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**ROLE OF THE AROMATIC HYDROCARBON RECEPTOR IN THE SUPPRESSION
OF CYTOCHROME P450 2C11 BY POLYCYCLIC AROMATIC HYDROCARBONS**

BY

Behnam Safa

**A thesis submitted in conformity with the requirements for the
degree of Master of Science
Graduate Department of Pharmacology
University of Toronto**

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ABSTRACT

Role of the aromatic hydrocarbon receptor in the suppression of cytochrome P450 2C11 by polycyclic aromatic hydrocarbons. Master of Science, 1998. Behnam Safa, Department of Pharmacology, University of Toronto.

The aromatic hydrocarbon receptor (AHR) mediates the induction of cytochromes P450 (CYP) of the CYP1A subfamily caused by polycyclic aromatic hydrocarbons (PAHs). CYP1A induction by PAHs is accompanied by down-regulation of CYP2C11, the predominant CYP expressed constitutively in the liver of male rats. We performed a structure-activity relationship study with a series of PAHs of the anthracene class in order to determine if the AHR is involved in CYP2C11 down-regulation. Anthracene, benz[a]anthracene, dibenz[a,c]anthracene, dibenz[a,h]anthracene, 7,12-dimethylbenz[a]anthracene, as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene decreased CYP2C11 immunoreactive protein levels to varying degrees in primary rat hepatocytes cultured on a laminin-rich extracellular matrix. The binding affinity of the PAHs for the rat liver cytosolic AHR correlated with the potency for transforming the cytosolic AHR to its DNA-binding form. In addition, the ability of the PAHs to suppress CYP2C11 correlated with both the AHR binding affinity and the AHR transformation potency. These results suggest that the AHR plays a role in the down-regulation of CYP2C11 caused by PAHs.

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ABBREVIATIONS

AH	Aromatic hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
AHR	Aromatic Hydrocarbon receptor
AHRE	Aromatic hydrocarbon responsive element
ANTH	Anthracene
ARNT	Aromatic hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
BA	Benz[a]anthracene
bHLH	Basic helix-loop-helix
BIS	N,N'-methylenebisacrylamide
Blotto	5%(w/v) skimmed milk powder in TNT
BP	Benzo[a]pyrene
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
°C	Degree Celsius
%C	Amount of BIS crosslinker expressed as a percent of the sum of acrylamide monomer and BIS
cDNA	Complementary DNA
cm	Centimetre
cpm	Counts per minute
CYP	Cytochrome P450
DB[a,c]A	Dibenz[a,c]anthracene
DB[a,h]A	Dibenz[a,h]anthracene
DCC	Dextran-coated charcoal
DNA	Deoxyribonucleic acid
DMBA	7,12-Dimethylbenz[a]anthracene
DMSO	Dimethylsulphoxide
dpm	Disintegration per minute
DRE	Dioxin-responsive element
DRE3	Dioxin-responsive element-3
EDTA	Ethylenediaminetetraacetic acid, disodium salt
fmol	Femtomole

x g	Relative centrifugal force
GH	Growth hormone
HCB	3,4,5,3',4',5'-Hexaclorobiphenyl
HEG	25 mM HEPES/1.5 mM EDTA/10% glycerol, pH 7.4
HEGD	25 mM HEPES/1.5 mM EDTA/10% glycerol/1 mM dithiothreitol, pH 7.4
HEPES	Hydroxyethane piperazine sulphate
HNF	Hepatic nuclear factor
Hsp 90	Heat shock protein 90 KDa
<i>in vivo</i>	in the live animal
i.p.	Intraperitoneal
kb	Kilobase
K_d	Apparent dissociation constant
kDa	Kilodalton
kg	Kilogram
M	Molar
MAb	Monoclonal antibody
MC	3-Methylcholanthrene
min	Minute
mg	Milligram
ml	Millilitre
MPL	Major late promoter
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nm	Nanometre
nmol	Nanomole
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo- <i>p</i> -dioxin
PCDF	Polychlorinated dibenzofuran
PB	Phenobarbital

QSAR	Quantitative structure-activity relationship
SDS	Sodium dodecyl sulphate
STR	Steroid/thyroid/retinoic acid receptor
t _{1/2}	Half-life
T ₄ PNK	T ₄ Polynucleotide Kinase
TCDD	2,3,7,8,-Tetracholorodibenzo- <i>p</i> -dioxin
TCDF	2,3,7,8,-Tetracholorodibenzofuran
TE8	10 mM TRIS-HCl/1 mM EDTA pH 8.0
TEMED	N,N,N',N',-Tetramethylethylenediamine
TNT	20 mM TRIS/137 mM NaCl/0.1% (v/v)Tween-20, pH 7.6
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
tRNA	Transfer ribonucleic acid
μg	Microgram
μl	Microlitre
μM	Micromolar
UV	Ultraviolet
v/v	Volume per volume
V	Volt
w/v	Weight per volume
XRE	Xenobiotic responsive element

1.0 INTRODUCTION

1.1 Statement of the Research Problem

The cytochromes P450 (CYP) constitute a large superfamily of heme-containing microsomal mono-oxygenases that are present at high levels in the liver of many species, including humans. These enzymes have an extraordinary range of substrate recognition and play a major role in the metabolism of xenobiotics as well as endogenous substances such as steroids, fatty acids, vitamins, bile acids, leukotrienes, thromboxanes and prostaglandins (Henderson and Wolf, 1992). An important characteristic of many of the cytochrome P450 species is inducibility by xenobiotics (Okey, 1990). Polycyclic aromatic hydrocarbons (PAHs), phenobarbital, ethanol, glucocorticoids and clofibrate are among the best studied inducers of various CYP enzymes. Regulation of CYP1A1 induction via the aromatic hydrocarbon receptor is the most completely understood system. In addition to induction, PAHs have also been reported to cause down-regulation of specific constitutively-expressed CYPs in the liver of male rats, namely CYP2C11 and CYP3A2. However, the mechanisms for this PAH-mediated suppression of constitutive CYPs are not understood. The research hypothesis on which this thesis is based, is that PAHs exert their suppressive effect at least partly through the aromatic hydrocarbon receptor (AHR). The goal of this research was to examine the role of the AHR in the suppression of CYP2C11 through a structure-activity relationship study using a series of structurally-related anthracene derivatives in primary cultures of rat hepatocytes maintained on a laminin-rich extracellular matrix. The ability of these compounds to suppress CYP2C11 immunoreactive protein in rat hepatocytes was compared with their affinity for the AHR and their ability to transform the receptor to its DNA-binding form.

1.2 Drug Metabolism

Humans are exposed daily to a wide variety of foreign substances absorbed across the lungs or skin or via ingestion. This exposure may be unintentional and

inescapable as for compounds present in food, drink and air or deliberate as drugs for therapeutic or "recreational" purposes. Some of these xenobiotics are innocuous, but many are capable of provoking various biological responses. Fortunately, we are equipped with a diverse array of enzymes that are able to act upon these xenobiotics, hence accelerating their elimination from the body. It is important to note that endogenously synthesized compounds such as steroids, fatty acids, prostaglandins etc. also are substrates for these enzyme systems.

Drug metabolism is normally divided into two phases: phase I (or functionalization reactions) and phase II (or conjugative reactions). Phase I reactions usually convert the parent compound to a more polar metabolite by introducing or unmasking a reactive functional group (-OH, -NH₂, -SH) on the molecule. If phase I metabolites are sufficiently polar, they may be readily excreted. However, many phase I products are not eliminated rapidly and undergo a subsequent phase II reaction in which a substrate such as glucuronic acid, sulfate, acetate, glutathione or an amino acid combines with the newly established functional group to form a highly polar conjugate which is readily eliminated (Gibson and Skett, 1994; Katzung, 1995; Ortiz de Montellano, 1995). The majority of phase I reactions are carried out by the cytochrome P450 superfamily of enzymes.

1.3 Cytochrome P450

1.3.1 General Properties

The term cytochrome P450 (CYP) refers to a superfamily of enzymes that contain a noncovalently bound heme prosthetic group. The name cytochrome P450 is derived from the spectral properties of this hemoprotein. In its reduced (ferrous) form, it binds carbon monoxide to form a complex that absorbs light maximally at 450 nm (Omura and Sato, 1964). The CYP superfamily consists of about 481 genes and 22

pseudogenes found in 85 eukaryotic (including vertebrates, invertebrates, fungi and plants) and 20 prokaryotic species (Nelson et al., 1993; Nelson et al., 1996). CYPs are present in the microsomal fraction of all mammalian cell types, except mature red blood cells and skeletal muscle cells (Okita and Masters, 1992). With the exception of the bacterial enzymes, all CYPs are membrane-bound. A few CYPs involved in steroid biosynthesis are found in the inner mitochondrial membrane while most CYPs are located in the phospholipid bilayer of the endoplasmic reticulum (Gonzalez, 1994).

The diversity of forms, as well as a relatively non-stringent active site, explain the long recognized broad substrate specificity of these enzymes. In fact CYPs have been described as the most versatile biological catalysts in existence (Ryan and Levin, 1990). The CYP system has far reaching effects in medicine. It is intimately associated with a) inactivation or activation of therapeutic agents, b) activation of certain substrates into carcinogenic, mutagenic or cytotoxic products and c) participation in steroid hormone biosynthesis and catabolism and metabolism of fatty acids and their derivatives (Okita and Masters, 1992). In essence, CYPs represent one of the major enzyme systems that determine the organism's capability of dealing with lipophilic drugs and chemicals.

1.3.2 Catalytic Cycle

The CYPs are designated as "phase I" drug metabolism enzymes, catalyzing the introduction of an atom of oxygen into a substrate. In essence CYPs act as terminal electron acceptors in a membrane bound electron transport chain. Reducing equivalents, derived from NADPH, are passed along to membrane bound CYP via the flavoprotein NADPH-cytochrome P450 reductase (Riddick, 1998). This process is thought to involve cytochrome b₅ in some cases (Okita and Masters, 1992). These reducing equivalents are required to allow binding of molecular oxygen to the ferrous heme and for subsequent activation of the oxygen, one atom of which is incorporated into the substrate. The overall reaction catalyzed by these enzymes is (Katzung, 1995):

In this scheme, R-H represents the oxidizable substrate and R-OH represents the hydroxylated product of the reaction.

Since only one of the two oxygen atoms is incorporated into the substrate, CYPs are referred to as monooxygenases. In addition to oxidation at carbon atoms, these enzymes also catalyze oxidation reactions at nitrogen and sulfur atoms (Henderson and Wolf, 1992). The oxidation reaction catalyzed by CYPs facilitates further metabolic processing by "phase II" enzymes to yield a hydrophilic excretable product. The role of CYPs is therefore central to the detoxification and elimination of lipophilic xenobiotics as well as endogenous compounds.

1.3.3 Multiplicity and Nomenclature of CYPs

In every animal species examined numerous CYPs are expressed. In humans at least 36 genes and 9 pseudogenes have been characterized, while in rats at least 54 genes and 6 pseudogenes have been characterized (Nelson et al., 1993; Nelson et al., 1996). The sequence similarity of CYPs has made it abundantly clear that the many forms of CYP enzymes have emerged due to gene duplication events from a common ancestral enzyme in the course of evolution (Okita and Masters, 1992). A nomenclature system based on amino acid sequence homology has been implemented to categorize the ever-growing number of CYPs (Nebert and Gonzalez, 1987; Nelson et al., 1996). The CYP superfamily is divided into families and subfamilies. CYPs within one family are usually defined as having at least 40% amino acid sequence similarity and the ones within a given subfamily are usually more than 55% identical (Nelson et al., 1996). Each cytochrome P450 is identified by the root symbol "CYP", followed by an

Arabic numeral denoting the gene family, a capital letter denoting the gene subfamily, and an Arabic numeral indicating the specific enzyme (e.g. CYP2C11) (Nebert and Gonzalez, 1987; Nelson et al., 1996).

All of the mammalian CYPs can be grouped into two categories, the biosynthetic forms and the "multipurpose" forms (Schenkman, 1992). The biosynthetic CYPs are involved in steroid biosynthesis. The "multipurpose" CYPs may or may not metabolize steroids, but they are responsible for the metabolism of a wide variety of chemicals and drugs, and often a number of endogenous substrates, including free fatty acids and prostaglandins. The "multipurpose" CYPs may be constitutively expressed (constitutive CYPs) or may require challenge with some chemical (inducer) for *in vivo* expression (nonconstitutive CYPs) (Schenkman, 1992). CYP1A2, CYP2E1 and CYP2C11 are examples of constitutive CYP isozymes, whereas CYP1A1, CYP2B1 and CYP3A1 are examples of nonconstitutive CYP isozymes in rats (Schenkman, 1992).

1.3.4 Regulation of Hepatic Microsomal CYP

In addition to multiplicity in form and diversity of substrate specificity, constituents of the CYP superfamily exhibit various modes of expression that may include developmental, tissue-specific, sex-dependent (hormonal), or inducer-specific regulatory patterns (Lu and West, 1980). The expression of many CYP forms is controlled by various external stimuli (drugs and chemicals) as well as internal stimuli. Exposure to any one of numerous substances may result in the induction of total CYP content as well as an alteration in the spectrum of the enzymes being expressed. This alteration is of clinical significance for two main reasons: a) alteration in efficacy of therapeutic agents, b) creation of an undesirable imbalance between rates of "toxification" vs. "detoxification" leading to activation and/or accumulation of undesirable compounds (Okey et al., 1986). Every CYP has its own regulatory circuit and with the advent of molecular biology techniques we have learned much about molecular

mechanisms controlling CYP expression. The regulation of CYPs has been documented to occur at multiple levels, involving both positive and negative-acting mechanisms, including protein stability, mRNA stability, rate of transcription as well as translational efficiency (Porter and Coon, 1991).

A distinguishing feature of the CYP superfamily is inducibility by both endogenous and exogenous compounds such as food components, alcohol, smoking, and physiological states such as starvation, etc. (Okey, 1990). Thus, PAHs, phenobarbital, ethanol, glucocorticoids, and clofibrate each induce their own spectrum of CYPs. Induction of some species of CYP is sometimes accompanied by the suppression of other CYP forms. The mechanism or mechanisms by which some agents concurrently induce one CYP isozyme and suppress another have not been studied in great detail. Over the years it has become clear that both the inductive and the suppressive effects might occur at many different levels in the CYP regulatory system.

1.3.5 Rat Hepatic Microsomal CYP

Various CYP forms have been isolated and/or cloned from rat tissue. To date at least 54 distinct functional rat CYP genes have been identified and classified into 12 families (Nelson et al., 1996). Rats have been used as experimental models because most is known about the regulation of constitutive CYPs in this species. Although rat CYPs are well characterized, a limitation of this model is that differences in CYP regulation exist between rats and humans, especially with respect to the sexual differentiation of hepatic CYP function. Although gender-related differences are known to occur in humans (Bonate, 1991), they do not appear to be as pronounced as in the rat. Certainly, sexual dimorphisms at the level of CYP isozyme expression have not been characterized in human liver. At present, the existence of human CYP forms orthologous to constitutive rat CYPs has not been established except in cases such as

CYP2E1 and CYP3A subfamily forms (Wrighton and Stevens, 1992). Thus extrapolation of regulatory mechanisms from rat to human may not always be possible, but in cases where rat and human orthologues clearly exist (CYP1A1 and CYP2E1), mechanisms of CYP regulation appear to be conserved. At present rat liver remains the best characterized system for the study of constitutive CYP isozyme expression.

