is expressed as early as gestational day-14 in ureter from C57BL/6 mice (Choi *et al.*, 2006).

There was a surprise when we studied AHR expression and function in embryonic tissue and cells derived from ''nonresponsive'' mouse strains. Recall that livers of adult nonresponsive strains are completely refractory to CYP1A induction by nonhalogenated ligands. In cytosol from tissues of embryos at 15–19 days gestation, not only were we able to detect specific binding of the potent agonist, [^{3}H]TCDD, we also were able to detect some specific binding of the nonhalogenated inducer, [^{3}H]3-MC, to AHR. Moreover, in primary cultures derived from embryos we found that dose–response curves for AHH induction by another PAH inducer, benz[*a*]anthracene, were essentially the same for cells from ''nonresponsive'' mice as they were for ''responsive'' mice (Harper *et al.*, 1991a). Ying Huang, a graduate student working with Patricia Harper and me, found that the AHR in embryonic cells was indistinguishable from the receptor expressed in adult liver (Huang *et al.*, 1995). The mechanistic explanation remains elusive for why embryonic cells from ''nonresponsive'' strains can, in fact, respond to PAHs, whereas adult tissues *in vivo* show strong separation into ''responsive'' and ''nonresponsive'' phenotypes. These experiments in embryonic cell cultures suggest that the cellular context has a significant influence on AHR function, possibly because the particular repertoire of proteins that interact with the AHR differs between embryonic cells versus cells from adult animals.

During postnatal development, Carlstedt-Duke *et al.* (1979) discovered that rat liver AHR levels are highest around weaning, then wane as animals age. This pattern of highest hepatic AHR prior to weaning was confirmed for rat by Kahl *et al.* (1980) and also found to hold true for mouse and rabbit. Tom Gasiewicz's laboratory at the University of Rochester extended the ontogenic findings by showing that although AHR levels drop in liver and lung after weaning, levels in thymus remain elevated for a longer period (Gasiewicz *et al.*, 1984). In rat prostate, AHR levels are high at birth but undergo a steep decline even before weaning (Sommer *et al.*, 1999). The general pattern holds for most rodent tissues: AHR levels are highest in the younger animals and decline with age. This pattern suggests that the main biological role of the AHR plays out during development. Later, the tools of molecular biology would further illuminate the fundamental biology of the AHR in relation to development (see below).

After the AHR was discovered in rodent livers, we and other laboratories surveyed a wide range of mammalian tissues to determine how broadly this new regulatory protein is distributed. By radioligand binding and SDG assays, Michelle Mason, a research assistant in my laboratory, detected AHR in liver, lung, kidney, intestine, thymus, and prostate of C57BL/6 mice and Sprague–Dawley rats. By treating mice *in vivo* with [^{3}H]TCDD she also found that the [^{3}H]TCDD $\times$ AHR complex could be recovered from nuclei of liver, lung, kidney not only in responsive C57BL/6 mice but also in ''nonresponsive'' DBA/2 mice (Mason and Okey, 1982). This was the first definitive evidence that nonresponsive mice do, in fact, possess an AHR that is competent to bind ligand and translocate into the nucleus.

Space does not permit a full accounting of each mammalian tissue that subsequently has been shown to express the AHR.

Suffice it to say that methods ranging from ligand binding to mRNA expression profiling reveal that the AHR can be detected in virtually all mammalian cells and tissues, albeit at widely varying levels.

A few words on phylogeny. From the earliest days of AHR research there has been the conundrum of why animals are endowed with a receptor whose main function appears to be binding of notoriously toxic and carcinogenic xenobiotic chemicals. In the precloning era, mapping the phylogenetic distribution of AHR was one avenue to trying to understand the receptor's ''purpose'' and evolutionary history.

Most toxicology is ''mammalocentric'' but we and other laboratories wanted to find out if the AHR was present in nonmammalian species as well as in a broad spectrum of mammals (Denison and Wilkinson, 1985). Chick embryo has a noble history as an excellent model system for studying AHH induction and other biochemical/toxic effects of halogenated aromatic hydrocarbons (Hamilton *et al.*, 1983; Poland and Glover, 1973a,b; Rifkind *et al.*, 1990). In collaboration with Christopher Wilkinson's laboratory (Cornell University), Mike Denison and I found that AHR is expressed in chick embryo as early as at 5 days of incubation and that levels in chick liver drop rapidly after hatching, reminiscent of the postnatal decline in AHR levels in rodents (Denison *et al.*, 1986a). Gail Bellward's laboratory (University of British Columbia) found, using our assay methods, that AHR is not confined to domestic fowl but also is detectable in feral bird species such as pigeon, heron, and cormorant (Sanderson and Bellward, 1995).

We also were interested in AHR expression in fish because trout are highly sensitive to biochemical and toxic effects of dioxin-like chemicals and PAHs. Our attempts to detect AHR with our standard [^{3}H]TCDD binding assay in trout liver proved fruitless, possibly because fish livers contain proteolytic enzymes (Hahn *et al.*, 1994) that are adapted to low temperatures and happily degrade the AHR, even at the 0–4° conditions of the binding assay. Angela Lorenzen, a graduate student in my laboratory, was first to gain solid evidence that the AHR exists not only in homeothermic vertebrate species but also in poikilothermic animals. She used a trout hepatoma cell line in culture to demonstrate binding of [^{3}H]TCDD to cytosolic AHR, translocation of the [^{3}H]TCDD • AHR complex into the nucleus and subsequent induction of AHH activity (Lorenzen and Okey, 1990). As we would find with recalcitrant AHRs in other systems, adding molybdate to the buffer was essential to stabilize trout AHR so that specific [^{3}H]TCDD binding could be detected.

Research over the past three decades indicates that AHR structure and function are remarkably diverse among vertebrates and invertebrates. Early physicochemical analyses suggested that molecular properties of the AHR are similar among laboratory mammals such as rat, mouse, guinea pig, and hamster (Gasiewicz and Rucci, 1984; Poellinger *et al.*, 1983); however, our physicochemical characterization indicated that rat and mouse AHRs are not identical (Denison *et al.*, 1986c).

Structural differences became even more apparent when both photoaffinity labeling and the development of anti-AHR antibodies allowed electrophoretic separation of AHRs from different animal species under denaturing conditions. These experiments indicate apparent molecular masses ranging from 95 kDa for the product of the mouse *Ahr^{b1}* allele to 146 kDa in trout (Hahn *et al.*, 1994; Landers *et al.*, 1989; Poland and Glover, 1987, 1990; Poland *et al.*, 1991; Prokipcak and Okey, 1990). Cloning and sequencing of *AHR* genes, beginning in the 1990s, confirmed the diversity of molecular masses among vertebrate AHRs and also revealed that most of the variation in AHR primary structure resides near the carboxy terminus of the protein (Gu *et al.*, 2000; Korkalainen *et al.*, 2001).

[^{3}H]TCDD binds with specificity and high affinity to AHR proteins from a wide range of vertebrate species. However, in invertebrates, specific [^{3}H]TCDD binding has not been detectable in any species out of the many tested (Denison *et al.*, 1985, 1986d; Hahn *et al.*, 1994). Mark Hahn's laboratory at the Woods Hole Oceanographic Institution has taken a leading role in demystifying phylogeny and evolution of the AHR (Hahn, 1998, 2002; Hahn *et al.*, 2006). He proposes that during the AHR's evolutionary history it has changed from a protein that does not bind ligand (invertebrates) to a protein that is a ligand-activated transcription factor (Hahn *et al.*, 2006). Among the Hahn laboratory's other key discoveries is the unexpected finding, from comparative genomics, that mammals seem impoverished in regard to how many *AHR* genes exist within the genome of an individual species. Genomic sequencing indicates that mammals have but a single *AHR* gene, whereas in certain fish or bird species there may be as many as two to five genomic sequences that are predicted to encode AHRs. For a full appreciation of AHR phylogeny and evolution see the comprehensive and authoritative reviews by Mark Hahn (Hahn, 1998, 2002; Hahn *et al.*, 2006).

The nearly ubiquitous occurrence of AHR in vertebrate tissues implies that this receptor has important biological functions. However, as we will see below, AHR knockout, at least in mice, is not lethal.

Humans, Too, Have AHR

After the discovery and initial characterization of AHR in rodent tissues, we and other laboratories were eager to determine if humans possess a similar receptor. Such knowledge would be valuable when attempts are made to incorporate mechanistic data on dioxin toxicity into human risk assessment. But the human AHR obstinately refused to cooperate. When we used assay methods that worked well in rodent cells and tissues, AHR abundance appeared to be very low or absent in clinical samples such as lung (Roberts *et al.*, 1986).

We screened many human cell lines and tissues with disappointing results. With human tissues, there is the challenge

of obtaining tissues of good quality while adhering to ethical requirements. Fortunately, human placenta is highly responsive to induction of CYP1A enzymes by cigarette smoke (Manchester *et al.*, 1984; Nebert *et al.*, 1969; Welch *et al.*, 1968). David Manchester (a pediatrician and clinical geneticist at the University of Colorado Health Sciences Center) had a long-standing interest in regulation of CYP1 enzyme induction in placenta and had set up a very effective protocol for obtaining placental tissues of high quality from smoking mothers and nonsmokers, so we collaborated with David's laboratory to investigate AHR in this responsive and available tissue.

We knew that molybdate was a very helpful ingredient in homogenizing buffers to stabilize various steroid hormone receptors but we had found that molybdate was not really necessary to stabilize AHR in rodent livers (Denison *et al.*, 1986b). Nevertheless, when we modified our procedures with human placenta by including molybdate in the homogenizing buffer, we found that molybdate was an elixir that finally permitted us to detect human AHR (Manchester *et al.*, 1987). As it turns out, placenta is the human tissue that is perhaps the most richly endowed with AHR (Manchester *et al.*, 1987; Okey *et al.*, 1997).

By adding molybdate to the buffer and making other adjustments, we were able to routinely detect AHR in a wide variety of human tissues. My graduate student, Angela Lorenzen (who also discovered AHR in trout), used the improved assay to demonstrate that human tonsils express significant AHR levels (Lorenzen and Okey, 1991); this is of potential relevance to human health since atrophy of immune organs such as the thymus is one of the most sensitive toxic responses to dioxin-like chemicals in rodents.

Human tissues are useful for obtaining a snapshot of AHR abundance in diverse tissues from different donors. However, tissue samples cannot provide much information on the function of AHR pathways. Thus, we applied our improved receptor assay to several human cell lines. My Toronto colleague, Patricia Harper, a genuine cell biologist, directed most of our studies in cell model systems.

In humans, skin is the most notable target for dioxin toxicity which manifests as chloracne (Geusau *et al.*, 2001; Panteleyev and Bickers, 2006), the disfiguring condition that came to world-wide attention with the poisoning of presidential candidate, Victor Yushchenko, in Ukraine in 2005 (Schecter *et al.*, 2006). As a surrogate for skin cells, we tested the human squamous cell carcinoma line, A431 and detected good levels of AHR. In the A431 cells (just as in our previous research with the mouse Hepa-1 cell line) exposure to TCDD provoked nuclear translocation of the AHR. Both TCDD and benz[*a*]-anthracene induced AHH activity in a classic sigmoidal dose–response fashion (Harper *et al.*, 1988).