1.3.6 Sexual Dimorphism of Hepatic CYPs in Rats

Gender differences in drug metabolism in rats have been known for more than 60 years. Male rats metabolize various anesthetics, sedatives, analgesics, steroids, etc. more rapidly than females (Ryan and Levin, 1993; Shapiro et al., 1995). Recent studies have demonstrated that this sexual dimorphism in drug metabolism results from the differential expression of possibly a dozen or so gender-dependent hepatic CYP forms (Shapiro et al., 1995). One female-specific isozyme, CYP2C12, and four male-specific isozymes, CYP2A2, CYP2C13, CYP2C11, and CYP3A2 have been purified to homogeneity from rat liver microsomes (Ryan and Levin, 1993). There are also CYP isozymes that show gender-predominance, that is while the enzyme can be found in the liver of both sexes its expression levels will be greater in either males or females (Shapiro et al., 1995). For example, CYP2A1 is expressed equally in immature male and female rats but its expression is decreased in male rats at puberty, and thus becomes female-predominant in adult rats (Waxman et al., 1985; Shapiro et al., 1995; Waxman and Chang, 1995).

Over the years, experiments using hypophysectomized rats have made it clear that the androgenic effects on hepatic gender-dependent CYP isozymes are mediated by the pituitary. Substantial evidence has accumulated which indicates that growth hormone (GH) is the pituitary factor responsible for maintaining these gender differences (Shapiro et al., 1995). Secretion of testicular androgens in the neonate during the first weeks of life predetermines or "imprints" a male pattern of metabolism

instead of a female pattern which becomes apparent at maturity (Ryan and Levin, 1993). Adult male rats are characterized by episodic release of growth hormone every 3.5-4 hrs with troughs of low, almost undetectable basal levels (Jansson et al., 1985; Shapiro et al., 1995). In female rats, the growth hormone pulses are more frequent, irregular and of lower magnitude than in males, whereas the interpeak (baseline) concentrations are higher and always measurable (Eden, 1979; Jansson et al., 1985; Shapiro et al., 1995).

In summary, gender differences in drug metabolism in the rat are determined by the differential expression of gender-determined CYP isozymes, which are regulated by the sexually dimorphic growth hormone plasma profiles (Shapiro et al., 1995).

1.3.7 CYP2C11

CYP2C11 is the major focus of this work. In order to understand the rationale and objectives of this thesis, it is essential to briefly summarize the properties and the regulation of CYP2C11. Rat CYP2C11 is the prototype of a gender-specific hepatic CYP enzyme. Levels of CYP2C11 are undetectable in female rats at any age but increase dramatically at maturation in male rats (Ryan and Levin, 1993). It is the predominant constitutive hepatic microsomal CYP in untreated male rats catalyzing steroid 16 α - and 2 α -hydroxylation (Swinney et al., 1987). CYP2C11 appears to be important in the metabolism of endogenous steroid hormones and the bioactivation of procarcinogens (Waxman, 1988; Butler et al., 1989). A schematic summary of the hormonal pathways involved in the regulation of CYP2C11 is shown in Fig. 1. The expression of CYP2C11 is male-specific and is dependent on exposure to testosterone in the neonate. It appears that neonatal imprinting by androgens is essential to establish the male-specific pulsatile pattern of growth hormone secretion, thus allowing the expression of CYP2C11 at puberty in male rats (Waxman, 1988; Kato and Yamazoe, 1993). It has been demonstrated that GH regulates the expression of CYP2C11 at the

Figure 1. Hormonal regulation of hepatic CYP2C11 expression.

[SS=somatostatin; GHRH=growth hormone releasing hormone; GH=growth hormone; TRH=thyrotropin releasing hormone; TSH=thyrotropin; T4=thyroxine, T3=triiodothyronine; LHRH= luteinizing hormone releasing hormone; LH=luteinizing hormone. (modified from Henderson & Wolf, 1992)]

level of transcription both *in vivo* and in primary cultures of rat hepatocytes (Legraverend et al., 1992; Sundseth et al., 1992).

CYP2C11 has been purified (Guengerich et al., 1982; Ryan et al., 1984; Waxman, 1984) and the entire amino acid sequence deduced from the cDNA (Yoshioka et al., 1987). The genomic sequence for *CYP2C11* has also been determined, (Morishima et al., 1987), and recently, a large portion (2.3 kb) of the 5'-flanking region was cloned (Strom et al., 1994). Potential binding sites for putative transcription factors exist. The gene contains a TATA box and a glucocorticoid-responsive element half-site. Potential binding sites for hepatic nuclear factors [HNF-1, -85 to -70], [HNF-3, -80 to -67; -54 to -44], and the ubiquitous nuclear factor 1 [NF1, -54 to -41] have been identified. The gene contains a HepG2-specific P450 2C factor motif [HPF1, -311 to -291] that is a conserved site found in many members of the CYP2C subfamily (Venepally et al., 1992). This sequence in the *CYP2C11* gene binds a variety of orphan nuclear receptors, but appears to be of minimal functional importance (Strom et al., 1995). The *CYP2C11* 5'-flank contains sequences that interact in a sex-dependent and GH-regulated manner with putative transcription factors (Sundseth et al., 1992; Strom et al., 1994); however, only three functional elements have been localized in the *CYP2C11* upstream region. An HNF-1-like binding site confers negative regulation on a heterologous thymidine kinase promoter in reporter gene assays (Strom et al., 1994); however, the identity of the potential *trans*-acting factor is not known. Two negative regulatory regions (silencer elements) spanning nucleotides -1230 to -1188 and -409 to -368 were identified and it was suggested that induction of CYP2C11 expression by intermittent GH may involve derepression of these silencer elements (Strom et al., 1994); however, the functional significance of these elements remains unknown.

1.4 Aromatic Hydrocarbons

The polycyclic aromatic hydrocarbons are a complex group of chemicals containing two or more aromatic rings. Members of this chemical class include the halogenated as well as the nonhalogenated aromatic hydrocarbons. These chemicals have received increasing attention over the past 25 years since the public has become increasingly aware of their presence and the risk they pose to human health and the environment (Nisbet and Lagoy, 1992; Poland and Bradfield, 1992). Indeed these compounds are extremely potent at producing a variety of toxic effects associated with alteration in growth and development of a variety of different cell types in experimental animals (Devito and Birnbaum, 1994). The structures of some of the representative aromatic hydrocarbons are shown in Fig. 2.

Polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), are ubiquitous environmental contaminants. Due to their chemical stability, lipophilicity and resistance to degradation they tend to persist in the environment and accumulate in the food chain (Poland and Knutson, 1982). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of this chemical class and produces a broad spectrum of toxic effects in laboratory animals including a wasting syndrome, thymic atrophy, chloracne, hepatotoxicity, immunotoxicity, reproductive toxicity, developmental toxicity, teratogenesis and carcinogenesis (Devito and Birnbaum, 1994).

Nonhalogenated polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (BP) are components of crude and refined oils, asphalt and coal tars and are formed as by-products during the incomplete combustion of organic material. As such they are commonly encountered as contaminants at hazardous waste sites (Nisbet and Lagoy, 1992). PAHs with four or more rings have been characterized as animal and human carcinogens. In fact the aromatic hydrocarbons in cigarette

Figure 2. Structures of selected aromatic hydrocarbons. Examples of toxic halogenated aromatic hydrocarbons (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), carcinogenic nonhalogenated polycyclic aromatic hydrocarbons (3-methylcholanthrene and benzo[*a*]pyrene) and dietary constituents (indolo[3,2-*b*]carbazole) are shown. (Okey et al., 1994b).

2,3,7,8-tetrachlorodibenzo-*p*-dioxin

indolo[3,2-*b*]carbazole

3-methylcholanthrene

benzo[*a*]pyrene

smoke have been implicated as causative agents of lung cancer (Wynder and Hoffmann, 1994). The mechanism associated with PAH-induced carcinogenesis involves the bioactivation of these compounds by CYPs resulting in production of reactive metabolites that bind covalently to cellular macromolecules (e.g. alkylation of DNA), leading to cytotoxicity, mutagenesis and tumor initiation (Chaloupka et al., 1993).

Risk assessment and decisions about the reduction of the adverse effects of any toxic chemical can be much more rational if the mechanism of its action is known. For aromatic hydrocarbons the first real insight came from the discovery of the aromatic hydrocarbon receptor, and elucidation of its role in mediating induction of CYP1A1.

1.5 Aromatic Hydrocarbon Receptor

The aromatic hydrocarbon receptor (AHR) was discovered by Poland and coworkers in 1976 as a cytoplasmic constituent in the liver of C57BL mice which bound [³H]TCDD specifically with very high affinity (Poland et al., 1976). It has achieved notoriety as the major site of action of aromatic hydrocarbons over the past 20 years (Okey et al., 1994b). There are several sources of evidence implicating the AHR as the primary mediator of toxicity produced by the aromatic hydrocarbons (Okey et al., 1994a; Okey et al., 1994b): (1) Quantitative structure-activity relationships (QSAR) have demonstrated that within a particular series of halogenated aromatics (polychlorinated biphenyls, polychlorinated dioxins, polychlorinated dibenzofurans) the toxicity of the individual isomers and congeners is closely related to the affinity with which the compounds bind to the AHR (Poland and Knutson, 1982; Safe, 1990; Whitlock, 1990). An excellent correlation between the capacity of several PAHs to displace [³H]TCDD from the AHR and the capacity to induce aryl hydrocarbon hydroxylase activity has also been demonstrated (Bigelow and Nebert, 1982). In contrast, Piskorska-Pliszczyńska *et al.* (1986) showed that there was no apparent correlation between the AHR binding affinity and CYP1A1 induction potency for the most active PAHs. It is clear that such

relationships may not apply when comparing ligands from different structural classes (Riddick et al., 1994b). (2) Several compounds that act as partial antagonists/partial agonists have been shown to inhibit or reduce the toxic and biochemical effects of the potent agonist TCDD (Merchant et al., 1990; Piskorska-Pliszczyńska et al., 1991). (3) Greater susceptibility to a wide range of toxic effects of TCDD in mice segregates with the *Ah^{b-1}* allele that encodes a 'high affinity' form of the AHR in the responsive mouse strains such as C57BL/6J. The AHR from 'nonresponsive' strains of mice, such as DBA/2J, binds TCDD with an affinity that is about 10-fold lower than in C57BL/6J mice (Okey et al., 1989). This difference in the affinity for TCDD is in the same range as the difference in the sensitivity between 'responsive' and 'nonresponsive' strains to CYP1A1 induction or toxicity from TCDD. The DBA/2J mice require TCDD doses which are approximately 1 log. unit higher than the doses required in C57BL/6J mice to achieve the same biologic/toxic effect (Nebert, 1989).

The AHR is expressed in several animal species, including rodents, humans, fish, birds and diverse mammalian cells in culture (Bunce et al., 1988; Henry et al., 1989; Bank et al., 1992). Within the same animal, the AHR is expressed in multiple organs (Henry et al., 1989), and the AHR mRNA demonstrates tissue-specific expression. For instance, in rats highest levels are found in lung, thymus, liver and kidney, whereas the lowest levels of AHR mRNA are found in heart and spleen (Carver et al., 1994). The broad distribution of AHR in vertebrate tissue implies an important and yet to be identified physiological function (Okey et al., 1994a). Although AHR is frequently referred to as a cytosolic receptor, its true location in the intact cell has been controversial. In cell homogenates, the unliganded receptor fractionates into the cytosol, and hence is often termed "cytosolic" (Denison et al., 1986a). Furthermore, immunofluorescence microscopy results also lend support to the cytosolic location of the unliganded AHR (Hord and Perdew, 1994; Pollenz et al., 1994). In general function the AHR acts very much like the steroid/thyroid/retinoic acid (STR) receptor superfamily which are "zinc finger" DNA-binding proteins that act as ligand-induced transcription

factors for transactivation of target genes (Evans, 1988). Indeed for some years it appeared likely that AHR was a member of the STR receptor superfamily. However, recent studies indicate that the structure of the AHR protein is significantly different from the STR receptor superfamily (Dolwick et al., 1993; Schmidt et al., 1993). The AHR has been cloned and sequenced (Burbach et al., 1992; Ema et al., 1992) and it has become clear that AHR's N-terminal sequence contains a basic helix-loop-helix (bHLH) domain that is required for recognition of specific DNA sequences, and is devoid of any significant sequence similarity with the STR receptor superfamily. The molecular mass of the cytosolic AHR isolated as a multimeric protein complex has been determined to be approximately 270 kDa in mice (Denison et al., 1986b). Cross-linking experiments have revealed that the cytosolic AHR complex is in fact a tetrameric receptor complex consisting of a ligand-binding subunit and three other proteins of approximately 96, 88, and 46 kDa relative molecular masses (Perdew, 1992). The 90-kDa heat shock protein (hsp90) has been identified as one of the proteins associated with the cytosolic form of the AHR complex (Perdew, 1988). Since hsp90 is an abundant cytosolic protein only occurring in a dimeric form it is reasonable to propose that the tetrameric cytosolic AHR complex may contain two hsp90 subunits (Chen and Perdew, 1994). Presently, the ~46 kDa protein remains unidentified, but recently described candidates include the immunophilin-like proteins termed AIP (AHR-interacting protein) (Ma and Whitlock, 1997) and ARA9 (AHR-activated-9) (Carver and Bradfield, 1997). Western blot analysis under denaturing conditions has shown that the mass of the AHR ligand-binding subunit varies from ~95 to 130 kDa, depending on the animal species and strain (Poland et al., 1991). It is not yet known whether variation in molecular mass of AHR bears any direct relationship to variation in receptor function among different animal species (Okey et al., 1994a).

Binding of a ligand triggers some alteration of the cytosolic receptor complex that converts the AHR to its DNA-binding form. AHR recovered from the nucleus of mouse hepatoma cells has a molecular mass of ~175 kDa, which does not correspond in size

to the ~95 kDa monomeric ligand-binding subunit from the cytosol. Hankinson and coworkers (Hoffman et al., 1991) cloned a protein termed the "aromatic hydrocarbon receptor nuclear translocator" (ARNT) and demonstrated that the nuclear AHR complex is in fact a heterodimer made up of the AHR binding subunit and ARNT (Reyes et al., 1992). The ARNT gene product also carries a basic helix-loop-helix domain at the N-terminus, allowing dimerization to the AHR and DNA binding. The AHR and ARNT proteins are structurally similar to each other and also to two additional proteins: the *Drosophila* neurogenic protein Sim, and the *Drosophila* circadian rhythm protein Per. These four proteins are characterized by a region of homology of approximately 250 amino acids referred to as the PAS domain (Per, AHR-ARNT, and Sim) (Okey et al., 1994b). There are now at least 11 members of the mammalian "PAS" family (Hogenesch et al., 1997). Mouse AHR and ARNT are 20% identical in the amino acid sequence. They also show a striking resemblance in overall structure (Fig. 3) (Burbach et al., 1992; Ema et al., 1992; Hankinson, 1995). There are three major domains within the AHR and ARNT molecule: (1) The basic helix-loop-helix motif is at the extreme N-terminus of the protein. The HLH domain is found in a group of transcriptional factors and is responsible for the dimerization with a homologous or heterologous partner molecule, whereas the basic amino acid sequence immediately N-terminal to the HLH recognizes and binds a specific DNA sequence. Sim but not Per, also possesses a bHLH motif. (2) The PAS domain, which contains two short stretches of repetitive sequence referred to as PAS A and PAS B repeats, is also in the N-terminal half of the protein. It has been shown that the PAS region serves as the dimerization domain. The PAS domain of AHR also plays a role in ligand and hsp90 binding. (3) A region near the C-terminus of the molecule that influences the ligand-dependent transformation of the AHR to its DNA binding form. AHR and ARNT both contain glutamine-rich regions near their carboxy termini. This is a common feature of proteins involved in transcriptional activation (Swanson and Bradfield, 1993).

murine AH receptor

human AH receptor

human ARNT

Figure 3. Schematic representation of the overall structure of the murine AHR, human AHR and human ARNT. There are three major domains within the AHR and ARNT molecule: 1) The basic helix-loop-helix motif (bHLH). 2) The PAS domain containing two stretches of repetitive sequences: PAS A and PAS B. 3) Q rich region at the C-terminus (Okey et al., 1994b)

Although no endogenous ligand has been identified, AHR binds diverse structural classes of environmental pollutants including both the halogenated aromatic hydrocarbons and the nonhalogenated PAHs (Poland and Knutson, 1982; Safe, 1990). The ligand-binding site is hydrophobic, and preferentially accommodates planar non-polar ligands having molecular dimensions approximating a 1.0 nm x 0.3 nm rectangle, exemplified by TCDD (Poland et al., 1976; Safe, 1986). However, to accommodate the high-affinity binding of substantially larger molecules such as polycyclic aromatic hydrocarbons a binding site of dimensions 1.4 nm x 0.7 nm must be postulated (Landers and Bunce, 1991). Ligand binding properties of the AHR have been studied in some detail and the structure-activity relationships (SAR) have implicated the AHR in a variety of biological responses elicited by aromatic hydrocarbons (Safe, 1986).

1.5.1 Molecular Mechanism of AHR and ARNT Function

The AHR is a soluble intracellular protein that functions as a ligand-activated transcription factor (Elferink and Whitlock, 1994). Prior to occupancy by a ligand the inactive AHR is thought to reside in the cytoplasm in a soluble complex with the heat shock protein hsp90 and possibly other proteins (Chen and Perdew, 1994). Hsp 90 seems to stabilize the unbound AHR in a ligand binding conformation and repress its inherent DNA-binding activity (Pongratz et al., 1992). The postulated sequence of events in the transformation of the cytosolic AHR to the DNA-binding state is shown in Fig. 4. Binding of a ligand such as TCDD triggers the dissociation of the hsp90 and translocation of the ligand-receptor complex into the nucleus in a temperature-dependent manner (Okey et al., 1980). The process by which ligand binding transforms the cytosolic AHR to its functional DNA-binding state is complicated and poorly understood. Phosphorylation of both AHR and ARNT appears to be important for generation of the functional DNA-binding complex (Perdew, 1991; Berghard et al., 1993). Although protein kinase C (PKC) seems a likely candidate to catalyze the

Figure 4. Postulated sequence of events in the transformation of cytosolic AHR to the DNA-binding state. "P" indicates a possible role for phosphorylation in receptor transformation. (Okey et al. 1994a)

phosphorylation events, recent evidence suggests that PKC is not required for transformation nor for DNA binding as assessed *in vitro* (Schafer et al., 1993). Within the nucleus the ligand-receptor complex forms a heterodimer with ARNT. Thus, the nuclear AHR complex is a unique ligand-induced heterodimer composed of two bHLH proteins. Both helices of the bHLH region of ARNT are absolutely required for dimerization with AHR and the deletion of either the A or B segments of the PAS region of ARNT slightly affects dimerization with AHR, while deletion of the complete PAS region severely affects dimerization. Thus, ARNT and presumably AHR, possess multiple domains for maximal heterodimerization (Reisz-Porszasz et al., 1994; Hankinson, 1995). Ultimately this heterodimer interacts with specific *cis*-acting DNA enhancer sequences known as AH-responsive elements (AHREs; also known as dioxin-responsive elements: DREs; or xenobiotic-responsive elements: XREs) (Okey et al., 1994a). Whitlock and coworkers (Denison et al., 1988a) originally demonstrated that the AHREs from the mouse *Cyp1a1* upstream region contained an invariant 7-bp core sequence, 5'- TNGCGTG-3' required for AHR binding. More recently, Yao & Denison (1992) derived an AHRE consensus sequence for AHR binding (5'- GCGTGNNNA/TNNNC/G-3') and Lusska *et al.* (Lusska et al., 1993) derived an AHRE functional consensus sequence (5'-T/GNGCGTGA/CG/CA-3'). It is believed that interaction of the AHR complex with the AHRE results in nucleosome disruption leading to a change in chromatin structure and increased promoter accessibility (Wu and Whitlock, 1992). It is through this interaction that the AHR can increase the rate of transcription of various genes. The mechanism of AHR-mediated regulation of gene transcription is depicted in Fig. 5.

1.5.2 Other Genes Whose Expression is Altered by Aromatic Hydrocarbons

As a ligand-activated transcription factor, the AHR activates the transcription of a large number of genes. However, much of our understanding about AHR's function

Figure 5. Current concept of the mechanism of AHR-mediated regulation of gene transcription. Genes that are under control of AHR are depicted as belonging to two major functional groups: [1] Induction of drug metabolizing enzymes (e.g. CYP1A1/2) can alter the biotransformation capacity for endogenous and exogenous substrates. [2] Gene products that regulate cell differentiation and proliferation presumably play fundamental roles in normal development and their aberrant regulation may underlie the toxic manifestations of TCDD and related aromatic hydrocarbons. Note that although ARNT is shown here as a cytosolic protein, the subcellular distribution of ARNT remains controversial. (modified from Okey et al., 1994b; and Riddick et al., 1994a).

stems from analysis of the mechanism by which TCDD induces the transcription of a member of the CYP superfamily known as CYP1A1. Other CYP isozymes regulated (at least in part) by the AHR include CYP1A2 and CYP1B1. Glutathione-S-transferase Ya, the TCDD-inducible UDP-glucuronosyltransferase UGT1*06, NAD(P)H:quinone oxidoreductase NQO₁, and aldehyde dehydrogenase ALDH3c are among the non-CYP drug-metabolizing enzymes regulated by AHR (Okey et al., 1994b). Induction of drug-metabolizing enzymes by AHR ligands leads to altered activation and detoxification of xenobiotics. This is of toxicological significance since CYP1A1 and 1A2 bioactivate PAHs and aromatic amines to ultimate carcinogens.

Several genes that encode growth and differentiation regulatory proteins such as epidermal growth factor receptor, plasminogen activator inhibitor-2, interleukin-1 β , and transforming growth factor α and β 2 also appear to be responsive to AHR agonists. It is likely that derangements in expression of these genes are involved in the toxic/carcinogenic effect of TCDD (Sewall et al., 1993). In contrast to the well-characterized induction of drug-metabolizing enzymes mediated by AHR, several of the growth-regulatory genes are down-regulated by aromatic hydrocarbons. Down-regulation of epidermal growth factor receptor (Lin et al., 1991a) and estrogen receptor (Lin et al., 1991b; Zacharewski et al., 1991; White and Gasiewicz, 1993) is at least partly AHR-mediated, although detailed molecular mechanisms are lacking. Progress has been made recently in determining the mechanisms of AHR-mediated down-regulation of two estradiol-induced genes, pS2 (Zacharewski et al., 1994; Gillesby et al., 1997) and cathepsin D (Krishnan et al., 1995). It is important to understand the molecular mechanisms by which aromatic hydrocarbons down-regulate gene expression. Although negative regulation of gene transcription is as vital as induction, our understanding of the mechanisms of suppression lags far behind that for transcriptional activation (Clark and Docherty, 1993). To gain insight into how foreign chemicals negatively regulate gene expression, we have examined the down-regulation of a member of the CYP superfamily, CYP2C11, caused by aromatic hydrocarbons.

1.5.3 CYP2C11 Suppression

The regulation of the male-specific CYP2C11 has been studied extensively. It has been established that administration of aromatic hydrocarbons to adult male rats causes down-regulation of CYP2C11 (Guengerich et al., 1982; Yeowell et al., 1987; Shimada et al., 1989; Jones and Riddick, 1996). However, CYP2C11 suppression is not a response that is specific to aromatic hydrocarbons. A variety of chemicals and pathophysiological stimuli have been shown to cause down regulation of CYP2C11: cytokines (Sakai et al., 1992; Morgan et al., 1994; Thal et al., 1994; Nadin et al., 1995), antineoplastic agents such as cisplatin (LeBlanc and Waxman, 1988), cyclophosphamide (LeBlanc and Waxman, 1990) and 5-fluorouracil (Stupans et al., 1995; Afsar et al., 1996), drugs of abuse such as morphine (Rane and Ask, 1992), cocaine (Badger et al., 1992), and ethanol (Badger et al., 1993), ethylbenzene (Yuan et al., 1992), plant indoles (Wortelboer et al., 1992), bacterial endotoxins (Morgan, 1989), diabetes (Thummel and Schenkman, 1990), liver cirrhosis (Murray et al., 1992), and partial hepatectomy (Ronis et al., 1992). The mechanism(s) by which such a diverse set of stimuli cause suppression of a single hepatic gene is not understood at this time. Several studies have shown that hepatic microsomal steroid 16 α -hydroxylation (a marker for CYP2C11) is lowered following treatment of rats with PB-type, MC-type, and glucocorticoid monooxygenase inducers (Waxman, 1984; Waxman et al., 1985). With respect to aromatic hydrocarbons, it has been demonstrated that the suppression of CYP2C11 catalytic activity is accompanied by decreased CYP2C11 apoprotein (Dannan et al., 1983; Waxman et al., 1985; Jones and Riddick, 1996) and mRNA (Yeowell et al., 1987; Jones and Riddick, 1996) indicating that the xenobiotics decrease the *de novo* synthesis of CYP2C11 rather than increasing the competition between CYP2C11 and concomitantly-induced CYP isozymes (e.g. CYP1A1,1A2) for limited cellular heme pools (Waxman et al., 1985; Yeowell et al., 1987; Emi and Omura, 1988; Shimada et al.,

1989; Ryan and Levin, 1993). At present, it is not known whether suppression of specific CYPs is linked to induction of other CYPs or whether distinctive mechanisms are operative.

1.6 Gap in the Knowledge Leading to the Research Hypothesis

Although the mechanism by which aromatic hydrocarbons cause induction of CYPs has been studied extensively, the mechanisms of xenobiotic-mediated suppression of CYPs are not understood. An attractive hypothesis is that suppression of CYP2C11 by aromatic hydrocarbons is also mediated by the AHR. However, an alternative hypothesis is that aromatic hydrocarbons exert their suppressive effect by disrupting normal endocrine pathways responsible for hepatic CYP2C11 regulation.

At present, evidence exists both for and against the involvement of the hepatic AHR in the suppression of CYP2C11 caused by halogenated and nonhalogenated aromatics. Support for the role of AHR comes from two sources: (a) limited structure-activity relationships with a series of polybrominated biphenyl congeners (Dannan et al., 1983) and polychlorinated biphenyls and dibenzofurans (Yoshihara et al., 1982) demonstrated correlations between suppression of CYP2C11 and stimulation of AHR-mediated responses such as CYP1A1/2 induction and thymic atrophy, and (b) α -naphthoflavone, an "inactive" AHR ligand, does not suppress CYP2C11 (Emi and Omura, 1988). In contrast, a study with 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) showed that suppression of CYP2C11 and induction of CYP1A1 could be distinguished on the basis of dose-response and time-course data (Yeowell et al., 1987), suggesting that the two processes may be mediated by different mechanisms.

1.6.1 Research Hypothesis

The hepatic AHR mediates the down-regulation of CYP2C11 caused by aromatic hydrocarbons.

1.6.2 Rationale for Study Design and Specific Objectives

Structure-activity relationships constitute one of the primary sources of evidence for the involvement of the AHR in the induction of CYP1A1 by aromatic compounds. Hence, in this thesis I have examined the role of AHR in the suppression of CYP2C11 using a series of structurally-related nonhalogenated anthracene derivatives. The chemical structures of these compounds as well as the reference compound MC are shown in Fig. 6. The IC₅₀ values for these compounds competing for binding of [³H]TCDD to the rat hepatic cytosolic AHR were determined as a measure of the relative binding affinities of the PAHs for the AHR. The AHR binding IC₅₀ values for MC, ANTH, BA, DB[a,c]A, DB[a,h]A and DMBA have been previously reported in the literature (Okey and Vella, 1982; Denison et al., 1986b; Piskorska-Pliszczyńska et al., 1986). However, the AHR binding affinity studies cited above involved the use of C57BL/6J mouse liver cytosol, C57BL/6J mouse and Spague-Dawley rat liver cytosol and Wistar rat liver postmitochondrial supernatant, respectively. Since our studies of CYP2C11 suppression are carried out in inbred Fischer 344 rats in order to avoid the complicating effects of polymorphic expression of other CYP2C subfamily members (eg. CYP2C13) in outbred rat strains (eg. Sprague-Dawley), I needed to confirm that the hepatic AHR from Fischer 344 rats displays similar affinity for the PAHs used in my study to rule out the possibility of important species and strain differences.

I determined the EC₅₀ values for each PAH for the transformation of the rat hepatic cytosolic AHR to its DNA-binding form. Previous work in this field has focused on halogenated aromatic hydrocarbons including dioxins and dibenzofurans (Santostefano et al., 1992). My study is the first to systematically examine the relationship between AHR binding affinity and transformation potency for a series of structurally-related nonhalogenated PAHs.

Figure 6. Structure of the nonhalogenated PAHs used in the structure-activity relationship study

Anthracene (ANTH)

Benz[a]anthracene (BA)

Dibenzo[a,c]anthracene (DB[a,c]A)

Dibenzo[a,h]anthracene (DB[a,h]A)

7,12-Dimethylbenzo[a]anthracene (DMBA)

3-Methylcholanthrene (MC)

It is well established that expression of CYP2C11 is subject to a complex endocrine regulation. Hence, in order to examine direct hepatic effects of aromatic hydrocarbons, I used primary cultures of rat hepatocytes maintained on a laminin-rich extracellular matrix as an in vitro cellular system in which xenobiotic effects can be assessed in the absence of functional endocrine circuits. This system supports stable and quantifiable levels of CYP2C11 protein expression (Guzelian et al., 1988; Liddle et al., 1992; Chen et al., 1995b)

In this thesis I have compared the ability of the PAHs to suppress CYP2C11 immunoreactive protein in rat hepatocytes with their potency for competing for [³H]TCDD binding to the rat hepatic cytosolic AHR and for transforming the receptor to its DNA-binding form. Such analysis was undertaken to resolve the issue of whether the AHR mediates the suppressive effects of aromatic hydrocarbons on the expression of CYP2C11.

2.0 MATERIALS AND METHODS

2.1 Source of Chemicals

[³H]2,3,7,8-Tetrachlorodibenzo-*p*-dioxin ([³H]TCDD) (specific activity, 40 Ci/mmol; radiochemical purity, >97%), was purchased from Chemsyn Science Laboratories (Lenexa, KS). Non-radioactive 2,3,7,8-tetrachlorodibenzofuran (TCDF) was generously provided by Dr.S.Safe (Texas A&M University, College Station, TX). Dibenz[a,h]anthracene (DB[a,h]A; chemical purity, 97%) and benz[a]anthracene (BA; chemical purity, 99%) were purchased from Aldrich Chemical Company (Milwaukee, WI). 3-Methylcholanthrene (MC; chemical purity, 98%), dibenz[a,c]anthracene (DB[a,c]A; chemical purity, 97%), anthracene (ANTH; chemical purity,>99%) and 7,12-dimethylbenz[a]anthracene (DMBA; chemical purity, 95%) were purchased from Sigma Chemical Company (St. Louis, MO).