Eve Roberts, a clinician hepatologist, undertook a period of basic research training in my laboratory and found that HepG2 cells, a human hepatoma cell line widely used as a model for human hepatic drug metabolism, express AHR and induction of CYP1A1 (Roberts *et al.*, 1990). In collaboration with William Waithe and Alan Anderson (L' Hotel dieu de Quebec), we found that human peripheral blood lymphocytes, after being immortalized for growth in culture, displayed the complete AHR-dependent regulatory mechanism for CYP1A1 induction (Waithe *et al.*, 1991)

The highest AHR concentration that we've detected in any human cell line is in LS180 colon carcinoma cells (Harper *et al.*, 1991b). A postdoctoral fellow in my laboratory, Wei Li, found that LS180 cells exhibit CYP1A2 induction by TCDD or 3-MC (Li *et al.*, 1998). Although CYP1A1 is highly inducible in a wide variety of mammalian cell lines, CYP1A2 expression in immortalized cells lines usually is silenced for reasons that are unclear. Thus, the LS180 cell line constitutes an opportunity to clarify factors that regulate basal CYP1A2 expression and its AHR-dependent induction.

After early frustrations in our search for human AHR it has been rewarding to see dozens of reports on AHR expression in a wide range of human tissues and cell types (summarized in Okey *et al.*, 1994a). Unquestionably, the AHR is available in humans to carry out many of the same functions (for better or worse) that it does in laboratory animals.

Astonishing Range of AHR Ligands

As we have seen, [^{3}H]TCDD became the ideal ligand for detection and characterization of the AHR. Quite clearly, however, the binding site is not just a ''dioxin receptor'' or ''TCDD receptor.'' Beginning with the first studies with [^{3}H]TCDD as radioligand, a multitude of chemicals has been tested to see if they can compete with [^{3}H]TCDD for specific AHR-binding sites. Competition studies are a mainstay in determining if a new chemical is a receptor ligand because the test chemical does not need to be radiolabeled. Through competition studies, the catalog of AHR ligands has been greatly expanded over the past three decades. However, because [^{3}H]TCDD binds with very high affinity and dissociates exceedingly slowly from the AHR, methods used to test for competition need to be carefully designed to prevent false negative conclusions, especially when weak ligands are tested (Denison and Nagy, 2003).

Based on the original experiments with TCDD and closely related compounds it appeared that the AHR ligand-binding site had rigid dimensions and that it could accommodate ligands only if they were highly planar. This view has changed dramatically as the catalog of ligands expanded via competition studies and high-throughput screening assays. Now the AHR is viewed as one of the ''promiscuous'' receptors; that is, a receptor that can effectively bind compounds of diverse shape and chemical properties. Among the ligands from exogenous sources: halogenated dioxins, dibenzofurans, and polychlorinated biphenyls (PCBs); nonhalogenated PAHs; flavones and carbinols of plant/dietary origin; therapeutic agents such as omeprazole (for an excellent review see Denison and Nagy, 2003).

As I mentioned in the Prelude, my original interest in enzyme induction was motivated by effects of the pesticide, DDT. Although we found that commercial grade *p,p′*-DDT has some ability to inhibit binding of [^{3}H]TCDD to the AHR (Okey *et al.*, 1979), it later would turn out that P450 induction by DDT *in vivo* most likely is due to binding of its metabolite, dichlorodiphenyldichloroethylene to the nuclear receptors CAR and PXR rather than to the AHR (Coumoul *et al.*, 2002; Wyde *et al.*, 2003). Thus, although the AHR is ''promiscuous,'' it is not the universal receptor for all environmental contaminants.

Endogenous Ligands (or Why did Evolution Endow us with a Receptor for Toxic Dioxins?)

The question of why a ''dioxin receptor'' arose in evolution is inextricably tied to the question of whether there is an endogenous AHR ligand that regulates ''normal'' physiologic functions. From the earliest days of AHR research there has been a keen interest in identifying the ever-elusive endogenous ligand. It might be more judicious to say endogenous ligands because there is no *a priori* reason why multiple endogenous agents from different chemical classes might not exist, given the AHR's promiscuous reputation for binding structurally diverse exogenous compounds (Denison and Nagy, 2003).

Progress in identifying candidate endogenous ligands was slow during the first two decades of AHR research. However, in recent years multiple endogenous agents have been shown to activate AHR pathways. For example, arachidonic acid (AA) metabolites are released in response to TCDD and it is possible that some AA metabolites, such as prostaglandins, may act as AHR agonists (Denison and Nagy, 2003; Rifkind, 2006). Bilirubin and related tetrapyroles, at high concentrations, can activate AHR, perhaps serving to induce glucuronosyltransferase enzymes that conjugate and remove the potentially toxic products of heme degradation (Denison and Nagy, 2003; Sinal and Bend, 1997). Ultraviolet (UV) irradiation photo-converts tryptophan into products that have high affinity for the AHR and are potent inducers of CYP1A1 (Denison and Nagy, 2003; Rannug and Fritsche, 2006). Tryptophan also can be converted by the enzyme, aspartate aminotransferase, into indole-3-pyruvate which spontaneously generates multiple compounds that can act as AHR agonists (Bittinger *et al.*, 2003).

One approach to identifying endogenous ligands is to prepare tissue extracts and assay their ability to activate an AHR-mediated reporter gene system. Song *et al.* (2002) employed this approach and isolated, from porcine lung, a compound whose structure was identified as 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE). Very recently Henry *et al.* (2006) confirmed that synthetic ITE is a potent AHR agonist which induces CYP1A1 in cell culture and *in vivo*; however, ITE does not produce dioxin-like teratogenic effects in mice. This is another instance which reminds us that high-affinity AHR ligands are not necessarily toxic. We need to find out why this is true for specific chemical cases and what features of a chemical ligand are necessary to elicit severe dioxin-like toxicity.

Of course, an obvious explanation for why a high-affinity ligand might lack toxicity is that the ligand is an antagonist rather than an agonist. Savouret *et al.* (2000) identified 7-ketocholesterol (7-KC) as an endogenous compound that competitively binds AHR and inhibits CYP1A1 induction by TCDD; they propose that 7-KC is a ''protective modulator'' of AHR function.

Another contender for the role of endogenous AHR agonist is modified low-density lipoprotein (LDL). In the 1990s hydrodynamic shear stress was found to induce CYP1A1 in cell culture and initially it was thought that induction was due to release of AA metabolites (Mufti and Shuler, 1996). However, very recently the Bradfield laboratory reported that hydrodynamic shear stress (mimicking blood flow in the vasculature) modifies an LDL in blood serum such that the LDL becomes an AHR activator (McMillan and Bradfield, 2007). This may provide a mechanism for the vital role which the AHR plays in vascular development (Lahvis *et al.*, 2005).

This brief overview of the range of exogenous and endogenous AHR ligands is intended simply to remind us of how little we understand about the diversity of AHR ligands and AHR functions. Chris Bradfield's laboratory divides AHR-mediated responses into three pathways: (1) adaptive responses (such as changes in xenobiotic metabolism); (2) toxic pathway; (3) developmental pathway (Walisser *et al.*, 2004b). Different ligands may selectively act upon one or more of these pathways for good or ill (Denison and Nagy, 2003).

To conclude this section, let's consider what happens if the AHR is activated in the *absence* of any ligand. Lorenz Poellinger's laboratory deleted the ligand-binding domain and thereby created an AHR that is constitutively active, i.e., it stimulates gene expression without any ligand (Kohle *et al.*, 2002; McGuire *et al.*, 2001). This led to the disturbing discovery that mice whose AHR is locked into the ''on'' state have a shortened life-span and frequently develop stomach cancer. However, stomach cancer is not commonly seen in laboratory animals exposed to dioxin-like chemicals and it is unclear why the constitutively active AHR causes stomach tumors, whereas the persistent AHR ligand, TCDD, is not an efficient gastric carcinogen. The constitutively active AHR in these mice does mimic some other toxic effects of TCDD, notably thymic atrophy and liver enlargement (Brunnberg *et al.*, 2006). An independently derived mouse strain, in which the constitutively active AHR is expressed specifically in T cells, also shows thymocyte loss without needing to be activated by TCDD (Nohara *et al.*, 2005).

In the above studies, constitutively active AHR was produced through molecular engineering by the investigators. In an intriguing recent review (Schlezinger *et al.*, 2006), David Sherr and colleagues summarize evidence which indicates that breast tumor cells frequently contain significant levels of nuclear AHR in the absence of any exogenous ligand. This ''constitutive

activation'' of AHR might account for elevated CYP1B1 levels in tumor cells and may be important in pathogenesis. It is not clear whether the nuclear AHR in breast tumors has been activated by an endogenous ligand or whether the receptor truly is ''constitutively activated'' but this model deserves further attention, both to understand the regulatory mechanisms and for its implications for mammary carcinogenesis.

Photoaffinity Labeling as the Path to Purification of AHR Protein

As mentioned above, early AHR characterization relied on reversible, high-affinity binding of [^{3}H]TCDD. Much was learned with this approach. However, with reversible ligands, unless biochemical procedures are gentle, the radioligand dissociates from the receptor and leaves the investigator in the dark. Clearly, it would be advantageous to permanently attach a radioligand to the AHR.

Photoactivation of [^{3}H]TCDD and [^{3}H]3-MC with UV light leads to a modest level of covalent attachment of these radioligands to the AHR (de Morais *et al.*, 1994; Landers *et al.*, 1989; Prokipcak and Okey, 1990). However, a photoaffinity ligand developed by Andrew Kende and Alan Poland (Poland *et al.*, 1986), 2-azido-3[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin, was much more efficient at covalent coupling to the AHR and possessed very high specific activity. Using this photoaffinity ligand and various methods of protein fractionation, two postdoctoral fellows in the Poland laboratory, Gary Perdew and Chris Bradfield, successfully purified AHR from liver of C57BL/6 mice (Bradfield *et al.*, 1991; Perdew and Poland, 1988). Most importantly, a partial N-terminal amino acid sequence was obtained from the purified protein. This, at last, opened the door to cloning the AHR.

Cloning of Mouse AHR: Revelation of a New Gene Family

The first cloning of mouse AHR cDNA was done the ''old-fashioned'' way. The laboratories of Chris Bradfield and Yoshiaki Fujii-Kuriyama each took advantage of the N-terminal protein sequence to design oligonucleotide probes that then were used to screen cDNA libraries (Burbach *et al.*, 1992; Ema *et al.*, 1992). Prior to AHR cloning, most of us in the AHR community expected the AHR sequence to be similar to that of various steroid receptors, many of which already had been cloned. As it turned out, despite the fact that the AHR and steroid receptors behave very similarly in their general mode of action, the AHR belongs to an entirely different gene family.

The closest relative to AHR, based on sequence, is the ARNT protein, cloned the year prior to AHR cloning in Oliver Hankinson's laboratory (Hoffman *et al.*, 1991). AHR and ARNT contain domains with high sequence similarity to each other and also domains with sequence similarity to two *Drosophila* proteins, Per and Sim (Burbach *et al.*, 1992; Ema *et al.*, 1992). ARNT, AHR, Per, and Sim became founding members of the PAS protein superfamily. Proteins such as AHR and ARNT which contain PAS domains constitute a subgroup within the broader basic helix–loop–helix (bHLH) superfamily of transcriptional regulators. bHLH/PAS proteins must dimerize in order to bind DNA and dimerization is a major function of the PAS domain (reviewed in Gu *et al.*, 2000; Kewley *et al.*, 2004). The bHLH/PAS superfamily expanded rapidly and now includes members with such diverse functions as clock proteins involved in circadian rhythms and multiple other physiologic roles (McDearmon *et al.*, 2006).

Purification of mouse AHR and subsequent cDNA cloning were enormous breakthroughs which allowed the very powerful techniques of molecular biology to lift AHR characterization to an entirely new level. Initial cloning was rapidly followed by identification of domains (Fig. 5) in the modular AHR protein that carry out key functions of ligand binding, dimerization with ARNT, DNA binding, and transactivation of gene expression (Dolwick *et al.*, 1993; Fukunaga *et al.*, 1995; Whitelaw *et al.*, 1993, 1994).

Since the initial cloning of mouse AHR cDNA, cloning has been accomplished for several other species including rat (Elferink and Whitlock, 1994), hamster (Korkalainen *et al.*, 2000), guinea pig (Korkalainen *et al.*, 2001), numerous other mammalian species, fish, and birds (reviewed in Hahn, 2002; Hahn *et al.*, 2006). Collectively, cloning studies from multiple vertebrate species reveal that sequence is highly conserved in the N-terminal region which contains bHLH/PAS domains whereas the C-terminal region is much more variable. Later in this review we will consider the impact of sequence variation on AHR function.

Cloning of human AHR cDNA in Chris Bradfield's laboratory (Dolwick *et al.*, 1993) was particularly important because this opened the possibility of determining, by molecular epidemiologic studies, whether human *AHR* polymorphisms might

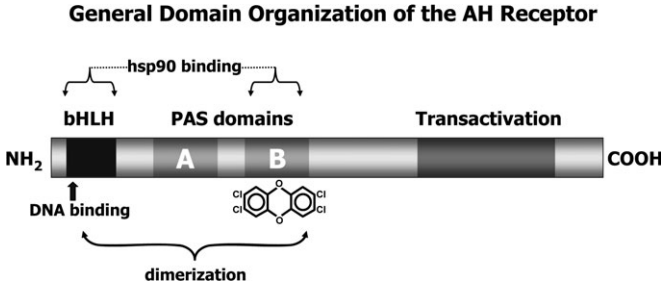

FIG. 5. General domain organization of the AHR. The AHR has modular organization in which specific functions are concentrated within particular domains. Ligands bind the PAS-B domain. Binding to hsp90 involves both the PAS-B domain and the bHLH domain. Dimerization with ARNT also involves both the HLH domain and the PAS domains. DNA binding and the nuclear-localization signal reside in the basic region of the bHLH domain. The transactivation function involves a large region toward the carboxy terminus and contains several subdomains (not shown) (Ko *et al.*, 1997; Reen *et al.*, 2002; Sogawa *et al.*, 1995). The carboxyl half of the AHR protein shows the greatest structural variation across different animal species whereas bHLH/PAS domains are highly conserved. (Modified from Gu *et al.*, 2000; Kewley *et al.*, 2004; Okey *et al.*, 1994b.)

be associated with the risk of adverse health effects from dioxin-like compounds (see below).

AHR: THE ESSENTIAL MEDIATOR OF TCDD TOXICITY

Receptor theory is the backbone of pharmacology. The principles of receptor function have provided mechanistic explanations of great utility in pharmacology and therapeutics for understanding drug properties such as specificity, potency, and efficacy.

Although Paul Ehrlich postulated in the late 19th century that pathogenic microbes possess receptors which might serve as fruitful targets for toxic therapeutic agents (toxic, that is, to the microbes), receptor theory is a relative latecomer to the field of mammalian toxicology. Despite this late start, receptor theory provides considerable explanatory power regarding mechanisms that underlie toxicities of certain important xenobiotic chemicals. The AHR was in the vanguard of research that linked soluble intracellular receptors to xenobiotic toxicity.

The confluence of multiple independent lines of evidence firmly establishes that essentially all major toxic effects of TCDD and related dioxin-like substances are mediated by the AHR. The earliest robust evidence that the AHR mediates dioxin toxicity arose from structure–activity analyses of dioxin congeners coupled with mouse genetics.

Ligand Structure in Relation to Toxicity

Quantitative structure–activity relationships. One aspiration of toxicologists is to be able to predict the toxicity of a compound from its chemical structure alone. There is a vast literature on the relationship between chemical structure and biological activity for a wide range of toxicant categories, including dioxin-like compounds. Indeed, quantitative structure–activity relationships (QSAR) constitute one of the foundations of dioxin research. QSAR principles permeate mechanistic models as well as dioxin risk assessment.

Prior to structure–activity evaluation of dioxins in relation to overt toxic endpoints, Alan Poland's laboratory showed that induction of AHH was strongly influenced by structure for small series of chlorinated congeners of dibenzo-*p*-dioxins, dibenzofurans, azoxybenzenes, and azobenzenes (Poland and Glover, 1973b; Poland *et al.*, 1976a). Later the Poland laboratory would show that within categories of dioxin congeners, brominated biphenyls, and PAHs, the compounds that have the highest affinity for the AHR also are the most toxic (Poland and Glover, 1980).

Structure–activity AHR studies took a quantum jump when the Canadian chemist, Stephen Safe, entered this field. In a true tour de force, he and his wife Lorna eventually synthesized essentially all congeners in the families of chlorinated dibenzo-*p*-dioxins, dibenzofurans, and PCBs so that individual pure congeners could be tested for biological activity. Stelvio Bandiera, an energetic Ph.D. student from the Safe laboratory (then at the University of Guelph, Ontario) spent a brief time in my laboratory (then at the University of Windsor) to learn the AHR binding assay and to begin testing a series of PCB congeners for their ability to compete with [^{3}H]TCDD for specific AHR sites. From this study we found that all PCB congeners tested had some ability to compete with [^{3}H]TCDD but that competitive potency varied widely. The most potent competitors were the coplanar congeners, 3,3′,4,4′,5-pentachlorobiphenyl and 3,3′,4,4′-tetrachlorobiphenyl, whereas congeners that were chlorinated in *ortho* positions were only weak competitors (Bandiera *et al.*, 1982).

Competitive binding studies with PCB congeners and concurrent measurements of CYP1A induction allowed preliminary modeling of the molecular features that confer specific, high-affinity binding and revealed the importance of such parameters as number of substituent groups, hydrophobicity, and electronegativity (Safe *et al.*, 1985). Subsequent collaborative studies between the Safe laboratory and my group suggested that the structural ligand features which are important for binding to human AHR differ somewhat from those that influence binding to receptors from laboratory species (Golas *et al.*, 1990). The relationship between ligand structure and toxicity has been thoroughly explored in multiple reviews by Safe (1986, 1990, 1998a,b) and continues to be an active area of investigation in his laboratory (Khan *et al.*, 2006).

Some puzzles remain regarding the relationship between affinity of a compound for the AHR and its potency and efficacy to stimulate downstream responses. TCDD is a far more potent AHH inducer than 3-MC—some 30,000 times more potent *in vivo* (Poland and Glover, 1974). Higher potency usually suggests higher affinity for the relevant receptor. However, when we resumed binding experiments with radiolabeled PAHs we found, by direct binding assays, that the affinity of [^{3}H]3-MC for AHR in rodent liver cytosols was very similar to the affinity with which [^{3}H]TCDD is bound (Okey and Vella, 1982). The vastly greater potency of TCDD is not explained simply by higher affinity. David Riddick, then a postdoctoral fellow in my laboratory, conducted a thorough step-by-step comparison of TCDD and 3-MC in the classic Hepa-1 cell model and found that 3-MC was essentially equal to TCDD in its affinity for cytosolic receptor, its ability to transform the AHR to a DNA-binding state and its initial induction of AHH. However, 3-MC is rapidly metabolized *in vivo* or in cells that have active CYP1 enzymes whereas TCDD is highly resistant to metabolism. This pharmacokinetic behavior accounts for part of the difference in potency between TCDD and 3-MC (Riddick *et al.*, 1994); but, in truth, there still is considerable mystery as to why the gap between TCDD and 3-MC spans many orders of magnitude.

AHR antagonists. Antagonists can be powerful tools to identify receptor functions. For several years in the AHR field, the best available ''antagonists'' also possessed an undesirable degree of agonistic activity. Nonetheless, these antagonists/

partial agonists were able to inhibit, at least to some extent, biochemical and toxic responses to TCDD *in vivo*, providing further evidence for the importance of AHR binding as an early step in toxicity (reviewed in Okey *et al.*, 1994a). Recently, there has been a great improvement in development of antagonists to probe AHR function. The laboratories of Steve Safe and Tom Gasiewicz identified flavone derivatives that have a much improved ratio of antagonist activity to agonist activity (Henry *et al.*, 1999; Lee and Safe, 2000).

In a very intriguing recent development, Kim *et al.* (2006b) screened a large chemical library for compounds that act as AHR antagonists. They identified a synthetic agent (code name: CH-223191) that has remarkable ability to block TCDD binding to the AHR and to prevent downstream events including CYP1A1 induction, hepatic toxicity and wasting *in vivo*. CH-223191 is devoid of agonist activity for the AHR and ER and appears to be of low toxicity; thus the authors propose that this compound might be clinically useful for chemoprevention of toxicity from dioxin-like compounds.

Pharmacogenetics in Rodents In Vivo

Mouse. Via breeding experiments with mice, Alan Poland's laboratory (Poland and Glover, 1980) conclusively demonstrated that two major *in vivo* manifestations of TCDD toxicity—thymic atrophy and cleft palate—segregate with the *Ah* locus, i.e., the locus that encodes the AHR (Okey *et al.*, 1979).

At that early stage in AHR research the AHR remained undetectable in dioxin-resistant mice such as the DBA/2 strain; hence, it was not possible to compare receptor properties between dioxin-susceptible and dioxin-resistant mouse strains. Subsequently, my laboratory developed techniques to characterize stubborn receptors and found that the product of the mouse Ahr^{b1} allele (from the prototype C57BL/6 mouse) has affinity for [^{3}H]TCDD that is about 10-fold higher than the affinity of the receptor encoded by the Ahr^{d} allele (from the prototype DBA/2 mouse) (Okey *et al.*, 1989). This relative difference in affinity for [^{3}H]TCDD was independently confirmed by *in vitro* heterologous expression of cloned variant AHR structures (Ema *et al.*, 1994) and with a different radioligand, 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin (Poland *et al.*, 1994).