Specific chemicals used in the sucrose density gradient assay were obtained from the following sources: ultra-pure sucrose (Beckman Instruments Inc., Fullerton, CA), Norit A charcoal (BDH Inc, Toronto, ON) and dextran (Sigma). Bovine serum albumin and bovine liver catalase were labeled with [¹⁴C]formaldehyde (Du Pont Company, Wilmington, DE) as described by Denison et al. (1986b) and used as standard internal markers during velocity sedimentation. Ready-Value® cocktail was purchased from Beckman Instruments Inc.

Specific chemicals used in the gel retardation assay were obtained from the following sources: [³²P] ATP (specific activity, >5000 Ci/mmol, radiochemical purity >95%) was purchased from Amersham Life Science Inc. (Arlington Heights, IL). A pair of complementary DNA oligonucleotides containing the dioxin response element (DRE3 of the mouse *Cyp1a1* gene) was synthesized by General Synthesis and Diagnostics (Toronto, ON). T4 Polynucleotide kinase was purchased from Pharmacia Biotech

(Uppsala, Sweden). Poly [d(l-C)] was from Boehringer Mannheim (Mannheim, Germany). Sephadex G-50 was purchased from Pharmacia Biotech (Uppsala, Sweden).

Specific chemicals used for the primary rat hepatocyte cultures were obtained from the following sources: collagenase B from *Clostridium histolyticum* was purchased from Boehringer Mannheim; bovine pancreas insulin, EGTA, penicillin G and Trypan blue were purchased from Sigma. Waymouth's MB 752/1 medium was purchased from Life Technologies (Gaithersburg, MD). Matrigel® basement membrane matrix was purchased from Collaborative Biomedical Products (Bedford, MA).

Specific chemicals used for immunodetection of CYP isozymes were obtained from the following sources: a monoclonal antibody (MAb) to rat liver CYP1A1 was prepared in the laboratory of Dr. H.V. Gelboin (National Cancer Institute, Bethesda, MD) by the hybridoma method of Kohler and Milstein (1975) as described by Park *et al.* (1982). A MAb against rat liver CYP2C11 was purchased from Oxford Biomedical Research Inc. (Oxford, MI) (Reik *et al.*, 1987). Reagents for SDS-PAGE and blotting as well as premixed molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA). Secondary antibody (sheep anti-mouse Ig-horseradish peroxidase conjugate) and enhanced chemiluminescence detection reagents were purchased from Amersham.

2.2 Animals

Adult male Fischer 344 rats (9-10 weeks old, 190-225 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were fed Purina rodent lab chow (No.5001) and water *ad libitum*, and were housed under controlled conditions (2 per cage; 22°C; 12-hr of light / 12-hr of dark cycle, with lights on at 0700h) in the Division of Comparative Medicine, University of Toronto. Five days were allowed for acclimatization prior to experimentation. Animals were cared for in accordance with the principles of the

Canadian Council on Animal Care and all animal experimentation was approved by the University of Toronto Animal Care Committee.

2.3 Cytosol Preparation

Rats were killed by decapitation. The liver of each animal was perfused *in situ* via the inferior vena cava with ice-cold HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10 % glycerol, 1 mM DTT, pH 7.4). The liver was removed, weighed, minced using a pair of surgical scissors and homogenized in 4 volumes of ice-cold HEGD buffer using a Potter-Elvehjem apparatus. Hepatic cytosol was prepared by differential centrifugation as described (Denison et al., 1986b). The homogenate was centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant was centrifuged at 106,000 x g for 1 hr at 4°C. The final supernatant representing the cytosolic fraction, was frozen immediately in liquid nitrogen and stored in the -80°C freezer until use.

2.4 Protein Determination

The protein concentration of the cytosol was determined by the method of Bradford (1976). Bovine serum albumin standard (1 mg/ml in water) was used to prepare the following standard solutions: 0 µg (no BSA), 10 µg (10 µl BSA), 20 µg (20 µl BSA), 30 µg (30 µl BSA), 40 µg (40 µl BSA), 50 µg (50 µl BSA). Bio-Rad Protein Reagent Concentrate was diluted with 4 vol of H₂O and filtered prior to use. Aliquots of cytosol (typically 5 µl) and the BSA standard solutions were diluted with 2.5 ml of diluted Bio-Rad reagent. After 15 min, the absorbance at 595 nm (with 465 nm as background reference) was determined using the Beckman DU-65 spectrophotometer. Blank solutions that lacked only BSA were defined as displaying zero absorbance. The Quant II Quadratic Soft-Pac Module (Beckman) was used to fit the standard curve via non-linear regression.

2.5 Competitive AHR Radioligand Binding Studies

2.5.1 Labeling of Cytosol for Velocity Sedimentation Analysis

$[^3\text{H}]\text{TCDD}$ and TCDF were supplied as solutions in toluene and benzene, respectively. These solutions were evaporated at room temperature under nitrogen and $[^3\text{H}]\text{TCDD}$ and TCDF were redissolved in DMSO. DMSO served as the solvent for all nonhalogenated PAHs as well.

Rat liver cytosol (two independent pools of cytosol, each prepared from 3 rats) was diluted to a final protein concentration of 4-5 mg/ml. Cytosol (500 μl) was incubated at 4°C for 1 h with 8 nM $[^3\text{H}]\text{TCDD}$ in either the presence or absence of various concentrations of a non-radiolabeled PAH competitor. Radioligand and competitors were added to the cytosolic incubation in 5- μl volumes. Nonspecific binding was defined by incubating cytosol with 8 nM $[^3\text{H}]\text{TCDD}$ in the presence of 100-fold molar excess of nonradioactive TCDF. The concentration of $[^3\text{H}]\text{TCDD}$ was always confirmed by liquid scintillation counting. Following the incubation, the cytosol was added to a dextran-coated charcoal (DCC) pellet (at 1 mg charcoal/mg cytosolic protein) in order to remove the excess unbound $[^3\text{H}]\text{TCDD}$. The pellets were formed by centrifugation of the DCC suspension (0.5% stock solution of DCC was prepared by adding 5 mg dextran and 5 mg charcoal per 1 ml of HEG) at 12,000 x g for 5 min in a Beckman Microfuge 12 and removing the HEG supernatant. After addition of cytosol, the DCC pellet was resuspended on a vortex mixer and the cytosol-DCC mixture was incubated on ice for 15 min. The DCC was then separated from the cytosol by centrifugation of the cytosol-DCC mixture at 12,000 x g for 3 min. The cytosol was then carefully separated from the DCC pellet. The protein concentration of the cytosol following the DCC treatment was determined by method of Bradford (1976) as outlined in section 2.4.

2.5.2 Velocity Sedimentation on Sucrose Gradients

The linear sucrose gradients used in velocity sedimentation were made from a stock sucrose solution of 66% (1710 g sucrose in 1 litre of HEG). 10% and 30% sucrose solutions prepared in HEGD were layered and then mixed using a BioComp Gradient Master. Gradients were frozen at -20°C for storage. 300 µl Aliquots of the labeled and DCC-treated cytosol were loaded onto linear 10-30% sucrose gradients along with the internal sedimentation markers [¹⁴C]BSA and [¹⁴C]catalase. The gradients were centrifuged at 372,000 x g_{av} for 2h at 4°C using the vertical tube rotor technique described by Tsui and Okey (1981). Following the centrifugation, the gradients were fractionated with an ISCO Model 640 Density Gradient Fractionator (Instrumentation Specialties Co., Lincoln, NE) into 25 x 200 µl fractions. 2.5 ml of Ready-Value® scintillation cocktail (Beckman) was added to each fraction and the radioactivity from [³H] and [¹⁴C] was determined by liquid scintillation counting. Binding of [³H]TCDD to AHR was localized to the 9S region of the gradient. The IC₅₀ (concentration of nonradioactive competitor required to reduce specific binding of [³H]TCDD by 50%) for each competitor was determined by interpolation from a plot of specific binding versus log₁₀ [competitor]. Each cytosol preparation used in competitive radioligand binding studies was first characterized in a saturation binding experiment using a range of concentrations of [³H]TCDD (0.25 - 15 nM). The concentration of specific AHR sites (B_{max}) and the apparent equilibrium dissociation constant (K_d) for each pool of cytosol, were determined by nonlinear regression analysis of saturation plots using the program Enzfitter.

2.6 Transformation of AHR to a DNA-binding Form

2.6.1 Annealing of DRE3 Oligonucleotides

Equal amounts (50 µg, 10 µl at 5 µg/µl) of a pair of complementary synthetic DNA oligonucleotides (DRE3-A and DRE3-B) with the sequences 5'-GATCTGGC TCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3' , containing the AHR binding site from DRE3 of the mouse *Cyp1a1* gene (General Synthesis and Diagnostics, Toronto, ON), were annealed in the following steps : 2 min at 88°C, 15 min at 65°C, 15 min at 37°C, 15 min at room temperature, 15 min at 4°C - the annealing buffer was 67 mM Tris-HCl, pH 7.6 containing 13 mM MgCl₂, 6.7 mM DTT, 1.3 mM spermidine and 1.3 mM EDTA. The mixture was diluted with TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to yield a dsDNA stock solution at 30 ng/µl (~2 pmol/µl), which was stored at -80°C.

2.6.2 5'-Endlabeling of the dsDRE3 Oligonucleotide

The dsDRE was end-labeled at the 5' end using T4 polynucleotide kinase (T4 PNK) and [γ -³²P]ATP . The following labeling reaction was set up: 5 µl of dsDRE (10 pmol) + 5 µl 10X T4 PNK buffer (0.5 M Tris-HCl pH 7.6, 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) + 25.5 µl H₂O + 12.5 µl [γ -³²P]ATP + 2 µl T4 PNK (20 units). Following a 45 minute incubation at 37°C the reaction was stopped by adding 2 µl 0.5 M EDTA and 50 µl TE8 and placing on ice. In order to extract the labeled dsDRE, 100 µl of Phenol/Chloroform/Isoamyl alcohol (25:24:1) was added, the mixture was vortexed and centrifuged at 12,000 x g for 5 minutes in an Eppendorf 5415C microfuge. The upper aqueous phase was removed. To the remaining organic phase, 50 µl TE8 was added, and the sample was vortexed and centrifuged as before, and the upper aqueous phase was carefully separated and pooled with the previous aqueous phase.

300 μ l of ice-cold absolute ethanol was added to the pooled aqueous phase, and precipitation of dsDRE was allowed to proceed for at least 1 hr at -20°C. Following a 30 min centrifugation at 12,000 x g, the supernatant was discarded and the pellet was resuspended in 100 μ l TE8. The sample was purified using a Sephadex G-50 spin column. In order to make a stock solution of the labeled dsDRE (100 ng/ml or 6.6 pmol/ml) HEGD was added to the eluate obtained to make the total volume up to 1.5 ml. 5 ml of Ready-Value® scintillation cocktail was added to a 5 μ l aliquot of the labeled dsDRE stock solution and the radioactivity [³²P] was determined by liquid scintillation counting. The specific activity of the oligonucleotide (cpm/fmol) was calculated assuming 100% recovery. Probe was stored in a shielded box at 4°C.

2.6.3 Preparation of Nondenaturing Polyacrylamide Gels

Protein-DNA complexes were analyzed by non-denaturing electrophoresis on a 1.5 mm 4% polyacrylamide gel. For each gel required the following solution was prepared: 34 ml H₂O + 4.5 ml 10 x TAE (0.4 M Tris-acetate, 0.01 M EDTA, pH 8.0) + 6.0 ml acrylamide/bis (30% T, 2.7% C, where %T represents the total percentage [w/v] of acrylamide monomer and N,N'-methylene bisacrylamide [BIS] crosslinker, and %C represents the amount of BIS crosslinker expressed as a percentage of the sum of acrylamide monomer and BIS) + 0.45 ml 10% ammonium persulfate + 22.5 μ l TEMED (N,N,N',N',-tetramethylethylenediamine). The final gel composition was as follows: acrylamide (4% T, 2.7%C), 40 mM Tris-acetate pH 8.0, 1mM EDTA, 0.1% ammonium persulfate, 0.05% TEMED. The gel sandwich (16 x 20 cm gels) was placed in a Gibco BRL V-16-2 Vertical Gel Electrophoresis System. The gels were "pre-electrophoresed" at 130 V for 2 h, with 1X TAE as the running buffer. A Gibco/BRL Model 250 power supply was used. The running buffer was continuously recirculated between the upper and lower reservoirs using a Gilson Minipuls pump.

2.6.4 Transformation of Cytosolic AHR

The gel retardation assay which measures the culmination of both AHR transformation and DRE3 binding, was performed essentially as described by Denison & Yao (1991). Rat hepatic cytosol (three independent preparations, each from an individual rat) prepared in HEGD with a protein concentration of ~ 20 mg/ml, was incubated with the various concentrations of [^3H]TCDD or nonradioactive PAH ligands for 2 h at room temperature. These preparations contained 85 to 108 fmol/mg protein specific [^3H]TCDD binding sites as detected with 15 nM [^3H]TCDD by sucrose gradient analysis. Vehicle controls (DMSO in the absence of ligand) were always included and compounds were added to the cytosolic incubation (25 μl) in 1- μl volumes. Poly[d(I-C)] (Boehringer Mannheim) was dissolved in HEGD to give a stock solution of 125 $\mu\text{g/ml}$. From the transformed cytosol a volume that contained 180 μg of protein was incubated with 0.31 μg of Poly[d(I-C)] at room temperature for 15 min. 100,000 cpm (0.25-0.8 ng) of the [^{32}P]DRE3 probe was added (the volume varied depending on the specific activity of the probe on the day of use) and the sample was incubated for a further 15 min at room temperature. The binding reaction was carried out in HEGD buffer containing 80 mM NaCl. Finally, 5 μl of 6 x sample loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll) was added. Preliminary experiments were performed to determine the incubation time, temperature, poly[d(I-C)] concentration, NaCl concentration and amount of cytosolic protein and/or AHR that yielded maximal TCDD-induced transformation and DRE3-binding of the F344 rat hepatic AHR.

2.6.5 Polyacrylamide Gel Electrophoresis and Autoradiography

Equal amounts of cytosolic protein (108 μg of protein) from each transformed sample were loaded and protein-DNA complexes were analyzed by electrophoresis on

nondenaturing, pre-electrophoresed polyacrylamide gels. The gel was run at 50-60 V for 15 min to allow samples to enter the gel and then at 130 V for ~2 hr. The gel was blotted onto a precut piece of filter paper and dried under vacuum on a Slab Gel Dryer Model SE 1160 (Hoefler Scientific Instruments, San Francisco, CA) for ~2 h at 80°C. The corners of the dried gel were marked for orientation with fluorescent paint. The dried gel and a sheet of Kodak XAR5 film were then placed in a Fisher Biotech X-ray cassette along with two Fisher Biotech L-Plus intensifying screens. The cassette was kept in the -80°C freezer during the exposure time (~4 hr). Films were developed using the x-ray development equipment of the Department of Molecular and Medical Genetics, University of Toronto.