The 10-fold lower affinity for TCDD in AHRs of mice carrying the Ahr^{d} allele leads to a proportional reduction of about 10-fold in sensitivity for many responses to dioxins ranging from CYP1A1 induction to lethality, thymic atrophy, teratogenicity, and hepatic porphyria (reviewed in Nebert, 1989; Okey *et al.*, 1994a,b, 2005a; Poland and Knutson, 1982), further implicating the AHR as a major determinant of dioxin toxicity.

Rat. Beginning in the mid-1980s Raimo Pohjanvirta and Jouko Tuomisto at the National Public Health Institute, Kuopio, Finland, astutely pursued their original observation that a colony of Han/Wistar rats in Kuopio was extraordinarily resistant to lethal effects of TCDD (Pohjanvirta and Tuomisto, 1987; Pohjanvirta *et al.*, 1987). They found that resistance in rat is inherited as an autosomal dominant trait (Pohjanvirta, 1990); reviewed in Pohjanvirta and Tuomisto, 1994; Tuomisto, 2005).

With the encouragement of his senior colleague, Jouko Tuomisto, Raimo came to my laboratory at the University of Toronto in 1995 with the goal of cloning the AHR from resistant rats since it seemed probable that their genetic resistance to dioxin toxicity would reside in some altered AHR property. Cloning efforts began in Toronto with input from a Ph.D. student in my laboratory, Judy Wong, along with my Toronto colleague, Patricia Harper. Our hope for a mechanistic understanding of the basis for dioxin resistance was fulfilled after Raimo's return to Kuopio when he completed the molecular genetic analysis which revealed that the *AHR* gene in resistant H/W(*Kuopio*) rats contains a mutation at an intron–exon boundary that unveils cryptic splice sites, leading to deletion of either 38 or 43 amino acids from the transactivation domain (TAD) of the AHR protein (Pohjanvirta *et al.*, 1998). Breeding studies by Jouni Tuomisto (son of Jouko Tuomisto) show that resistance to TCDD lethality in rat segregates with the variant Han/Wistar form of the AHR (Tuomisto *et al.*, 1999), again strongly linking the AHR to dioxin toxicity. Note that in rat, *resistance* to TCDD toxicity is inherited as an autosomal dominant trait, whereas in mouse it is *susceptibility* that is inherited as a dominant trait (Okey *et al.*, 2005a). We will further explore the utility of the resistant rat model for understanding mechanisms of dioxin toxicity in a later section of this review.

Birds. Evidence that molecular properties of the AHR are a major determinant of susceptibility to TCDD was strengthened and extended phylogenetically by recent studies in birds. Chickens are much more sensitive to biochemical and toxic effects of dioxin-like compounds than are terns. Mark Hahn's laboratory found that the reduced sensitivity of terns can be attributed to the fact that tern AHR has a lower binding affinity for TCDD than does chicken AHR; consequently, tern AHR has lesser ability to transactivate gene expression (Karchner *et al.*, 2006).

AHR Knockout Clinches the Case

Cloning of mouse AHR and deciphering the genomic organization of the *Ahr* gene locus (Mimura *et al.*, 1994; Schmidt *et al.*, 1993) predictably led to efforts by several laboratories to knock out *in vivo* AHR function by targeted gene disruption. Laboratories led by Frank Gonzalez (NIH) (Fernandez-Salguero *et al.*, 1995), Chris Bradfield (University of Wisconsin) (Schmidt *et al.*, 1996), and Yoshiaki Fujii-Kuriyama (then at Tohoku University, Japan) (Mimura *et al.*, 1997) all succeeded in creating *Ahr*-null mice.

These knockouts provide unequivocal evidence that the AHR is crucial to TCDD-induced toxicity. Although the phenotype differs somewhat among *Ahr*-null mice from the three

laboratories (Lahvis and Bradfield, 1998), in each case knocking out the AHR conferred striking resistance to toxicities such as lethality, immunotoxicity, hepatotoxicity, carcinogenicity, and teratogenesis (Fernandez-Salguero *et al.*, 1995; Lin *et al.*, 2001; Mimura *et al.*, 1997; Peters *et al.*, 1999; Thurmond *et al.*, 1999). *Ahr*-null mice also are highly resistant to carcinogenicity from BP (Shimizu *et al.*, 2000).

Note, however, that there is a special exception to the ''rule'' that the absence of AHR protects from dioxins. Chris Bradfield's lab showed that in the case where the mouse *mother* is *Ahr*-null, the embryo is at greater risk of teratogenesis, probably due to decreased TCDD clearance leading to increased TCDD exposure *in utero* (Thomae *et al.*, 2004). (In this experiment the embryos were dioxin-susceptible because they were heterozygous ($Ahr^{+/-}$) at the *Ahr* locus.)

The most recent experiments with mice whose AHR pathways have been engineered shows that, not only is the AHR essential to dioxin toxicity, it is the AHR's transcriptional regulatory function that is linked to toxicity. The Bradfield laboratory introduced a mutation which disrupts the AHR domain responsible for nuclear translocation and binding to AHRE. TCDD is able to bind to cytosolic AHR in these mice; but, since they lack nuclear binding of the TCDD–AHR complex, they are highly resistant to hepatotoxicity, thymic atrophy, and cleft palate when exposed to TCDD (Bunger *et al.*, 2003). Moreover, as also shown by the Bradfield laboratory, if mice are engineered so that they have hypomorphic *ARNT* alleles and thus express low levels of ARNT protein, they too are highly resistant to TCDD toxicity in accord with the AHR's inability to alter gene expression when its dimerization partner is deficient (Walisser *et al.*, 2004a). ''Mouse engineering'' in the Bradfield laboratory further reveals that the cell types involved in AHR-related signaling during liver development (endothelial/hematopoietic cells) differ from the cell type (hepatocytes) involved in adaptive and toxic responses to dioxins (Walisser *et al.*, 2005).

Knockouts and Toxicity as Windows on ''Normal'' AHR Functions

AHR knockout studies are valuable, not only because they demonstrate that dioxin toxicity is AHR-dependent, but also because they shed some light on normal physiologic functions of the AHR. One immediate finding from the knockout experiments was that absence of a functional AHR is not lethal in mice (Fernandez-Salguero *et al.*, 1995; Mimura *et al.*, 1997; Schmidt *et al.*, 1996). On the other hand, global knockout of the AHR's dimerization partner, ARNT, leads to fetal death at midgestation, probably due to failed vascular development since the ARNT protein is an essential participant in hypoxic signaling by dimerizing with HIF-1α (rather than the AHR) (Kozak *et al.*, 1997; Maltepe *et al.*, 1997).

In the absence of exposure to dioxins or other exogenous ligands, *Ahr*-null mice, although viable, do show abnormalities in liver development, vascular development, immune function (reviewed in Lahvis and Bradfield, 1998), and fertility (Baba *et al.*, 2005). Curiously, the defects in vascular development that develop in mice which are hypomorphic for AHR or ARNT can be substantially prevented by exposing these mice to TCDD in utero; possibly the potent TCDD stimulus is an effective substitute for a weaker endogenous AHR ligand that lacks sufficient activating power when levels of AHR or ARNT are low (Walisser *et al.*, 2004a). It is a truism in biomedical sciences that ''pathology illuminates physiology.'' Perhaps by determining which systems suffer adverse effects from dioxins we might unveil still more about normal physiologic roles that the receptor plays. This prospect has been nicely elaborated by K. Walter Bock in a recent commentary (Bock and Kohle, 2006).

Dioxin Toxicity as the Consequence of Aberrant Gene Expression

Toxicity from many drugs and environmental chemicals is due to their enzymatic conversion into reactive metabolites which covalently attack vital cellular proteins or DNA (Liebler and Guengerich, 2005). TCDD, however, is highly resistant to metabolism in most organisms; it is the parent molecule that does the dirty work. Evidence summarized in the preceding section shows that the AHR is indispensable to major forms of dioxin toxicity and that the AHR must be transcriptionally competent in order for TCDD toxicity to occur.

Our working hypothesis, and that of several other laboratories, is that since the AHR's main biological function is transcriptional regulation, TCDD toxicity results from TCDD's ability to dysregulate genes that are under control of the AHR. The questions, then, are (1) what is the full spectrum of genes that the AHR regulates? (2) Which of these genes does TCDD dysregulate in a manner that leads to toxicity?

We have known for about two decades that the AHR regulates enzymes in the CYP1 family along with several other drug-metabolizing enzymes (reviewed in Nebert *et al.*, 2000). Founding members of the ''AH gene battery'' were identified by what now would seem old-fashioned—the ''one-gene-at-a-time'' approach. The first hint that genes other than the ''usual suspects'' could be induced by TCDD came from pioneering efforts of Thomas Sutter and William Greenlee who used differential hybridization as an unbiased open-ended method to detect novel dioxin-responsive genes and identified new AHR-responsive genes that play roles in growth and differentiation (Sutter *et al.*, 1991). Their experiments (Sutter *et al.*, 1994) also led to identification of a new P450, CYP1B1, which was independently discovered by Colin Jefcoate's laboratory via protein purification (Savas *et al.*, 1994).

The invention of gene expression arrays and their rapid technical improvement makes it possible to interrogate essentially the entire transcriptome in one experiment. Several laboratories, including ours, have applied array technology to

search for dioxin-responsive and AHR-regulated genes with the hope of uncovering key pathways to dioxin toxicity (Boverhof *et al.*, 2005, 2006a; Fletcher *et al.*, 2005; Guo *et al.*, 2004; Karyala *et al.*, 2004; Martinez *et al.*, 2002; Ovando *et al.*, 2006; Puga *et al.*, 2000).

Nathalie Tijet and Paul Boutros, research associates in my lab, used gene array methods in wildtype mice versus *Ahr*-null mice to identify the battery of genes that is regulated by TCDD in the absence of dioxins as well as the batteries of genes that are dioxin-responsive either in presence of AHR or in *Ahr*-null mice (Tijet *et al.*, 2006). Expression of over 450 ProbeSets is altered in liver by TCDD in an AHR-dependent manner. Another intriguing finding is that many genes are ''down-regulated'' (expression repressed) by TCDD, also in an AHR-dependent manner. Of course, throughout most of the history of AHR research the emphasis has been on ''upregulation'' (induction), not on downregulation. This array study also showed that very few genes have their expression altered by TCDD unless the AHR is present, further strengthening the concept that dioxin toxicity is the result of AHR-mediated dysregulation of gene expression.

The power of gene expression profiling on arrays is enticing. Yet, in a curious way, it led to some pessimism about whether array studies would be helpful in solving the mechanism of dioxin toxicity. The problem is that hundreds of genes respond to TCDD. As stated by Alvaro Puga: ''arriving at a sound understanding of the molecular mechanisms governing the biological outcome of TCDD exposure promises to be orders of magnitude more complicated than might have been previously imagined'' (Puga *et al.*, 2000). How can we tell which genes are really central to dioxin toxicity?