2.6.6 Quantitative analysis

The fluorescent paint markings were used to align the resulting autoradiograph with the dried gel. The ligand-inducible radioactive band of interest was cut out and placed into a scintillation mini-vial. Ready-Value® cocktail (5 ml) was added to each vial and the radioactivity [^{32}P] was determined by liquid scintillation counting. The difference in radioactivity between ligand- and DMSO-treated samples was used to calculate the amount of ligand-inducible DRE3 binding. Relative quantitation was confirmed by Phosphorimager® analysis (Molecular Dynamics, Sunnyvale, CA). By assuming that one molecule of [^{32}P]DRE binds one molecule of AHR, it was possible to express DRE3 binding in units of fmol/mg protein. Transformation efficiency expresses DRE3 binding as a percentage of AHR B_{max} determined by sucrose density gradient saturation analysis. Various concentrations of each ligand were used to transform AHR in three independent rat liver cytosol preparations. The EC₅₀ (concentration of ligand required to cause 50% of maximum transformation) for each ligand was determined by interpolation from a plot of DRE3 binding versus log₁₀ [ligand].

2.7 Modulation of CYP2C11 Expression by PAHs in Primary Cultures of Rat Hepatocytes

2.7.1 Hepatocyte Isolation and Cell Culture

Primary cultures of rat hepatocytes were established using methods described by Liddle *et al.* (1992) and Li *et al.* (1991). A single-pass (noncirculating) perfusion system was set up. Stringent sterile techniques were applied throughout the cell isolation procedure. Rats were anesthetized by an i.p. injection of sodium pentobarbital (Somnotol®) at 40 mg/kg. Anesthesia was maintained throughout the surgery using a beaker lined with halothane-soaked gauze placed in front of the rat's nose. The abdominal area was doused with 70% ethanol. The skin was cut from 1 cm above the pubis to the rib cage. A low midline incision in the abdominal wall was made, and broad cuts to the right and left were made to open the abdominal cavity. The viscera were moved to the right exposing the portal vein. A catheter was inserted into the portal vein and secured in place by a ligature. Perfusion with warm (37°C), oxygenated (95% O₂, 5% CO₂), calcium-free (containing EGTA) Krebs (10 mM dextrose, 25 mM NaHCO₃, 5.6 mM KCl, 120 mM NaCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 0.5 mM EGTA) was started immediately using a variable flow mini-pump. The thoracic cavity was opened, the major vessels were cut and the liver was perfused, with 200 ml of Krebs at a flow rate of 20 ml/min, until well blanched. Perfusion was switched to warm (37°C), oxygenated collagenase/culture medium (35 mg of collagenase in 250 ml of Waymouth's medium) and continued at a rate of 20 ml/min (~200 ml) until the liver appeared well-digested. The liver was dissected out, placed in a Falcon tube containing ~40 ml collagenase/culture medium. The liver was minced finely using sterile scissors and filtered through a sterile, stainless steel cell strainer (Sigma). The cells were centrifuged at 500 rpm for 2 minutes. The supernatant was aspirated and the cell pellet was washed twice with the culture medium. The final cell pellet was gently resuspended

in 15 ml of the culture medium. The cell count and viability were assessed using the Trypan Blue Method.

Each 60 mm cell culture plate was coated with 200 μ l of Matrigel® (diluted to 7.5 mg/ml with culture medium). The cells were plated in 3 ml of culture medium at a density of $\sim 3.5 \times 10^6$ cells/60 mm dish. The medium was replaced with 3 ml of fresh medium 4 hrs after plating and every 48 hrs thereafter. The culture medium was Waymouth's medium (752/1) containing insulin (0.15 μ M) and penicillin G (100 units/ml). Cultures were maintained in an incubator at 37°C in an atmosphere containing 5% CO₂. Hepatocytes were cultured for five days before beginning treatment to allow recovery of stable CYP2C11 expression (Liddle et al., 1992; Chen et al., 1995b). Hepatocytes were treated with the following concentrations of AHR ligands: [³H]TCDD (10 nM), MC (1 μ M), or ANTH, BA, DB[a,c]A, DB[a,h]A, DMBA (all at 10 μ M). These concentrations have been shown to either cause maximal CYP1A1 induction in mouse hepatoma cells (Riddick et al., 1994b) or to cause significant CYP1A1 induction in rat hepatoma cells (Piskorska-Pliszczyńska et al., 1986) without overt signs of toxicity. Control cells were treated with the vehicle DMSO (0.1% in culture medium). Radiolabeled TCDD was used to confirm the low concentration used.

2.7.2 Microsome Preparation from the Hepatocyte Cultures

Forty-eight hours following ligand treatment, hepatocytes were harvested by scraping as described (Liddle et al., 1992). Medium was aspirated from each culture dish and replaced with 2 ml of ice-cold phosphate buffered saline (PBS). Cells and matrigel were scraped from the plates using a Falcon cell scraper and transferred to a 50 ml Falcon tube, then allowed to stand on ice for ~ 45 min to dissolve the matrigel. Cells were then collected by centrifugation at 750 x g for 5 min. The supernatant was aspirated and the cells were washed twice with cold PBS. The final cell pellet was resuspended in 200 μ l of HED (25 mM HEPES, 1.5 mM EDTA, 1mM DTT at pH 7.4)

and allowed to swell for 15 min on ice. Hepatocytes pooled from ten culture plates were homogenized using a Dounce Homogenizer (Bellco, Vineland, NJ.). An equal volume of HED2G (25 mM HEPES, 1.5 mM EDTA, 20% glycerol (v/v), 1mM DTT at pH 7.4) was added to the cell homogenate. The homogenates were centrifuged at 9,000 x g for 20 min at 4°C and the resulting supernatant was centrifuged at 106,000 x g for 1 hr at 4°C. The microsomal pellet was resuspended in 200 µl of microsome storage buffer (10 mM Tris, 20% glycerol, 1 mM EDTA, at pH 7.4), frozen in liquid nitrogen and stored at -80°C. Microsomal protein concentration was determined by the method of Bradford (1976), as described in section 2.4.

2.7.3 Immunodetection of CYP1A1 and CYP2C11

Microsomal samples were prepared as described above. Microsomal protein samples were diluted 2-fold with 2 x sample buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.002% bromophenol blue) followed by heating in boiling water for 4 min. Microsomal proteins were resolved by SDS-PAGE by the method of Laemmli (1970). Equal amounts of microsomal protein (varied from 1 to 8 µg depending on experiment) were loaded onto 1 mm thick, polyacrylamide gels. Electrophoresis was carried out in a Bio-Rad Mini-Protean II gel apparatus attached to a Bio-Rad Model 1000/500 power supply. Discontinuous polyacrylamide gels (100 x 75 x 1 mm) of the following composition were employed: stacking gel - 4% T, 2.7% C, 0.125 M Tris-HCl pH6.8, 0.1% SDS, 0.05% ammonium persulfate, 0.1% TEMED, resolving gel - 10% T, 2.7% C, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate, 0.05% TEMED. The electrophoresis tank running buffer consisted of 0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS. The gels were run at a constant voltage of 200 V for ~ 45 min. Pre-stained low molecular mass standards were separated on each gel. A standard kit (Bio-Rad) containing phosphorylase B (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and

lysozyme (18.5 kDa) was employed. Proteins on the gel were transferred to nitrocellulose (Hybond-ECL[®], Amersham) electrophoretically as described by Towbin *et al.* (1979). The transfer was carried out in a Bio-Rad Mini Trans-Blot Electrophoretic Transfer cell using the Bio-Rad Model 200/2.0 Constant Voltage Power supply at 100 V for one hour with a prefrozen Bio-Ice cooling unit. Transfer buffer consisted of 0.025 M Tris-HCl (pH 8.3), 0.192 M glycine, and 20% (v/v) methanol. Efficiency of transfer was assessed using Ponceau S stain. Non-specific binding sites on the nitrocellulose membrane were blocked by shaking in 20 mM Tris/137 mM NaCl/0.1% Tween-20, pH 7.6 (TNT) containing 5% (w/v) skim milk powder (Blotto) overnight (~18 hrs) at 4°C. Nitrocellulose sheets were washed with TNT 4 x 10 min at room temperature. Primary antibody was diluted in Blotto and incubated for 1 hr at room temperature. MAb 1-31-2 (anti-CYP1A1) was used at a 1:1,000 dilution and MAb PM30 (anti-CYP2C11) was used at a 1:500 dilution. Nitrocellulose sheets were washed 4 X 10 min in TNT and then exposed to sheep anti-mouse Ig-horseradish peroxidase (Amersham) diluted (1:1,000) in Blotto at room temperature for 1 hr. The nitrocellulose sheets were washed in TNT as described above. Enhanced chemiluminescence was carried out by mixing equal volumes of ECL detection reagents 1 and 2, and addition of ECL developing solution to nitrocellulose sheets for ~ 1 min. Nitrocellulose sheets were drained, wrapped in Saranwrap and placed in Fisher Biotech autoradiography cassettes (no intensifying screens were used) with Hyperfilm ECL (Amersham) for the appropriate time period. The following approximate exposure times were used: CYP1A1 (15 seconds) and CYP2C11 (10 min). The films were then developed using the x-ray development equipment of the Department of Molecular and Medical Genetics, University of Toronto. For relative quantitative analysis of CYP2C11, films were scanned using a LKB 2222-020 UltroScan XL Laser Densitometer (Pharmacia LKB Biotechnology, LKB Producter AB, Bomma, Sweden). CYP1A1 immunoreactive protein was monitored as a positive control response, but densitometric quantification was not possible because the signals obtained were often beyond the linear response range.

2.8 Statistical analysis

Where appropriate, data are presented as means $\pm$ standard deviation. Statistical significance of the linear correlation data was determined by the Pearson correlation test. In all cases, a result was considered statistically significant if $P \leq 0.05$.

3.0 RESULTS

The overall objective of this study was to examine the role of the AHR in the suppression of CYP2C11 caused by a series of structurally-related PAHs in primary cultures of rat hepatocytes, a cellular system removed from the complex interplay of endocrine pathways responsible for hepatic CYP2C11 expression. I employed a structure-activity relationship approach which required a determination of the binding affinity of each PAH for the rat hepatic AHR and the potency of each PAH for transformation of the AHR to its DNA-binding form.

3.1 Characterization of Hepatic AHR of F344 Male Rat

Two pools of hepatic cytosol prepared from the liver of male Fischer 344 rats were used for the AHR competitive binding assays. The apparent equilibrium dissociation constant (K_d) and the binding capacity (B_{max}) of the hepatic AHR were determined for these two separate cytosolic preparations using a range of concentrations of [3H]TCDD by the method of sucrose density gradient analysis. Sucrose gradient analysis demonstrated that [3H]TCDD bound specifically to the ~9 S form of the AHR in rat hepatic cytosol (Fig. 7). Specificity of the binding was confirmed by the observation that the radioactive peak in the ~9 S region (fractions 10-15) of the sucrose gradients was eliminated by incubation in the presence of a 100-fold molar excess of non-radioactive TCDF.

The K_d and the B_{max} were determined by nonlinear regression analysis of saturation plots (Fig. 8) and the results are summarized in Table 1.

F344 HEPATIC CYTOSOL	K_d (nM)	B_{max} (fmol/mg)
POOL # 1	4.2	43.1
POOL # 2	2.0	30.1

TABLE 1. - [3H] TCDD Binding Characteristics of Fischer 344 Rat Hepatic AHR.

Figure 7. Binding of $[^3\text{H}]\text{TCDD}$ to rat liver cytosolic AHR. Cytosol from rat liver (~5 mg protein/ml) prepared in HEGD buffer was incubated with 15 nM $[^3\text{H}]\text{TCDD}$ at 4°C for 1 hr in the absence or presence of a 100-fold molar excess of nonradioactive TCDF. Samples were analyzed by velocity sedimentation on sucrose gradients. $[^{14}\text{C}]$ Formaldehyde-labeled bovine serum albumin (BSA; 4.4 S) and catalase (Cat; 11.3 S) were added to gradients as internal sedimentation markers (arrows).

Figure 8. Saturation plots for two pools of cytosol (a) and (b). Cytosol preparations used in competitive radioligand binding studies were first characterized in a saturation binding experiment using a range of concentrations of [3 H]TCDD (0.25-15 nM): Bmax and Kd for each pool of cytosol were determined by nonlinear regression analysis of the saturation plots.

a)

b)

3.2 Relative Binding Affinity of PAHs for the Rat Hepatic AHR

In order to determine whether the ability of MC and anthracene derivative compounds to suppress CYP2C11 expression is related to their AHR binding affinity, we determined the potency of each ligand for competing with [^3H]TCDD for binding to the rat hepatic cytosolic AHR. For example, Fig. 9 represents the competition by various concentrations of DB[a,c]A with [^3H]TCDD. The competition curves for all of the compounds are summarized in Fig. 10. The IC₅₀ value for each compound was determined using two independent pools of cytosol. The values are summarized in Table 2.

TABLE 2. - IC₅₀ Values for Competition for [^3H]TCDD Binding to the Rat Hepatic AHR by PAHs.

	POOL #1 (nM)	POOL #2 (nM)	MEAN (nM)
DB[a,h]A	5.0	8.1	6.6
MC	12.7	9.3	11
DB[a,c]A	15.7	9.3	12
BA	85.7	20.4	53
DMBA	140.0	201	170
ANTH	>100000	>100000	>100000

The following rank order of affinity was observed, from highest to lowest affinity: DB[a,h]A, MC, DB[a,c]A, BA, DMBA, ANTH.

3.3 AHR Transformation Potency

In order to determine whether the ability of these compounds to suppress CYP2C11 expression was related to their AHR transformation potency, we examined

Figure 9. Sucrose density gradient analysis representing the competitive binding of DB[a,c]A and [^3H]TCDD to rat liver cytosolic AHR. Cytosol was incubated with 8 nM [^3H]TCDD in the absence (a) or presence of various concentrations of nonradioactive DB[a,c]A (b-f). The specific binding in the 9 S peak was determined by sucrose density gradient analysis. Non-specific binding was determined by incubating cytosol with 8 nM [^3H]TCDD in the presence of 100-fold molar excess of TCDF (a-f).

Figure 10. Competition by nonradioactive PAHs for specific binding of 8 nM $[^3\text{H}]\text{TCDD}$ to rat liver cytosolic AH receptor. Cytosol from rat liver (~ 5 mg protein/ml) was incubated with 8 nM $[^3\text{H}]\text{TCDD}$ at 4°C for 1 hr in the absence or presence of concentrations of nonradioactive PAHs (MC, ANTH, DB[a,c]A, DB[a,h]A, DMBA, BA) indicated on the abscissa. Specific binding in the 9 S peak was determined for each sample by sucrose density gradient analysis. Data shown represent results for a single cytosol preparation, and IC_{50} values shown represent the mean of determinations from two independent cytosol preparations. In the example shown, the mean concentration of AH receptor sites detected with 8 nM $[^3\text{H}]\text{TCDD}$ was 39 fmol/mg protein.

ligand-induced transformation of the rat hepatic cytosolic AHR using a gel retardation assay in which binding of transformed AHR to a ^{32}P -labeled double-stranded DRE oligonucleotide is measured. As mentioned before, the AHR requires heterodimerization with ARNT for DNA binding and ARNT is known to be a nuclear protein; however, subcellular fractionation results in the appearance of ARNT in the cytosolic fraction (Pollenz et al., 1994), and thus the hepatic cytosol contains all the ingredients required for ligand-induced transformation of the AHR to its DNA-binding form. In order to enhance the signal to noise ratio we designed a series of experiments to optimize the conditions used in the gel retardation assay. These included optimization of the time of transformation, incubation temperature for the transformation reaction and the amounts of Poly [d (I - C)] and NaCl required for DNA binding by the AHR-ARNT complex. In all of these preliminary experiments, rat liver cytosolic AHR was transformed by incubation in the presence of 20 nM TCDD.