Which Genes Matter in Dioxin Toxicity? The Dioxin-resistant Rat as a Model to Identify Genes that are Most Relevant to Dioxin Toxicity

The dioxin-resistant H/W (*Kuopio*) rat strain discovered by Raimo Pohjanvirta and Jouko Tuomisto (reviewed in Pohjanvirta and Tuomisto, 1994) provides a potentially powerful model for discriminating between genes whose dysregulation is crucial to TCDD lethality versus those genes that are TCDD-responsive but not involved in lethality. The H/W rat strain is more than 1000-fold resistant to TCDD-induced lethality compared with standard laboratory strains due to the deletion in the AHR TAD described above. The TAD deletion does not interfere with AHR-mediated induction of genes in the conventional AH gene battery such as *CYP1A1*, *CYP1A2*, *CYP1B1*, or *UGT1A1* (Okey *et al.*, 2005b). Many chemical agents that induce CYP1A1 do not cause dioxin-like toxicity (Hu *et al.*, 2007). We hypothesize that the TAD deletion selectively alters the AHR's ability to dysregulate particular genes that are the key players in dioxin toxicity. In collaboration with Raimo Pohjanvirta and Jouko Tuomisto, two skilled and highly motivated Ph.D. students in my laboratory, Monique Franc and Ivy Moffat, employ gene expression arrays to determine which genes are affected differently by TCDD in dioxin-sensitive rats (which have wildtype AHR) versus the highly resistant H/W strain (carrying the TAD deletion). These array studies reveal that most TCDD-responsive genes are affected both in sensitive rat strains and in resistant rat strains. However, some genes are affected only in sensitive rats or only in resistant rats. We are performing postarray follow-up studies to attempt to determine if any of the strain-specific responsive genes can be linked to major dioxin toxicities (Franc *et al.*; Moffat *et al.*; Pohjanvirta *et al.*, in preparation). It is possible that lethality and other major forms of dioxin toxicity are the result of concomitant dysregulation of multiple genes rather than due to changes in a single vital gene; thus we also are exploring whether dysregulated genes are arranged into specific functional pathways.

Human AHR Variability and its Effect on Responses

As described above, AHR polymorphisms in rodents can have a profound impact on the animals' response to dioxin-like chemicals (reviewed in Okey *et al.*, 2005b). Hence, it is worthwhile and important to determine whether genetic variation also exists in the *AHR* gene in human populations and, if so, whether human *AHR* polymorphisms have significant functional consequences. Human variation is an important issue from the perspective of human risk assessment because regulations need to be constructed to protect the most vulnerable members of the population. In collaboration with David Manchester, we found that there is a greater than 10-fold range of variation in the human population in the affinity with which TCDD binds to AHR in placental cytosol but we have not been able to identify any genetic polymorphisms that account for this variation in binding affinity (reviewed in Harper *et al.*, 2002; Okey *et al.*, 2005a).

Our interrogation of the dbSNP (single nucleotide polymorphism) database revealed that the density of SNPs across the full locus is lower for the human *AHR* gene than for genes in the formal nuclear receptor superfamily (Okey *et al.*, 2005a). Conservation of gene structure often suggests that the gene plays an essential biological role and that evolution does not tolerate structural variations that interfere with function. As described earlier, knockout of the *Ahr* gene is not lethal in mice. The apparently high degree of sequence conservation in the human *AHR* raises the question of whether human AHR plays some vital role for which there are no backup pathways in our species (Okey, 2005).

Judy Wong and Maria Lam, graduate students working jointly with me and with my Toronto colleague Patricia Harper, diligently searched for novel human *AHR* polymorphisms and tested function by transfecting AHR variants into host cells in culture. The first human *AHR* polymorphism, at codon 554, was discovered by Kawajiri *et al.* (1995) and has been the most extensively studied. In our assays, the codon 554

polymorphism, on its own, had no significant effect on CYP1A1 induction by TCDD. However, the TAD wherein the codon 554 polymorphism resides also contains polymorphisms at codon 570 and codon 517 (reviewed in Harper *et al.*, 2002). Our experiments indicate that the 554Lysine/570Isoleucine combination or the 554Lysine/570Isoleucine/517Serine haplotype are poor at sustaining induction of the native *CYP1A1* gene by TCDD in cell culture (Wong *et al.*, 2001b). The 554Lysine/570Isoleucine/517Serine haplotype seems to be confined to persons of recent African origin and occurs with low frequency in the population (Wong *et al.*, 2001b); thus it would be difficult to conduct epidemiologic studies to determine how this variant affects health outcomes.

If human susceptibility to dioxin toxicity or to biochemical effects of dioxins is found to be associated with *AHR* polymorphisms, this would facilitate risk assessment by helping to establish whether there are individuals in the human population who might be unusually susceptible to dioxin toxicity due to genetic variation in their AHR. A few recent epidemiologic studies (Table 1) suggest that the *AHR* genotype affects relative risk for health outcomes such as breast cancer, lung cancer, hypertension, and birth weight in offspring of smoking mothers but these studies require replication before firm conclusions can be drawn. To date, the overall impact of genetic variation in human AHR on biochemical responses or health outcomes appears modest.

AHR Activity: Balancing the Good and the Not-so-Good

Given that in the absence of the AHR there essentially is no toxicity from dioxin-like chemicals, it might be tempting to conclude that humans (and other vertebrates) would be better off without an AHR. As an added bonus, not having an AHR prevents cancer induction by BP, presumably because CYP1A1 can no longer be induced and thus is not available to bioactivate BP into its ultimate carcinogenic form (Shimizu *et al.*, 2000). From an evolutionary perspective, there must be advantages to possessing an AHR. The adaptive and developmental roles elaborated by the Bradfield laboratory (Walisser *et al.*, 2004b), on balance, appear to outweigh the deleterious effects of the AHR's role in mediating toxicity.

Early in this review I described how my interest in enzyme induction arose from our discovery that the pesticide, DDT, could protect rats from cancers when they were exposed to the PAH carcinogen, DMBA (Silinskas and Okey, 1975), presumably because DDT stimulates enzymes that aid the rat to detoxify and eliminate the carcinogen. The fact that induction of P450s might protect animals from xenobiotic toxicants was contrary to the prevailing view in the 1960s, 1970s and 1980s that increased P450 activity is predominantly harmful to animals exposed to precarcinogens because P450s can bioactivate pretoxicants and precarcinogens into chemically reactive intermediates that covalently bind cellular macromolecules, thereby initiating mutagenesis, carcinogenesis, teratogenesis, and a host of related pathologies. There is no question that the ''reactive metabolite'' model explains how many chemicals become harmful.

But it is equally true that the strategy of using enzyme inducers as chemopreventive agents to reduce cancer risk has been recognized for several decades (reviewed in Conney, 2003a; Okey, 1990, 2005). Recently, a renewed appreciation has developed for the benefits conferred by AHR-mediated enzyme induction (reviewed in Nebert and Dalton, 2006; Nebert *et al.*, 2004). Eventually, it might be possible to use pharmacologic or molecular tools to manipulate AHR pathways to protect from dioxin-like chemicals. However, agents that are protective in one biological context may enhance toxicity or carcinogenesis in another setting. Our current understanding of AHR pathways is too primitive to allow us to safely tip the balance strongly in the direction of protection.

Implications of a Receptor-Mediated Mechanism for Risk Assessment

Just how great is the risk to human health from dioxin-like chemicals? There is no clear consensus on the answer to this vital question, particularly for the cancer endpoint. Dioxin risk assessment is fraught with controversy (Cole *et al.*, 2003; Connor and Aylward, 2006; Gough, 2003; Guzelian *et al.*, 2006; Starr, 2003; Tuomisto, 2005) and I don't presume that my experience in AHR research can cast much additional light into this dark corner. Nevertheless, a few reflections might be in order because of the central role the AHR plays in dioxin toxicity.

We established that the AHR mechanism is available in humans to potentially mediate the same functions that it does in laboratory animals, albeit with notable differences in species sensitivity. Dioxin-like chemicals are the first major toxicant class to undergo extensive risk assessment founded on the premise that their toxicity is initiated by binding to a soluble receptor—in this case, the AHR. The United States Environmental Protection Agency's Draft Dioxin Reassessment (highlights and summary at US-EPA, 2004) has been a lightning rod for criticism on specific points but also a useful catalyst to stimulate thoughtful and vigorous debate on risk assessment for the important dioxin class of toxicants. Lessons learned in the AHR/dioxin system also may facilitate risk assessment for environmental toxicants that act on other nuclear receptors such as the ER or androgen receptor.

Classic receptor theory, in conjunction with empirical observations in multiple receptor systems, makes certain predictions about how receptor-mediated responses ought to behave. Implications of receptor theory for dioxin risk assessment have been clearly enunciated in previous commentaries by Poland (1996, 1997). Among the issues raised by a receptor-mediated mechanism are the following.

Dose–response relationships and thresholds. The cloudiest and most contentious issue in dioxin risk assessment concerns the shape of the dose–response curve for adverse effects of

TABLE 1
Reported Effects of Human *AHR* Polymorphisms on Various Outcomes

Polymorphic site(s)	Assay or endpoint	Outcome	Ethnic group	Reference
		AHR coding region variants		
R554K	AHH induction in PBL	No effect	Japanese	(Kawajiri *et al.*, 1995)
R554K	EROD induction in PBL	Increased with 554K	Caucasian	(Smart and Daly, 2000)
R554K	CYP1A1 level in lung	No association	Finnish	(Anttila *et al.*, 2000)
R554K	CYP1A1 level in lung	No association	Caucasian	(Anttila *et al.*, 2001)
R554K	EROD activity in lung	No association	Caucasian	(Smith *et al.*, 2001)
R554K	CYP1A1 induction in PBL	No effect	mouse cell	(Wong *et al.*, 2001a)
R554K	EROD induction in PBL	No effect	French	(Cauchi *et al.*, 2001)
R554K	Lung cancer risk	No association	French	(Cauchi *et al.*, 2001)
R554K	Chloracne	No association	Caucasian	(Wanner *et al.*, 1999)
R554K	Bladder cancer risk	No association	Chinese	(Zhang *et al.*, 2002)
R554K	Endometriosis risk	No association	Japanese	(Watanabe *et al.*, 2001)
R554K	Micropenis risk	No association	Japanese	(Fujita *et al.*, 2002)
R554K	Reporter gene induction	No effect	human cell	(Koyano *et al.*, 2005)
R554K	Soft tissue sarcoma survival	Shortened with 554K	Caucasian	(Berwick *et al.*, 2004)
R554K	Breast cancer risk	Decreased with 554A	Chinese	(Long *et al.*, 2006)
R554K	Sperm Y:X chromosome ratio	No association	Swedish	(Tiido *et al.*, in press)
R554K	Blood pressure in smokers	No effect	French	(Gambier *et al.*, 2006)
R554K	Chloracne	No effect	Taiwanese	(Tsai *et al.*, 2006)
R554K (maternal)	Recurrent pregnancy loss	No association	Japanese	(Saijo *et al.*, 2004)
R554K (maternal)	Offspring size from smoking mothers	Decreased with 554R/R*	Japanese	(Sasaki *et al.*, 2006)
R554K	DNA damage in PBL from coke oven workers	Increased with 554K	Chinese	(Chen *et al.*, 2006)
R554K; V570I	EROD induction in PBL	Increased with 554K	African	(Smart and Daly, 2000)
R554K; V570I	CYP1A1 induction	Decreased	Mouse cell	(Wong *et al.*, 2001b)
P517S;R554K; V570I	CYP1A1 induction	Decreased	Mouse cell	(Wong *et al.*, 2001b)
K401R	Reporter gene induction	Reduced induction with 401R	Human cell	(Koyano *et al.*, 2005)
N487D	Reporter gene induction	Reduced induction with 487D	Human cell	(Koyano *et al.*, 2005)
I514T	Reporter gene induction	No effect	Human cell	(Koyano *et al.*, 2005)
K17T/R554K	Reporter gene induction	No effect	Human cell	(Koyano *et al.*, 2005)
K17T	Reporter gene induction	No effect	Human cell	(Koyano *et al.*, 2005)
		AHR promoter region variants†		
− 552 T > C	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
− 385 A > G	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
− 230 G > A	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
− 196/− 195 ΔGG	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
− 195 ΔG	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
− 137 C>T	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
+ 119 C > G	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
+ 157 G > A	Reporter gene induction	No effect	Human cell	(Racky *et al.*, 2004)
+ 157 G > A	EROD induction in PBL	No effect	French	(Cauchi *et al.*, 2001)
		Combination of polymorphisms		
R554K and *CYP1A1* T3801C	Blood pressure	Increased with 554K + *CYP3801C* in ex-smokers and nonsmokers	French	(Gambier *et al.*, 2006)
Ex1 + 185A > G and‡ IVS7 + 33T > G and Ex10 + 501G > A (= R554K)	Lung cancer risk in smokers	Increased with GGG haplotype	Korean	(Kim *et al.*, 2006a)