3.3.1 Optimization of Incubation Temperature

The gel retardation assay measures the culmination of both AHR transformation and DRE3 binding. Hence, the transformation of the AHR into its DNA-binding form is an essential first step for good quality gel retardation assays. Incubation temperature is an important determinant for this transformation process. I conducted an experiment in order to determine the incubation temperature that would maximize the transformation of the AHR for the gel retardation assay. Transformation of the AHR was carried out at the following incubation temperatures : 0°C (on ice), 22°C (room temp.), 30°C and 37°C. Fig. 11 shows the relationship between temperature and AHR transformation (TCDD-treated sample minus DMSO control). Based on these results, I decided to carry out the transformation reaction at room temperature. Although incubation at 30 °C resulted in slightly greater transformation than 22 °C, the simplicity of carrying out the

Figure 11. The relationship between incubation temperature and AHR transformation. The ordinate represents the counts per minute (cpm) obtained by subtracting the DMSO control cpm from the TCDD-treated sample cpm. Data shown represent results for a single cytosol preparation, and the experiment was repeated with an independent cytosol preparation with similar results.

Figure 12. The relationship between length of the transformation incubation period and AHR transformation. The ordinate represents the counts per minute (cpm) obtained by subtracting the DMSO control cpm from the TCDD-treated sample cpm. Data shown represent results for a single cytosol preparation, and the experiment was repeated with an independent cytosol preparation with similar results.

incubation at room temperature out-weighs the slight gain in transformation at 30 °C.

3.3.2 Optimization of the Length of Incubation

There are two factors working against each other: the degradation of the receptor as it is maintained at room temperature vs. the optimal length of time that allows for maximal transformation of the AHR to its DNA-binding form. I tested the following incubation times : 0, 15 min, 30 min, 1hr, 2 hr, 3 hr, 4hr, 5 hr, 6 hr. The result is summarized in Fig. 12 . Increasing the incubation period enhanced the transformation of the AHR. This effect was observed for the incubation periods of up to 2 hr. Incubation periods longer than 2 hr did not produce enhanced transformation of the AHR. As a result, a standard transformation period of 2 hr was employed.

3.3.3 Poly [d (I-C)] Titration

Poly [d(I-C)] is a non-specific sequence of DNA which is added to the final reaction mixture prior to the addition of the DRE3. The role of Poly[d(I-C)] is to bind to various DNA-binding proteins, hence reducing the chance of non-specific interaction of other DNA-binding proteins with the DRE3 sequence. However, addition of Poly[d(I-C)] in excess would result in the undesirable effect of decreased AHR-DRE3 interaction. Hence, we designed an experiment in order to determine the optimal concentration of Poly[d(I-C)] that would reduce the nonspecific binding of other DNA-binding proteins to the DRE3 sequence while maintaining the highest AHR-DRE3 binding. Based on the results, presented in Fig. 13, we determined that 1.0 µl of Poly[d(I-C)] (125 ng) generated the optimal TCDD-induced signal. Since 80 ug of cytosolic protein was present in the final reaction mixture, 1.7 ng of Poly[d(I-C)]/ µg of protein was used in all the subsequent experiments.

Figure 13. The relationship between amount of poly [d(I-C)] in the final reaction mixture and AHR-DRE3 binding. The ordinate represents the counts per minute (cpm) obtained in the DMSO control and TCDD-treated sample. Data shown represent results for a single cytosol preparation, and the experiment was repeated with an independent cytosol preparation with similar results.

Figure 14. The relationship between NaCl concentration in the final reaction mixture and AHR-DRE3 binding. The ordinate represents the counts per minute (cpm) obtained by subtracting the DMSO control cpm from the TCDD-treated sample cpm. Data shown represent results for a single cytosol preparation, and the experiment was repeated with an independent cytosol preparation with similar results.

3.3.4 Optimization of NaCl Concentration

The interaction of DNA-binding proteins with DNA is governed by ionic interactions. Hence, the ionic strength of the final reaction mixture is an important factor that needs to be adjusted in order to obtain the optimal AHR-DRE3 interaction. Fig. 14 demonstrates that a concentration of 80 mM NaCl in the final reaction mixture produces the optimal AHR-DRE3 binding.

3.3.5 Concentration-Response for each Ligand

The potency of each ligand for activation of the rat hepatic AHR to its DNA-binding form was assessed using gel retardation assays. It has been shown that *in vitro* transformation of the cytosolic AHR constitutes an effective quantitative method for comparison of AHR activation by different ligands (Cuthill et al., 1991; Denison and Yao, 1991; Santostefano et al., 1992; Riddick et al., 1994b). Incubation of rat liver cytosol with a ^{32}P -labeled DRE3 probe results in the formation of at least two prominent protein-DRE3 complexes, one being ligand-inducible (indicated by an arrow in Fig. 15) and the other(s) being noninducible protein-DRE3 complexes (i.e. present in both DMSO- and ligand-treated cytosol). Previous work (Denison and Yao, 1991) has demonstrated that the ligand-inducible band represents the interaction of the transformed rat liver cytosolic AHR with the DRE3 sequence. Fig. 15 illustrates the ability of a maximally-effective concentration of each ligand to activate the hepatic AHR to its DNA-binding form.

Concentration-response experiments were performed for each of the ligands with three independent cytosol preparations. The results of a representative concentration-response experiment are shown in Fig. 16, with the effects of all ligands being expressed relative to the maximum response produced by the reference compound TCDD. MC and all of the anthracene derivatives were able to activate the AHR to

Figure 15. Transformation of rat liver cytosolic AHR to its DRE3-binding form By $[^3\text{H}]$ TCDD and nonhalogenated PAHs. Cytosol from rat liver (~ 20 mg protein/ml) was incubated at room temperature for 2 hr with DMSO or one of the following ligands: 20 nM $[^3\text{H}]$ TCDD, 1 μM DB[a,h]A, 1 μM MC, 1 μM DB[a,c]A, 10 μM BA, 10 μM DMBA, 750 μM ANTH. Following incubation, cytosol was mixed with ^{32}P -labeled DRE3 oligonucleotide, and the formation of protein-DNA complexes was analyzed by gel retardation assay. The arrow indicates the ligand-inducible interaction of the AHR with DRE3, and F indicates the free DRE3 probe.

Figure 16. Concentration-dependent transformation of rat liver cytosolic AH receptor to its DRE3-binding form by [³H]TCDD and nonhalogenated PAHs. Cytosol from rat liver (~20 mg protein/ml) was incubated at room temperature for 2 hr with DMSO or the concentrations of [³H]TCDD or nonradioactive PAHs indicated on the abscissa, and samples were analyzed by gel retardation. The ligand-inducible complexes were excised from the dried gel, and the amount of radioactivity was determined by liquid scintillation spectrometry. DRE3 binding is expressed as a percentage of the maximum response produced by TCDD. Data shown represent results for a single cytosol preparation, and EC₅₀ values shown represent the means (± standard deviation) of determinations from three independent cytosol preparations. The EC₅₀ value for TCDD represents the mean of determinations from two independent cytosol preparations. In the example shown, the maximum TCDD-inducible DRE3 binding was 18 fmol/mg protein, representing a transformation of 21% of the total AH receptor content of 85 fmol/mg protein for this cytosolic preparation as detected by sucrose density gradient analysis with 15 nM [³H]TCDD.

varying degrees. Table 3 summarizes the potency (EC₅₀) of each ligand for transforming the AHR to its DNA-binding form.

TABLE 3.- EC₅₀ Values for Transformation of The Rat Hepatic AHR to its DNA-Binding Form by PAHs.

	CYTOSOL#1 (M)	CYTOSOL#2 (M)	CYTOSOL#3 (M)	MEAN (M)
DB[a,h]A	1.0 x 10 ⁻⁹	6.0 x 10 ⁻⁹	2.0 x 10 ⁻⁹	3.0 x 10 ⁻⁹
MC	3.0 x 10 ⁻¹⁰	2.0 x 10 ⁻⁹	2.0 x 10 ⁻¹⁰	8.3 x 10 ⁻¹⁰
DB[a,c]A	1.0 x 10 ⁻⁸	1.0 x 10 ⁻⁸	2.0 x 10 ⁻⁸	1.3 x 10 ⁻⁸
BA	2.0 x 10 ⁻⁶	2.0 x 10 ⁻⁶	1.0 x 10 ⁻⁶	1.7 x 10 ⁻⁶
DMBA	1.0 x 10 ⁻⁶	3.0 x 10 ⁻⁶	8.0 x 10 ⁻⁷	1.6 x 10 ⁻⁶
ANTH	5.0 x 10 ⁻⁴	6.0 x 10 ⁻⁴	6.0 x 10 ⁻⁴	5.7 x 10 ⁻⁴

The following rank order of potency (from highest to lowest potency) was observed based on the mean EC₅₀ values: MC, DB[a,h]A, DB[a,c]A, DMBA, BA, ANTH.

3.4 CYP2C11 Suppression in Primary Hepatocyte Cultures

In vivo administration of aromatic hydrocarbons to adult male rats causes down-regulation of CYP2C11 expression (Guengerich et al., 1982; Yeowell et al., 1987; Shimada et al., 1989; Jones and Riddick, 1996). At least a portion of this response may be due to disruption of endocrine pathways that are responsible for the maintenance of hepatic CYP2C11 expression *in vivo* (Shimada et al., 1989; Yeowell et al., 1989). In order to examine the effects of PAHs on the expression of CYP2C11 in an experimental system free of endocrine influences, we cultured primary rat hepatocytes on a laminin-rich extracellular matrix (matrigel) that assists in the maintenance of CYP2C11 expression (Guzelian et al., 1988; Liddle et al., 1992). We confirmed the previous work (Liddle et al., 1992) which indicated that between day 5 and day 7 of culture, CYP2C11

Figure 17. Immunoblot illustrating the level of microsomal CYP2C11 immunoreactive protein in primary cultures of rat hepatocytes maintained on matrigel for various time periods. -10 μ g of microsomal protein was loaded for each sample and the blot was probed using monoclonal antibody PM30 directed against CYP2C11. The designation "0 days in culture" represents freshly isolated rat hepatocytes.

Figure 18. The level of microsomal CYP2C11 immunoreactivity in the primary cultures of rat hepatocytes as a function of days in culture. These data were derived from a densitometric analysis of the immunoblot shown in Fig. 17. CYP2C11 immunoreactivity is expressed as a percentage of the maximum immunoreactivity observed in freshly isolated hepatocytes.

expression is maintained at a relatively stable level of 10 to 25 % of the level observed in freshly isolated hepatocytes. The immunoblot and the time-course plot derived from densitometer scanning of the blot are shown in Fig. 17 and Fig. 18, respectively. This level of expression is comparable to that observed *in vivo* in the absence of growth hormone pulses (Liddle et al., 1992). Thus, my study was designed to examine the ability of PAHs to act directly on hepatocytes to suppress CYP2C11 from a "basal" level of expression that represents 10 to 25% of the *in vivo* expression level. Preliminary experiments with TCDD, MC, and DB[a,c]A showed that CYP2C11 suppression was much greater after 48 hr than after 24 hr of exposure.

Immunoblot analysis of hepatocyte microsomal fractions prepared on day 7 indicated that treatment of cultures with PAHs on day 5 resulted in marked suppression of CYP2C11 immunoreactive protein. A representative immunoblot showing the response to TCDD, MC and DB[a,c]A is shown in Fig. 19a and the results for all PAHs are summarized in Fig. 19b. Reference compounds, TCDD (10 nM) and MC (1 μ M), suppressed CYP2C11 protein expression by 66% and 77%, respectively. All of the anthracene derivatives (10 μ M) caused some degree of CYP2C11 suppression with the following rank order of activity: DB[a,c]A (74% suppression); DB[a,h]A (65%); BA (58%); DMBA (40%); ANTH (37%).

Induction of CYP1A1 immunoreactive protein by the PAHs was monitored as a positive control response (Fig. 19a). All PAHs, including ANTH, caused detectable CYP1A1 induction. In general, there appeared to be a direct relationship between CYP2C11 suppression and CYP1A1 induction, although it was not possible to obtain reliable quantitative estimates of CYP1A1 induction, because the chemiluminescent signals were often beyond the linear response range.

Figure 19. Effect of aromatic hydrocarbons on the expression of microsomal CYP2C11 immunoreactive protein in primary cultures of rat hepatocytes. (a) A representative immunoblot analysis of microsomal protein (8 μ g) using monoclonal antibody 1-31-2 directed against CYP1A1 [top] and monoclonal antibody PM30 directed against CYP2C11 [bottom]. Rat hepatocytes were treated on day 5 of culture with 0.1% DMSO (Control), 10 nM [3 H]TCDD, 1 μ M MC, or 10 μ M DB[a,c]A and microsomes were prepared 48 hr after treatment.

(b) Summary of the quantitative analysis of CYP2C11 immunoreactivity by laser densitometry. Rat hepatocytes were treated on day 5 of culture with 10 nM [^3H]TCDD, 1 μM MC, 10 μM ANTH, 10 μM BA, 10 μM DB[a,c]A, 10 μM DB[a,h]A, or 10 μM DMBA and microsomes were prepared 48 hr after treatment. Results for ligand-treated hepatocytes are expressed as a percentage of the CYP2C11 expression observed for DMSO-treated hepatocytes in each individual experiment. Data with error bars represent the means ($\pm$ standard deviation) of determinations from three to five independent experiments. Data without error bars represent the means of determinations from two independent experiments.

(b)

3.5 Correlation Analysis

The results of the correlation analysis are presented in Fig. 20. This analysis included MC and all anthracene derivatives as members of the nonhalogenated class of aromatic hydrocarbons. There was a direct linear correlation (Fig. 20a) between the AHR binding affinity and the AHR transformation potency for the six ligands with a correlation coefficient of $r=0.92$ ($p < 0.01$). In addition, both the AHR binding affinity (Fig. 20b) and AHR transformation potency (Fig. 20c) correlated with the ability of the six ligands to cause suppression of CYP2C11 immunoreactive protein in primary cultures of rat hepatocytes. These results suggest that the ability of PAHs to bind to and activate the cytosolic AHR are important determinants of their ability to suppress CYP2C11 expression in cultured hepatocytes.

Figure 20. Correlation between (a) AH receptor binding affinity and AH receptor transformation potency, (b) AH receptor binding affinity and CYP2C11 suppression, and (c) AH receptor transformation potency and CYP2C11 suppression, for the six nonhalogenated PAHs examined in this study (MC, ANTH, BA, DB[a,c]A, DB[a,h]A, DMBA). AH receptor binding affinity is expressed as the mean of the $-\log_{10}IC_{50}$ (M), as shown in Figure 10. AH receptor transformation potency is expressed as the mean of the $-\log_{10}EC_{50}$ (M), as shown in Figure 16. CYP2C11 suppression is expressed as the mean percentage decrease relative to the corresponding DMSO control, as shown in Figure 19b. An estimated AH receptor binding IC_{50} value for ANTH of 10^{-4} M has been used for the purpose of these correlations. The equations of the lines of best fit were generated by least-squares linear regression analysis. Statistical significance of the correlation was determined by the Pearson correlation test.

(a)

(b)

(c)

4.0 DISCUSSION

In the present study, the role of the AHR in the suppression of CYP2C11 in primary hepatocyte cultures was studied. In essence, my study is a structure-activity relationship analysis. The affinity of a series of structurally-related anthracene derivatives for the AHR and their potency for transformation of the AHR to its DNA-binding form was correlated with the ability of the compounds to suppress CYP2C11 immunoreactive protein in cultured hepatocytes. These results suggest that the AHR plays a role in the down-regulation of CYP2C11 caused by PAHs.