Note. Arg/Arg (abbreviated R) is considered to be the wildtype for human AHR and lysine the variant (abbreviated K). EROD = ethoxyresorufin *O*-deethylase; PBL = peripheral blood lymphocytes. Mouse cell = activity tested with human AHR constructs transfected into an AHR-deficient mouse cell line. Human cell = activity tested with human AHR constructs transfected into a human cell line.

*The effect of polymorphism at codon 554 of the *AHR* gene on birth weight and birth length was greatest effect when the 554 polymorphism was present in combination with a variant allele in the *CYP1A1* gene.

†There is considerable confusion regarding the location of these promoter variants. For example, variant rs7796976 has been identified variously as being located at position + 185 (Kim *et al.*, 2006a); Entrez dbSNP build 126, or at position + 157 (Cauchi, 2001; Racky, 2004), whereas the current refseq (NM_001621.3, 14 January 2007) places this SNP at + 124.

‡Ex1 + 185A > G is rs7796976; IVS7 + 33T > G is rs2074113; and Ex10 + 510G > A is rs2066853 (R554K).

dioxins at environmentally relevant exposure levels. This is coupled with the question of whether a threshold exists, below which there is no significant increase in risk for cancer or other adverse health effects. Analytical improvements provide the means to measure human exposure at ever-lower levels (Reiner *et al.*, 2006) but also place more demands on the risk assessment process to know what these ultralow levels mean for human health.

Dose–response relationships lie at the heart of pharmacology and are underpinned by receptor theory. Receptor theory and numerous empirical precedents predict that responses should be sublinear at low doses and that a practical threshold should exist (Poland, 1997). The EPA Dioxin Reassessment draft document (US-EPA, 2004) concludes that AHR-mediated biochemical responses ''are likely to demonstrate low-dose linearity''—in other words, no threshold. However, the National Academies of Science review of the draft document holds that ''EPA's decision to rely solely on a default linear model lacked adequate scientific support'' (NatAcadSci, 2006). The heart of the problem in defining the shape of the dose–response curve at environmentally relevant levels is that valid, experimentally derived data are virtually impossible to obtain at these ultralow exposure levels; hence, the risk assessment continues to default to a linear multistage model. Some investigators such as Michael Schwarz and Klaus Appel believe that there is sufficient evidence to move away from the linear model for dioxins; they propose ''for regulatory purposes the application of a so called 'practical threshold' for the carcinogenic effect'' (Schwarz and Appel, 2005). As stated by others (Poland, 1997; Schwarz and Appel, 2005), the current linear multistage approach to risk is a policy decision rather than a scientific decision based on actual data from responses at very low dioxin levels. Any clever new approach that can experimentally resolve the nature of the dose–response curve at ultralow *in vivo* levels clearly would be welcomed but the prospects for such a breakthrough seem dim.

The toxicity of mixtures: toxic equivalency factors and toxic equivalencies. Dioxin-like chemicals exist in the environment as complex mixtures including polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and PCBs along with a multitude of nonhalogenated AHR ligands. For real-world risk assessment it's necessary to take into account the contributions made by the combination of AHR ligands, not just TCDD alone. Thus, was born the concept of using ''toxic equivalency factors'' (TEFs) in which each congener is assigned a TEF that reflects the best estimate of its toxicity relative to that of 2,3,7,8-TCDD. Overall toxicity of a mixture is termed the TEQ (toxic equivalency) which is assumed to be equal to the sum of the concentrations of individual congeners multiplied by their TEFs (Safe, 1997; Van den Berg *et al.*, 2006).

The central premise in the TEF/TEQ approach is that the cumulative toxic effects of components in a mixture are additive. There is experimental evidence to support the assumption of additivity (Viluksela *et al.*, 1998; Walker *et al.*, 2005). However, receptor theory predicts that compounds which compete for the same receptor site will antagonize each other if the mixture contains partial agonists; some studies have, indeed, found antagonism (Haag-Gronlund *et al.*, 1998; Hestermann *et al.*, 2000; Safe, 1998a; Van Birgelen *et al.*, 1994). In other words, the TEQ approach can, at least sometimes, overestimate the toxic potency of a mixture.

Assignment of the appropriate TEF for each congener is challenging because different biochemical and toxic responses may have very different dose–response curves, even within the same animal (Starr *et al.*, 1999). Therefore, the relative potency estimates for dioxin-like compounds are undergoing continual refinement (Budinsky *et al.*, 2006; Haws *et al.*, 2006). Although some aspects of the TEQ concept are at variance with expectations from receptor theory, in practice the TEQ approach is pragmatic and appears to make reasonably accurate predictions about the overall toxicity of mixtures as reflected in the most recent report from the World Health Organization Expert Panel on TEFs (Van den Berg *et al.*, 2006).

Reversibility. In principle, responses mediated by interaction of a ligand with its receptor are reversible when the ligand is withdrawn because response depends upon continued presence of the ligand–receptor complex. For most therapeutic agents the duration of response is a few hours or few days. Drug effects wane and terminate as metabolism fosters elimination of the drug. To the pharmacokineticist (accustomed to thinking of clearance in units of hours or days), TCDD is a bizarre compound because its half-life in humans is measured in years. Therefore, in humans this AHR ligand potentially is available to stimulate responses almost in perpetuity. Perhaps, then, TCDD-induced responses should be considered essentially irreversible (Schwarz and Appel, 2005) even though they are receptor-mediated. TCDD remains on board in humans for a very long time but, in truth, we know relatively little about its ability to access the AHR and continue to drive biochemical responses or overtly toxic responses. We do know that the skin disorder, chloracne, that occurs in some persons exposed to high dioxin levels can persist for years (Geusau *et al.*, 2001; Panteleyev and Bickers, 2006).

Tissue specificity. For therapeutic agents that act via receptors there is an expectation that the effect will be limited to a particular ''target'' tissue or cell type, i.e., those cells that have the appropriate receptor. As described above, the AHR has a remarkably broad pattern of expression in vertebrate species, tissues, and cell types. In principle, therefore, almost all vertebrate tissues could be targets for toxic effects of dioxin-like chemicals. However, particular cell types and tissues are preferentially damaged by dioxins and these are not necessarily the same across various mammalian species. It is not simply a matter of the level of AHR expressed in each tissue. Just as we do not yet fully understand the basis for species differences in susceptibility to dioxin-like chemicals, we also have a profound

lack of insight into what makes certain tissues highly susceptible to dioxin toxicity and what makes some ''privileged'' tissues highly resistant.

Enduring Mysteries

Dioxin toxicity continues to be an intriguing and high-profile jumble of science, policy, politics, economics, and societal values (Stone, 2007). After 30 years of AHR research, where has the voyage taken us in regard to understanding dioxin toxicity? I titled this essay ''. . . to the *Shores* of Toxicology'' because, although the sturdy and trusty AHR ship has brought us tantalizingly close to our objective of understanding mechanisms of gene regulation and dioxin toxicity, in truth, we have only just landed on the shore. Our quest for the mediator of dioxin's biochemical and toxic effects has let us glimpse many important and fundamental AHR attributes through gaps in the fog. But, as is perpetual in science, beyond the shore lies *terra incognita*.

We all love a good mystery when it's presented to us as a clever work of fiction in books or on screen. However, the scientist in us wants the answer—preferably as soon as possible (and before anyone else solves the puzzle). Research on the AHR and dioxin toxicity retains many unknowns. I believe that we have partial answers (as addressed throughout this review) to the questions below; but these issues beg for a more comprehensive understanding to place our science on a firmer footing.

What accounts for the striking differences in susceptibility to dioxin toxicity among mammalian species? Is susceptibility determined mainly by properties of the AHR?. One of the durable mysteries regarding dioxin toxicity is why there are such profound differences in susceptibility among mammalian species (Bock and Kohle, 2006). I know of no instance in small-molecule toxicology, other than TCDD, where species differences in sensitivity span several orders of magnitude. I also know of no other small-molecule toxicant that exerts such a broad range of severe toxicities in a wide variety of laboratory animals and yet has so few conclusively demonstrated serious adverse effects on human health. The sensitivity of standard mammalian laboratory species to dioxin toxicity is truly alarming. Extrapolation of animal toxicity data to humans is a traditional mainstay in risk assessment. However, the toxic endpoints (e.g., wasting, death, hepatic toxicity, teratogenesis) that are hallmarks of dioxin toxicity in laboratory species are not prominent in humans exposed to dioxins through occupation, accident or diet.

The US-EPA draft Dioxin Reassessment Scientific Highlights state that ''humans may fall in the middle of the range of sensitivity for individual effects among animals'' and that ''humans, in general, are neither extremely sensitive nor insensitive to the individual effects of dioxin-like compounds as compared to other animals.'' In my opinion, the available evidence from epidemiology and from assessment of biochemical responses to TCDD locates humans towards the dioxin-resistant end of the spectrum among mammalian species. We have measured TCDD binding to AHR along with CYP1A1 induction in several human cell lines in comparison with the benchmark mouse cell line, Hepa-1 (which carries the Ahr^{b1} allele). As illustrated in Figure 6, both the receptor occupancy curve and the dose–response curve for subsequent induction of CYP1A1 are shifted about one log unit to the right for human cells versus mouse Hepa-1 cells. Multiple human cell lines that we tested all are 5- to 10-fold less sensitive than Hepa-1 mouse cells for receptor affinity and/or for CYP1A1 induction (Harper *et al.*, 1988, 1991b; Roberts *et al.*, 1990; Waithe *et al.*, 1991). Ramadoss and Perdew (2004) used the photoaffinity ligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin and also found, by studies with expressed receptor proteins in intact cells, that at a given radioligand concentration, human AHR bound about 10-fold less ligand than did the product of the mouse Ahr^{b1} allele.

Studies on human cell lines provide AHR characteristics for only a few donors. Hence we took advantage of the high AHR abundance in human tonsils and human placenta to measure [^{3}H]TCDD binding affinity (*K*d) in human populations. Within these populations there was greater than a 10-fold range in binding affinity (Harper *et al.*, 2002; Lorenzen and Okey, 1991; Okey *et al.*, 1997). Many donors exhibited low affinity with *K*d values near that of AHR from nonresponsive mice. Connor and Aylward (2006) have used the term ''impaired'' to describe the apparently lowered functionality of human AHR and this appears apt for many samples. However, some individuals in the human population express an AHR that has affinity for TCDD near that of susceptible rodents such as C57BL/6 mouse or Sprague–Dawley rats (Harper *et al.*, 2002; Lorenzen and Okey, 1991).

Do structural features of the AHR account for its varied functions in different animal species? In certain laboratory animal models, described earlier (such as C57BL/6 vs. DBA/2 mice or the Han/Wistar(*Kuopio*) rat), sensitivity or resistance to TCDD clearly is related to properties of the AHR (Okey *et al.*, 2005b). However, there also are differences in susceptibility between laboratory species that remain unexplained. For example, AHRs have been cloned from guinea pig (Korkalainen *et al.*, 2001) and hamster (Korkalainen *et al.*, 2000); this cloning provides hints to how differences in AHR structure might affect AHR function and dioxin sensitivity but we still do not truly understand why guinea pig is highly susceptible to TCDD lethality, whereas hamster is exceptionally resistant.

The primary structure, determined by cDNA cloning or genomic sequencing, is now available for many mammalian species. However, the AHR protein has stubbornly resisted attempts to crystallize it and to obtain high-resolution 3-D structures. Homology modeling of the AHR ligand-binding domain gives some insight into the means by which the AHR recognizes ligands (Pandini *et al.*, 2007) and provides support for previous findings (Ema *et al.*, 1994; Poland *et al.*, 1994) that

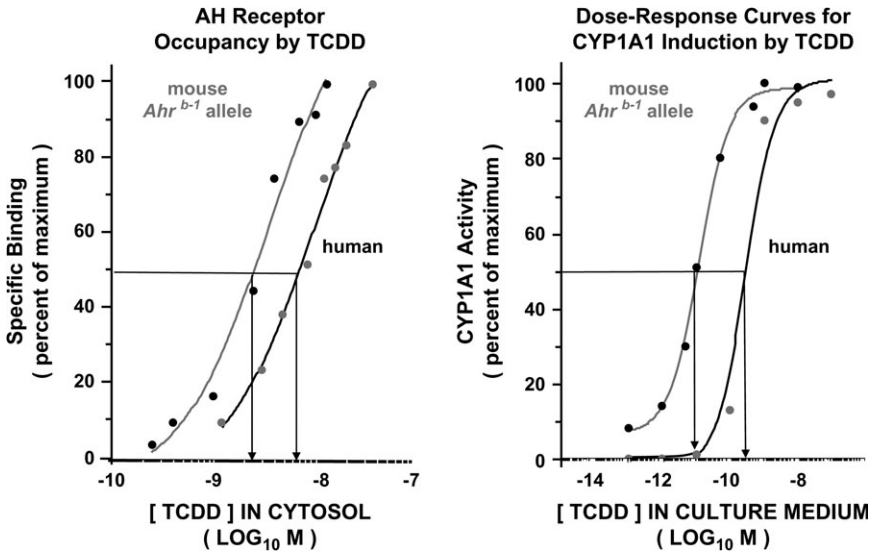

FIG. 6. Comparison of a human cell Line with a mouse cell line for TCDD receptor binding and CYP1A1 induction. The left-hand panel represents the extent of [^{3}H]TCDD-specific binding to cytosol from the mouse Hepa-1 cell line (Ahr^{b1} allele) and the human LS180 cell line over a wide range of radioligand concentrations. The right-hand panel represents dose–response curves for induction of CYP1A1 (measured as aryl hydrocarbon hydroxylase activity) in these same cell lines. The shift-to-the-right of about one log unit in the occupancy curve for TCDD binding to AHR (left panel) is similar in magnitude to the shift in the downstream response, CYP1A1 induction (right panel). Although the curves for CYP1A1 induction differ between mouse Hepa-1 cells and human LS180 cells in proportion to receptor occupancy, this does not necessarily indicate that all AHR-mediated gene responses will show the same difference between species. (Data replotted from Harper *et al.*, 1991b.)

substitution of valine for alanine at position 375 substantially reduces the affinity of TCDD binding. However, much remains to be done in solving the crystal structure to a resolution that will permit real understanding of AHR interactions with ligands, other proteins, and DNA and how these might differ among species.

Overall it appears that some portion of the relative insensitivity of the human species likely is due to the lower affinity with which human AHR binds TCDD when compared with affinities in most rat and mouse strains (Harper *et al.*, 2002) but it is unlikely that the difference in affinity alone accounts for all the differences in toxic outcomes in humans versus rodent models. There also are likely to be species differences and individual differences in pathways downstream of initial ligand binding which strongly influence dioxin susceptibility but these have not been extensively explored.

Production of ''AHR-humanized mice'' provides one strategy to determine whether properties of the AHR itself are the main determinant of sensitivity to biochemical and toxic effects of dioxins. Moriguchi *et al.* (2003) (Tsukuba, Japan) succeeded in inserting human AHR into mice in place of the normal mouse *Ahr* alleles. Mice that express human AHR *in vivo* are less susceptible to TCDD-induced cleft palate than mice that are homozygous for either the mouse Ahr^{b1} ''high affinity'' allele or the mouse Ahr^{d} ''low-affinity'' allele. These AHR-humanized mice open the opportunity to further dissect similarities and differences between mouse and human AHRs in their ability to drive biochemical, cellular, and toxic responses to dioxins and other AHR ligands.

Why don't all high-affinity AHR ligands produce dioxin-like toxicity?. Simple receptor theory predicts that all chemicals that are agonists for the same receptor should produce responses that qualitatively are the same. The difference among agonists should lie in the quantitative parameters of potency and efficacy, not the qualitative nature of the response elicited. As described above, there is a plethora of known AHR agonists from a very broad variety of chemical classes. Fortunately, few of these agents provoke dioxin-like toxicity. For example, several compounds of plant/dietary origin are potent AHR agonists in various *in vitro* assays but they do not mimic TCDD in regard to toxicity (Denison *et al.*, 2002). The usual explanation for this discrepancy between AHR activation and toxicity is a pharmacokinetic one: many natural products that bind the AHR are rapidly metabolized and have short half-lives *in vivo*. Yet pharmacokinetics alone cannot fully explain what properties make an AHR agonist highly toxic. Some insight into this puzzle may come about from determining how each ligand induces a specific conformation in the AHR protein and how this conformation permits the liganded AHR to selectively recruit coactivators or corepressors that tune the spectrum of ultimate responses with great subtlety. For example, Henry and Gasiewicz (2003) have shown that agonists produce a different conformation of the AHR protein than do antagonists. Stephen Safe has pioneered the concept of ''SAhRMS''; that is, selective AHR modulators whose structures elicit AHR-mediated responses that are therapeutically beneficial rather than toxic (Liu *et al.*, 2006; Safe and McDougal, 2002). Structure–activity relationships for the AHR, its ligands and responses have been

studied for more than 25 years but there still is much to learn in this area.

Which specific genes are dysregulated by dioxins in a fashion that leads to major toxicities?. As described earlier, there is strong evidence that the major forms of dioxin toxicity are due to dysregulation of gene expression mediated by the AHR but we do not know which specific genes are the key to different toxic manifestations. This is a major focus of current research in my laboratory. It is unlikely that a single molecular or biochemical response triggers the myriad diverse toxic outcomes from dioxin exposure. Varied toxic endpoints probably share some initial triggering events but each endpoint (wasting, lethality, hepatotoxicity, immunotoxicity, reproductive toxicity, developmental toxicity, etc.) likely is due to distinct (but overlapping) sets of dysregulated genes. Fortunately, there are many laboratories using expression arrays to map the sets of genes that respond to dioxin-like chemicals at different times, at different doses and in various species, tissues, and cells. Interpretation of these large data sets is not trivial but we can anticipate that central principles will begin to emerge from this concerted effort.

What is the full extent of the AHR's normal biological function – during development and in adult life and what is the full range of endogenous ligands?. Answers to these questions are inextricably linked to understanding dioxin toxicity. Toxic responses help to illuminate physiologic functions but, in turn, a more mature understanding of the AHR's physiologic roles will greatly aid toxicology. Is there a single ''master'' endogenous ligand? Or do physiologic functions of the AHR involve different ligands in different tissues at different times (Bock and Kohle, 2006)?

What factors steer AHR-mediated responses to one or more of the three general paths enunciated by the Bradfield laboratory (adaptive, developmental, toxic)?. How does signaling that begins with ligand binding in cytoplasm end up producing divergent responses that sometimes foster normal development, sometimes help adapt to external challenges, or sometimes are overtly toxic (Walisser *et al.*, 2004a)? The choice of pathway is strongly influenced by the nature of the chemical ligand but we need to know much more about how varied characteristics of the biological substrate shape the ultimate response. For example, what is the repertoire of coactivators and corepressors within a particular cell type? Are the components in the signaling pathway stable over time or are they altered by other endogenous and exogenous stimuli?

How important are protein–protein interactions in the AHR's normal biological functions and in toxicology?. In the ''standard model'' of dioxin toxicity (described above), dioxin-like chemicals must bind the AHR which then dimerizes with ARNT and alters gene transcription. Several investigators have shown that the AHR undergoes direct protein–protein interactions, not only with ARNT, but also with other key regulatory proteins. Most notably, when activated by TCDD, the AHR forms a complex with the retinoblastoma protein (RB) which inhibits transcription of E2F-dependent genes leading to subsequent arrest of the cell cycle (reviewed in Huang and Elferink, 2005; Marlowe and Puga, 2005; Puga *et al.*, 2002). In this instance, interaction of the AHR with the RB protein represses gene expression rather than the conventional enhancement of gene expression observed following dimerization with ARNT. In addition to direct interactions with the RB protein, the AHR also has been shown to interact with NF-κB, a powerful regulator of diverse cell functions (Tian *et al.*, 1999, 2002).

Literature on the AHR's protein–protein interactions and role in the cell cycle is derived almost entirely from experiments done in cell culture. Responses of cells in their normal *in vivo* context can differ substantially from what is seen with individual cell types alone in culture. It is important to determine whether the responses in cell culture accurately reflect how cells respond *in vivo* to signals via AHR protein–protein pathways. This is especially relevant to the potent tumor promoting activity of TCDD which is tied to altered cell proliferation and apoptosis (Bock and Kohle, 2005). As a step toward understanding *in vivo* effects of TCDD and AHR on cell cycle and cell proliferation the Elferink laboratory recently employed the regenerating rat liver model and found that when AHR activation is sustained by TCDD exposure, hepatic growth is attenuated (Mitchell *et al.*, 2006). However, it is not yet clear whether alteration of cell proliferation *in vivo* is dependent primarily on the ''standard'' transcriptional activity of the AHR or whether it is driven by protein–protein interactions such as with RB protein. If *in vivo* experiments can be designed that can distinguish between these two regulatory arms, they would be of great value for understanding normal biological functions of the AHR along paths of dioxin toxicity.