The role of the AHR in mediating the induction of CYP1A1 by aromatic hydrocarbons is well established (Birnbaum, 1994; Okey et al., 1994b; Hankinson, 1995). The AHR appears to regulate the expression of several other genes, including those coding for drug-metabolizing enzymes and proteins that play key roles in cell growth and differentiation (Sutter and Greenlee, 1992; Okey et al., 1994a). Whereas the mechanism by which the AHR mediates the transcriptional induction of genes such as *CYP1A1* is at least partially understood, the mechanisms by which aromatic hydrocarbons act to down-regulate the expression of specific genes are not well characterized. CYP2C11 is down-regulated following *in vivo* administration of aromatic hydrocarbons to adult male rats (Guengerich et al., 1982; Waxman et al., 1985; Emi and Omura, 1988; Shimada et al., 1989; Jones and Riddick, 1996); however, the role of the AHR in this process has remained controversial. My structure-activity relationship study implicates the AHR in the suppression of CYP2C11 caused by a series of PAHs in primary rat hepatocyte cultures.

Much work has been devoted to the elucidation of the hormonal factors involved in the control of hepatic CYP2C11 expression in rats *in vivo* (Zaphiropoulos et al., 1989; Kato and Yamazoe, 1993; Waxman and Chang, 1995). Neonatal androgen imprinting and adult circulating levels of serum testosterone are important for determining the

male-specific hepatic expression of CYP2C11 in adult rats. Treatment of male rats with MC or HCB decreases serum testosterone levels as well as hepatic CYP2C11 catalytic activity and mRNA, similar to castration (Yeowell et al., 1987; Yeowell et al., 1989). However, androgen replacement could elicit full CYP2C11 recovery in castrated rats but not in HCB-treated rats, indicating that testosterone depletion alone is not responsible for the effects of HCB on hepatic CYP2C11 expression (Yeowell et al., 1989). The main endocrine regulator of hepatic CYP2C11 expression involves the pulsatile pattern of pituitary growth hormone release characteristic of adult male rats, and a previous study (Shimada et al., 1989) has suggested that the decrease in CYP2C11 protein levels caused by MC may be controlled by growth hormone-mediated processes. Recent work has clearly established that the male-specific pulsatile pattern of growth hormone secretion controls the expression of the *CYP2C11* gene at the transcriptional level (Legraverend et al., 1992; Sundseth et al., 1992). I was interested in determining whether aromatic hydrocarbons can suppress CYP2C11 expression in an isolated hepatocyte system, in which the regulation of CYP2C11 is divorced from the complex interplay of endocrine factors.

A major limitation on the use of rat hepatocyte cultures in studies of drug metabolism and toxicity is the rapid loss of CYP expression observed during the early stages of primary culture (Paine, 1990). Several different approaches have been used with varying degrees of success to maintain hepatocyte differentiation and the expression of constitutive CYP isozymes in primary culture. Hepatocytes cultured on a laminin-rich extracellular matrix (matrigel) with insulin as the only hormone have demonstrated stable CYP2C11 expression at levels comparable to those observed *in vivo* in the absence of growth hormone pulses (Guzelian et al., 1988; Liddle et al., 1992; Chen et al., 1995b). This culture system has been used successfully in recent years to examine the regulation of members of the CYP2C subfamily by growth hormone (Legraverend et al., 1992), CYP2C11 suppression by cytokines and interferons (Chen et al., 1995a; Chen et al., 1995b), as well as the constitutive expression of a variety of

CYP forms (Kocarek et al., 1993). To my knowledge, this is the first demonstration that AHR ligands cause marked suppression of CYP2C11 immunoreactive protein expression in isolated hepatocyte cultures, suggesting that this effect can be at least partially explained by events at a cellular level. I am aware of a study (Wortelboer et al., 1992) that demonstrated a decrease in testosterone 2 α - and 16 α -hydroxylase activity (selective CYP2C11 catalytic markers) in cultured rat hepatocytes in response to acid reaction products of indole 3-carbinol, a group of compounds with relatively low AHR binding affinity (Jellinck et al., 1993). The present study has addressed the ability of several PAHs to down-regulate only that component of CYP2C11 expression that is not under the control of endocrine factors. We do not yet know the contribution that direct hepatic effects play in the overall CYP2C11 suppression caused by PAHs *in vivo*.

I determined the IC₅₀ values for the PAHs as a measure of the relative binding affinities for the rat AHR of these compounds. Similar data have been previously reported in the literature (Okey and Vella, 1982; Piskorska-Pliszczyńska et al., 1986; Denison et al., 1986b). Those studies reported AHR binding IC₅₀ values for MC, ANTH, BA, DB[a,c]A, DB[a,h]A and DMBA; my data are in general agreement with these published reports. I reasoned that it was important to include AHR binding affinity measurements in my study for two main reasons: (a) My studies of CYP2C11 suppression are carried out in inbred Fischer 344 rats in order to avoid the complicating effect of polymorphic expression of other CYP2C subfamily members in outbred rat strains. The AHR binding affinity studies cited above involved use of C57BL/6J mouse liver cytosol (Okey and Vella, 1982), C57BL/6J mouse and Sprague-Dawley rat liver cytosol (Denison et al., 1986b), and Wistar rat liver postmitochondrial supernatant (Piskorska-Pliszczyńska et al., 1986). I wanted to confirm that the hepatic AHR from the Fischer 344 rat displays similar affinity for the range of PAHs used in my study in order to rule out the possibility of important species and strain differences. (b) For correlation analyses, I believe strongly that it is preferable to compare AHR transformation and CYP protein regulation data with AHR binding data generated in our own laboratory,

rather than relying on data from previous publications by different investigators under somewhat different conditions.

My study examined the suppression of CYP2C11 caused by a single high concentration of PAHs that would be expected to saturate AHR occupancy and cause maximum biological responses in the absence of overt cytotoxicity. It is also important to point out that CYP2C11 suppression was studied at a single time-point (48 hr) for each PAH. Differences in metabolism of the PAHs may alter the time-frame for the peak effect of each compound. In addition, CYP2C11 suppression may be partly caused by metabolites of the parent PAHs. The extent of CYP2C11 suppression is a measure of efficacy and thus may not be expected to correlate with AHR affinity and transformation potency. However, my data indicate that AHR affinity correlates with AHR transformation potency (Fig. 20a), AHR transformation potency predicts AHR transformation efficacy (data not shown; $r = 0.769$, $P = 0.074$), and AHR transformation efficacy predicts CYP2C11 suppression efficacy (data not shown; $r = 0.778$, $P = 0.069$). For these PAH ligands, there is clearly a relationship between potency and efficacy for AHR-mediated responses that rationalizes my comparison of CYP2C11 suppression to AHR affinity and transformation potency. It is important to point out that the correlations shown in Fig. 20 plot CYP2C11 suppression on a linear scale whereas AHR affinity and transformation potency are plotted on logarithmic scales. Thus, relatively large differences between ligands in AHR affinity and transformation potency translate into relatively small differences in CYP2C11 suppression efficacy. The molecular basis for this effect is not known, but the data shown in Fig. 16 confirm that relatively large differences in AHR transformation potency correspond to relatively small differences in AHR transformation efficacy. My results indicate that relatively low concentrations of ANTH (10 μM) can cause a decrease in CYP2C11 immunoreactive protein in cultured hepatocytes (37 %). According to the in vitro studies, this concentration of ANTH is unable to significantly bind to or cause AHR transformation, suggesting the possibility

of a non-AHR-mediated component in CYP2C11 suppression by PAHs at the cellular level.

For halogenated aromatic hydrocarbons such as the dioxins, dibenzofurans, and biphenyls, structure-activity relationships have firmly established the role of the AHR in mediating the toxicity of these compounds as well as induction of CYP1A1 (Poland and Knutson, 1982; Safe, 1986). Recent studies have examined the relationship between AHR binding affinity and AHR transformation potency for representative halogenated aromatics. For a series of six dioxin and dibenzofuran congeners that differ in AHR binding affinity by <15-fold, Santostefano *et al.* (1992) reported EC₅₀ values for *in vitro* AHR transformation that differ by >840-fold. Differences in AHR transformation potency approximated the relative potencies of these congeners as CYP1A1 inducers. Complementary work (Harris *et al.*, 1990) with the same series of congeners indicated a linear correlation between AHH induction and nuclear AHR levels, suggesting that for halogenated aromatic hydrocarbons, the structure-activity relationships are due to ligand-dependent transformation of the AHR to its DNA-binding form. Much less work in this regard has been done with nonhalogenated PAHs. Several reports exist on the ability of selected PAHs to compete with [³H]TCDD for binding to the cytosolic AHR from rodent liver (Bigelow and Nebert, 1982; Okey and Vella, 1982; Okey *et al.*, 1984; Denison *et al.*, 1986b; Piskorska-Pliszczyńska *et al.*, 1986). These studies and my present work confirm that DB[a,h]A, MC, and DB[a,c]A are very potent competitors, BA and DMBA are weaker competitors, and ANTH is a very poor competitor for [³H]TCDD binding. To my knowledge, the present study is the first to systematically examine the relationship between AHR binding affinity and AHR transformation potency for a series of structurally-related nonhalogenated PAHs. My work indicates that the AHR binding affinity is a good predictor of the ability of a PAH to transform the cytosolic AHR to its DNA-binding form. Using TCDD, MC, and benzo[a]pyrene as ligands, Cuthill *et al.* (1991) concluded that there exists a correlation between the affinity of a ligand for the AHR and its ability to activate the AHR *in vitro* to its DNA-binding form. However, a

large quantitative discrepancy remained between differences in AHR transformation potency and CYP1A1 induction potency for these three ligands. Riddick *et al.* (1994b) recently showed that TCDD is approximately 1,000-fold more potent than MC as an AHH inducer in mouse hepatoma cells, despite the fact that the two ligands display very similar AHR binding affinity and transformation potency *in vitro*. These results confirm that structure-activity relationships for AHR ligands break down when comparing ligands from different structural classes, largely because of differences in the rates at which the ligands are metabolized in intact cells.

My results indicate that the AHR plays a role in the down-regulation of CYP2C11 caused by aromatic hydrocarbons. This conclusion is in agreement with earlier *in vivo* studies that showed a correlation between CYP2C11 suppression and CYP1A1/2 induction for a series of polybrominated biphenyls (Dannan *et al.*, 1983) and polychlorinated biphenyls and dibenzofurans (Yoshihara *et al.*, 1982). Yeowell *et al.* (1987) demonstrated that the time-course and dose-response for CYP2C11 suppression differed from that for CYP1A1 induction, indicating that the effects of HCB on the two CYP proteins may involve different mechanisms. However, these results do not preclude the possibility that both events are AHR-mediated. The differences in time-course may be due to differences in the half-lives of the relevant mRNAs and/or proteins (Hargrove and Schmidt, 1989), and it is clear that parallel dose-response curves for different biological responses can be separated by as much as several orders of magnitude and still be mediated by the same receptor (Okey *et al.*, 1995). It is important to point out that a rat liver cytosolic 4 S carcinogen-binding protein may also be involved in the transcriptional induction of CYP1A1 expression in response to nonhalogenated aromatic hydrocarbons (Houser *et al.*, 1985). I am not aware of any studies that have addressed the role of this protein in CYP2C11 down-regulation. A final point that may argue against a role for the AHR is that suppression of CYP2C11 is not a response that is specific to aromatic hydrocarbon exposure; a variety of chemical and patho-physiological signals have been shown to cause CYP2C11 down-regulation

with selected antineoplastic agents (LeBlanc and Waxman, 1988; LeBlanc and Waxman, 1990) and cytokines and inflammatory mediators (Morgan, 1989; Morgan, 1993; Chen et al., 1995b) being the best characterized negative regulators.

4.1 Future Research Directions

The molecular mechanism by which aromatic hydrocarbons cause CYP2C11 suppression is not understood. It is clear that these compounds suppress CYP2C11 expression at a pre-translational level involving a decrease in the level of CYP2C11 mRNA (Yeowell et al., 1987; Emi and Omura, 1988; Shimada et al., 1989; Yeowell et al., 1989; Jones and Riddick, 1996). It is not known whether this decrease in mRNA level is due to alterations in mRNA stability and/or processing or a decrease in the rate of transcription of the *CYP2C11* gene. Nuclear run-on assays can be used to address the role of transcriptional events in this suppressive response. It will be interesting to determine the molecular mechanisms by which the direct and/or indirect involvement of the AHR contributes to the down-regulation of *CYP2C11* gene expression. It is theoretically conceivable to suppress the expression of AHR in the cultured hepatocytes using an antisense approach. This model could be used to study the suppression of CYP2C11 by PAHs and provide direct demonstration of the role of AHR.

CYP2C11 cDNA (Yoshioka et al., 1987) and genomic (Morishima et al., 1987) clones were first isolated by the group of Fujii-Kuriyama. Investigations of the transcriptional regulation of CYP2C11 expression will be greatly facilitated by the cloning and sequencing of approximately 2.3 kb of the 5'-flanking region of this gene (Strom et al., 1994). Potential binding sites for putative transcription factors exist. The gene contains a TATA box and a glucocorticoid-responsive element half-site. Potential binding sites for hepatic nuclear factors [HNF-1, -85 to -70], [HNF-3, -80 to -67; -54 to -44], and the ubiquitous nuclear factor 1 [NF1, -54 to -41] have been identified. The gene contains a HepG2-specific P450 2C factor motif [HPF1, -311 to -291] that is a

conserved site found in many members of the CYP2C subfamily (Venepally et al., 1992). This sequence in the *CYP2C11* gene binds a variety of orphan nuclear receptors, but appears to be of minimal functional importance (Strom et al., 1995). The *CYP2C11* 5'-flank contains sequences that interact in a sex-dependent and GH-regulated manner with putative transcription factors (Sundseth et al., 1992; Strom et al., 1994); however, only three functional elements have been localized in the *CYP2C11* upstream region. An HNF-1-like binding site confers negative regulation on a heterologous thymidine kinase promoter in reporter gene assays (Strom et al., 1994); however, the identity of the potential *trans*-acting factor is not known. Two negative regulatory regions, (silencer elements) spanning nucleotides -1230 to 1188 and -409 to -368, were identified and it was suggested that induction of CYP2C11 expression by intermittent GH may involve derepression of these silencer elements (Strom et al., 1994); however, the functional significance of these elements remains unknown. I searched the *CYP2C11* 5'-flank for potential regulatory elements that may play a role in the response to aromatic hydrocarbons. My search reveals that the *CYP2C11* gene contains several sequences that closely resemble the AHREs found in the regulatory region of the *CYP1A1* gene. Furthermore, a putative AHRE [-1530 to -1519] containing a perfect match to the AHR-binding consensus sequence [5'-GCGTGNN(A/T)NNN(C/G)-3'] (Yao and Denison, 1992) provides a potential binding site for the transformed AHR. This adds support to my hypothesis that CYP2C11 suppression by aromatic hydrocarbons is at least in part mediated directly by the AHR. It is conceivable that AHRE-like sequences can function as transcriptional "silencer" elements in the context of the *CYP2C11* gene. One can use ³²P-labeled double-stranded oligonucleotides from the upstream sequences of the *CYP2C11* gene containing the AHRE and AHRE-like elements in gel retardation experiments to evaluate direct interaction of the AHR with the *CYP2C11* gene. If the binding of the AHR to the *CYP2C11* gene is demonstrated, the next step will be to determine whether this interaction is responsible for the altered expression of CYP2C11. One can test the function (or lack thereof) of putative AHRE-like sequences

from the *CYP2C11* 5'-flank by transient transfection of luciferase (LUC) reporter plasmids. A series of *CYP2C11*-LUC constructs can be made corresponding to the full 5'-flank or various lengths of the 5'-flank (created by progressive 5'-deletion) of the *CYP2C11* gene. Each of these sequences would be incorporated into a plasmid containing a LUC reporter gene under the control of a promoter with high basal activity. Using this approach one should be able to identify areas of the *CYP2C11* 5'-flank that mediate reporter gene silencing in response to AHR ligands. It would be particularly interesting to see whether any of the AHRE-like sequences are present within the silencing regions.