Full circle: how did AHR and ER pathways come to be so intertwined?. As I described in the opening to this story, my first independent research was devoted to interactions of ''MC-type'' PAHs (AHR agonists) with the ER. When our research on AHR took off, my laboratory discontinued work on ER. Little did we realize that interactions between AHR pathways and ER pathways would become an area of keen interest to toxicologists. In the late 1980s, Michael Gallo's laboratory and Stephen Safe's laboratory tested the effects of TCDD on uterine tissue and found that TCDD was predominantly antiestrogenic (Umbreit and Gallo, 1988; Umbreit *et al.*, 1988); reviewed in (Safe and Wormke, 2003).

However, emphasis over the past few years has shifted to the ability of AHR ligands to act as estrogens rather than antiestrogens, stimulated in large part by a paper from Ohtake *et al.* (2003) who postulated that ligand-activated AHR functions as a coactivator of ERα to stimulate estrogen targets. Subsequent studies in other laboratories indicate that TCDD

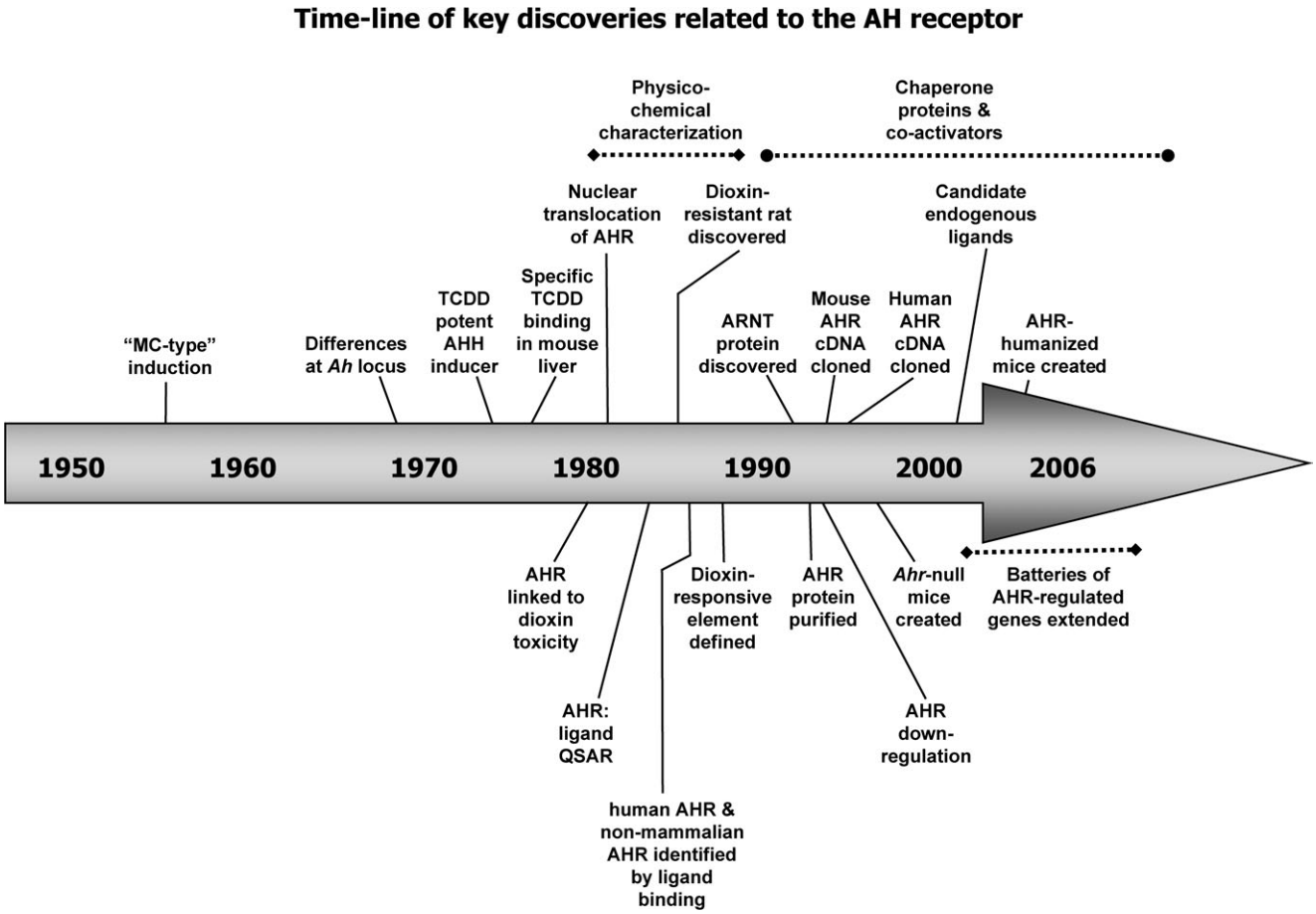

FIG. 7. Time-line of key discoveries related to the AHR. See the text for details.

and several additional AHR agonists can directly bind and activate ER rather than requiring the AHR to function as a coactivator (Abdelrahim *et al.*, 2006; Boverhof *et al.*, 2006b; Shipley and Waxman, 2006).

So perhaps the passing structural similarities between AHR agonists and estrogens (Fig. 1) do, in fact, permit AHR ligands to cross-activate the ER. However, activation of ER by AHR ligands such as 3-MC, BP, or PCBs requires concentrations in the micromolar range (Liu *et al.*, 2006). Environmental and dietary exposures to dioxin-like compounds do not typically lead to plasma concentrations in the micromolar range. Thus, although AHR ligands can, in principle, activate ER, it is not established that these interactions are important for endocrine function, reproduction, and toxicology.

Finally, just to show that cross-talk in signaling pathways rarely is simple and straightforward, the ARNT protein has been shown to be a coactivator of ER-dependent transcription (Brunnberg *et al.*, 2003), whereas TCDD inhibits some estrogenic responses such as upregulation of ERβ by ERα (Kietz *et al.*, 2004). Moreover, for some genes such as *CYP1A1*, AHR appears to be able to recruit ERα into the promoter region, perhaps explaining the estrogen-dependent nature of CYP1A1

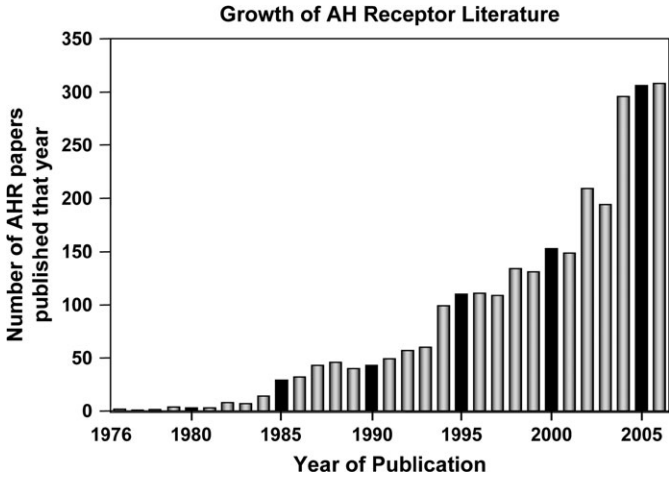

FIG. 8. Growth of literature related to the AHR. Data from 1980 through 2006 represent the number of papers retrieved for each year from a PubMed search using the terms: ''aryl hydrocarbon receptor'' or ''dioxin receptor.'' The AHR is not necessarily the primary focus of each of these papers nor has the number of AHR publications been normalized to the total number of papers published in the biomedical literature for each year. The number of AHR papers listed from 1976 until 1980 is based on my reprint files of papers that appeared during the inaugural years of research in the AHR field.

induction in some cell lines (Matthews and Gustafsson, 2006; Matthews *et al.*, 2005) although it also has been reported that the ERα which is recruited to the *CYP1A1* promoter functions as a repressor of transcription rather than as a coactivator (Beischlag and Perdew, 2005).

At present, it is impossible to make a simple summary of how AHR and ER pathways intersect. Determining the resultant of the push–pull interactions between AHR and ER pathways, under conditions of real-world exposure, is important and should keep investigators in this field busy for several years to come.

CONCLUSION: RETURN ON INVESTMENT

We frequently tell our students (and, sometimes, granting agencies and governments) that if we understand the mechanism by which a toxicant acts, we will be able to make a much more accurate and valid assessment of the risk which that toxicant poses to human health and health of the ecosystem. As stated by Conolly (2002), research on mechanisms of toxic action serves to reduce uncertainty in risk assessment. However, mechanistic understanding is not, on its own, sufficient to define risk—a point eloquently made by Prof. Jouko Tuomisto in his Deichmann Lecture in 2004 (Tuomisto, 2005). Nevertheless, I believe that the AHR research community can legitimately claim, with pride (and without apology or embarrassment), that our collective mechanistic discoveries during the past 30 years (Fig. 7) are providing an invaluable scientific foundation for assessing human health risks.

Research on the AHR shows no signs of waning (Fig. 8). Much remains to be done, both in basic AHR science and regarding its role in environmental health. It's tempting, when concluding an overview such as this, to make predictions about the future of the field. Of course such prognostications carry their own risk—that of being nothing more than fool-hardy, ill-informed failures. Given the pace of current science, I'm confident, however, that we will not need to wait another 30 years for solutions to most of the mysteries listed above. We also should anticipate a few more surprises, twists, and turns in the AHR story along the way.

ACKNOWLEDGMENTS

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I want to pay special tribute to the extraordinary contributions made over the last three decades of ''AHR adventures'' by my graduate students and post-docs and, particularly, to my long-term Toronto colleagues, Dr Patricia Harper and Dr David Riddick, and to my valued Finnish collaborators and friends, Prof. Jouko Tuomisto and Prof. Raimo Pohjanvirta. I truly am indebted to all of you.

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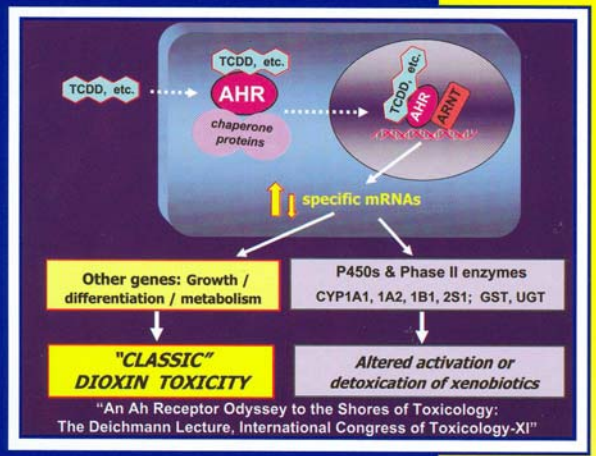

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