Alternatively, if the ligand-AHR-ARNT complex does not bind to the 5'-flanking region of the *CYP2C11* gene and does not suppress its expression, one can entertain the hypothesis that aromatics convey their suppressive effect by interfering with a specific mechanism which is normally responsible for constitutive expression of *CYP2C11*. For example, it has been recently shown that most b/HLH factors bind as dimers to the palindromic E box motif [5'-CACGTG-3']. ARNT forms a homodimer that binds to the E box core sequence and this interaction activates transcription of genes driven by the E box motif (Antonsson et al., 1995; Sogawa et al., 1995). Although there is some similarity between the E box motif and the AHRE core, the AHR-ARNT heterodimer does not bind to the E box sequences (Mason et al., 1994). The orientation of the heterodimeric AHR complex on its DNA recognition sequence is such that ARNT binds to the 5'-GTG-3' half site, which is equivalent to the E box half site (Bacsi et al., 1995). ARNT homodimer was shown to bind to the E box core sequence CACGTG in adenovirus major late promoter (MLP) and in cotransfection experiments with MLP fused to a chloramphenicol acetyltransferase (CAT) reporter gene, ARNT markedly increased CAT expression (Antonsson et al., 1995). This raises the interesting possibility that ARNT homo- or heterodimeric pairs may have roles in controlling distinct batteries of genes. As suggested by Swanson et al. (1995), in the absence of AHR agonists, ARNT homodimer may play a role in the regulation of a second battery of

genes, possibly through interactions at E boxes, that may be down-regulated in the presence of TCDD-like compounds. Interestingly, the 5'-flanking region of *CYP2C11* contains at least two CACGTG sequences (Strom et al., 1994) and the ARNT homodimer may play a role in stimulating the constitutive expression of *CYP2C11*. Thus, activated AHR may sequester ARNT protein, thereby limiting the ability of ARNT homodimers to stimulate genes such as *CYP2C11* via E box elements. Thus, during induction of *CYP1A1* there may not be enough ARNT available for the stimulation of the regular expression of *CYP2C11* and it would therefore appear to be suppressed. In fact the time-course for the induction *CYP1A1* by MC is directly reciprocal to the time course for suppression of *CYP2C11* by MC (Jones and Riddick, 1996) lending support that this proposed mechanism for modulation of *CYP2C11* by an ARNT homodimer is feasible. There are two conditions, studied to a very limited extent to date, that must exist in order for this mechanism to be operative in intact cells: (1) ARNT is a low abundance protein and must be limiting in comparison to AHR following ligand treatment, as suggested by two recent studies (Whitelaw et al., 1993; Holmes and Pollenz, 1997), and (2) ARNT-ARNT interactions are weaker than AHR-ARNT interactions as shown by Swanson et al. (1995). This hypothesis could be tested by examining the ARNT homodimer-E box interaction in a series of gel retardation and transfection/reporter gene studies. In gel retardation experiments, ARNT homodimers could be identified by antibody super-shift assays.

The present study has not addressed the contribution that endocrine disruption plays in the suppression of *CYP2C11* caused by aromatic hydrocarbons *in vivo*. Clearly it will be important to determine the effects of aromatic hydrocarbons on growth hormone levels and secretion pattern and to examine the ability of these compounds to interfere with growth hormone signal transduction pathways at the cellular and molecular levels. There is overwhelming evidence that GH is the primary regulator of the hepatic *CYP2C11* expression *in vivo*. Aromatic hydrocarbons may alter growth hormone secretory profiles and/or interfere with GH signal transduction pathways that

operate to maintain CYP2C11 expression. Current understanding of the mechanisms by which the extracellular GH signal is transduced to the nucleus to alter gene expression is not complete, but the role played by STAT proteins (signal transducer and activator of transcription) has been the subject of recent investigation (Ihle, 1995; Rosen et al., 1995; Ihle, 1996). It seems that the GH signal triggers dimerization of the cell surface GH receptor, which in turn activates a tyrosine kinase called JAK2, a member of the Janus kinase family. JAK2-mediated phosphorylation of specific STAT proteins facilitates STAT dimerization, translocation to the nucleus and binding to enhancer elements with a conserved core sequence [5'-TTNCNNNAA-3']. Recent work indicated that a protein related to the prolactin-activated mammary gland factor STAT5 may serve as the intracellular regulator of male-specific hepatic gene transcription in response to pulsatile GH (Waxman et al., 1995; Wood et al., 1995). It has been shown that partial to complete restoration of CYP2C11 is possible in hypophysectomized rats by subcutaneous injection of GH twice daily, a schedule that mimics the adult male secretory pattern. One can use hypophysectomized rats to determine whether or not aromatic hydrocarbons have the ability to interfere with the ability of intermittent GH administration to restore CYP2C11 expression and whether aromatic hydrocarbons interfere with the GH signal transduction pathways at the cellular and molecular levels.

4.2 Summary and Overall Significance

The present structure-activity relationship study suggests that the AHR mediates at least a component of the suppression of CYP2C11 expression caused by PAHs. Aromatic hydrocarbons are ubiquitous environmental contaminants that pose significant threats to human health and the CYP system plays critical roles in the biotransformation of xenobiotics and endobiotics. This work contributes to our understanding of the mechanisms by which aromatic hydrocarbon pollutants and carcinogens cause suppression of a specific hepatic drug-metabolizing enzyme. This work has a number of

potentially significant implications. (1) This work will contribute to our understanding of how exposure to xenobiotics can alter the delicate balance of enzymes involved in the metabolism of drugs, carcinogens, and endogenous hormones which is of great clinical significance. (2) From a broader perspective, our studies of the down-regulation of CYP2C11 may constitute a useful model system to aid in our understanding of the molecular mechanisms by which aromatic hydrocarbons act to suppress the expression of other genes. This is of special interest because several of the genes that are down regulated play roles in cell growth and differentiation. (3) From a basic research perspective this work will provide insight into the mechanisms by which mammalian gene expression can be negatively regulated by foreign chemicals.

5.0 REFERENCES

Afsar, A., Lee, C., and Riddick, D. S. 1996. Modulation of the expression of constitutive rat hepatic cytochrome P450 isozymes by 5-fluorouracil. *Can J Physiol Pharmacol* **74**: 150-156.

Antonsson, C., Arulampalam, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. 1995. Constitutive function of the basic helix-loop-helix/PAS factor Arnt - Regulation of target promoters via the E box motif. *J Biol Chem* **270**: 13968-13972.

Bacsi, S. G., Reisz-Porszasz, S., and Hankinson, O. 1995. Orientation of the heterodimeric aryl hydrocarbon (dioxin) receptor complex on its asymmetric DNA recognition sequence. *Mol Pharmacol* **47**: 432-438.

Badger, T., Irby, D., Mercado, C., Shahare, M., Huang, J., Ronis, M., Crouch, J., and Thomas, P. 1992. Effects of chronic cocaine on the hepatic monooxygenase system: CYP 2C11. *FASEB J* **6**: A988.

Badger, T. M., Ronis, M. J. J., Lumpkin, C. K., Valentine, C. R., Shahare, M., Irby, D., Huang, J. A., Mercado, C., Thomas, P., Ingelman-Sundberg, M., and Crouch, J. 1993. Effects of chronic ethanol on growth hormone secretion and hepatic cytochrome-P450 isozymes of the rat. *J Pharmacol Exp Ther* **264**: 438-447.

Bank, P. A., Yao, E. F., Phelps, C. L., Harper, P. A., and Denison, M. S. 1992. Species-specific binding of transformed Ah receptor to a dioxin responsive transcriptional enhancer. *Eur J Pharmacol* **228**: 85-94.

Berghard, A., Gradin, K., Pongratz, I., Whitelaw, M., and Poellinger, L. 1993. Cross-coupling of signal transduction pathways - The dioxin receptor mediates induction of cytochrome P-450IA1 expression via a protein kinase-C-dependent mechanism. *Mol Cell Biol* **13**: 677-689.

Bigelow, S. W., and Nebert, D. W. 1982. The Ah regulatory gene product. Survey of nineteen polycyclic aromatic compounds' and fifteen benzo[a]pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicol Lett* **10**: 109-118.

Birnbaum, L. S. 1994. The mechanism of dioxin toxicity: Relationship to risk assessment. *Environ Health Perspect* **102**: 157-167.

Bonate, P. L. 1991. Gender-related differences in xenobiotic metabolism. *J Clin Pharmacol* **31**: 684-690.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Bunce, N. J., Landers, J. P., and Safe, S. H. 1988. Kinetic models for association of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin with the Ah receptor. *Arch Biochem Biophys* **267**: 384-397.

Burbach, K. M., Poland, A., and Bradfield, C. A. 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci USA* **89**: 8185-8189.

Butler, M. A., Guengerich, F. P., and Kadlubar, F. K. 1989. Metabolic oxidation of the carcinogens 4-aminobiphenyl and 4,4'-methylenebis(2-chloroaniline) by human hepatic microsomes and by purified rat hepatic cytochrome P-450 monooxygenases. *Cancer Res* **49**: 25-31.

Carver, L. A., and Bradfield, C. A. 1997. Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. *J Biol Chem* **272**: 11452-11456.

Carver, L. A., Hogenesch, J. B., and Bradfield, C. A. 1994. Tissue specific expression of the rat Ah-receptor and ARNT mRNAs. *Nucleic Acids Res* **22**: 3038-3044.

Chaloupka, K., Harper, N., Krishnan, V., Santostefano, M., Rodriguez, L. V., and Safe, S. 1993. Synergistic activity of polynuclear aromatic hydrocarbon mixtures as aryl hydrocarbon (Ah) receptor agonists. *Chem Biol Interact* **89**: 141-158.

Chen, H. S., and Perdew, G. H. 1994. Subunit composition of the heteromeric cytosolic aryl hydrocarbon receptor complex. *J Biol Chem* **269**: 27554-27558.

Chen, J. Q., Nikolova-Karakashian, M., Merrill, A. H., and Morgan, E. T. 1995a. Regulation of cytochrome P450 2C11 (CYP2C11) gene expression by interleukin-1, sphingomyelin hydrolysis, and ceramides in rat hepatocytes. *J Biol Chem* **270**: 25233-25238.

Chen, J. Q., Strom, A., Gustafsson, J. A., and Morgan, E. T. 1995b. Suppression of the constitutive expression of cytochrome P-450 2C11 by cytokines and interferons in primary cultures of rat hepatocytes: Comparison with induction of acute-phase genes and demonstration that CYP2C11 promoter sequences are involved in the suppressive response to interleukins 1 and 6. *Mol Pharmacol* **47**: 940-947.

Clark, A. R., and Docherty, K. 1993. Negative regulation of transcription in eukaryotes. *Biochem J* **296**: 521-541.

Cuthill, S., Wilhelmsson, A., and Poellinger, L. 1991. Role of the ligand in intracellular receptor function - Receptor affinity determines activation in vitro of the latent dioxin receptor to a DNA-binding form. *Mol Cell Biol* **11**: 401-411.

Dannan, G. A., Guengerich, F. P., Kaminsky, L. S., and Aust, S. D. 1983. Regulation of cytochrome P-450. Immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. *J Biol Chem* **258**: 1282-1288.

Denison, M. S., Fisher, J. M., and Whitlock, J.P. 1988a. The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. *J Biol Chem* **263**: 17221-17224.

Denison, M. S., Fisher, J. M., and Whitlock, J.P. 1988b. Inducible, receptor-dependent protein-DNA interactions at a dioxin-responsive transcriptional enhancer. *Proc Natl Acad Sci USA* **85**: 2528-2532.

Denison, M. S., Harper, P. A., and Okey, A. B. 1986a. Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin - Codistribution of unoccupied receptor with cytosolic marker enzymes during fractionation of mouse liver, rat liver and cultured Hepa-1c1 cells. *Eur J Biochem* **155**: 223-229

Denison, M. S., Vella, L. M., and Okey, A. B. 1986b. Structure and function of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Species differences in molecular properties of the receptors from mouse and rat hepatic cytosols. *J. Biol. Chem.* **261**: 3987-3995.

Denison, M. S., and Yao, E. F. 1991. Characterization of the interaction of transformed rat hepatic cytosolic Ah receptor with a dioxin responsive transcriptional enhancer. *Arch Biochem Biophys* **284**: 158-166.

Devito, M. J., and Birnbaum, L. S. 1994. Toxicology of dioxins and related chemicals. *In Dioxins and Health. Edited by A. Schecter.* Plenum Press, 233 Spring St, New York, pp. 139-162.

Dolwick, K. M., Schmidt, J. V., Carver, L. A., Swanson, H. I., and Bradfield, C. A. 1993. Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol* **44**: 911-917.

Eden, S. 1979. Age and sex related differences in episodic growth hormone secretion in the rat. *Endocrinology* **105**: 555-560.

Elferink, C. J., and Whitlock, J. P. 1994. Dioxin-dependent, DNA sequence-specific binding of a multiprotein complex containing the Ah receptor. *Receptor* **4**: 157-173.

Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N., Gotoh, O., Funae, Y., and Fujii-Kuriyama, Y. 1992. cDNA cloning and structure of mouse putative Ah receptor. *Biochem Biophys Res Commun* **184**: 246-253.

Emi, Y., and Omura, T. 1988. Synthesis of sex-specific forms of cytochrome P-450 in rat liver is transiently suppressed by hepatic monooxygenase inducers. *J Biochem* **104**: 40-43.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889-895.

Gibson, G. G., and Skett, P. 1994. Introduction to drug metabolism. (2nd edition). Chapman and Hall, London.

Gillesby, B. E., Santostefano, M., Porter, W., Safe, S., Wu, Z. F., and Zacharewski, T. R. 1997. Identification of a motif within the 5' regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression. *Biochemistry* **36**: 6080-6089.

Gonzalez, F. J. 1994. Regulation of xenobiotic-metabolizing cytochromes P450. *In* Assessment of the use of single cytochrome P450 enzymes in drug research. *Edited by* M. R. Waterman and M. Hildebrand. Springer-Verlag, Berlin, pp.21-42.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, K. S. 1982. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β -naphthoflavone. *Biochemistry* **21**: 6019-6030.

Guzelian, P. S., Li, D., Schuetz, E. G., Thomas, P., Levin, W., Mode, A., and Gustafsson, J.-A. 1988. Sex change in cytochrome P-450 phenotype by growth hormone treatment of adult rat hepatocytes maintained in a culture system on matrigel. *Proc Natl Acad Sci USA* **85**: 9783-9787.

Hankinson, O. 1995. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**: 307-340.

Hargrove, J. L., and Schmidt, F. H. 1989. The role of mRNA and protein stability in gene expression. *FASEB J.* **3**: 2360-2370.

Harris, M., Zacharewski, T., Piskorska-Pliszczyńska, J., Rosengren, R., and Safe, S. 1990. Structure-dependent induction of aryl hydrocarbon hydroxylase activity in C57BL/6 mice by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related congeners: mechanistic studies. *Toxicol Appl Pharmacol* **105**: 243-253.

Henderson, C. J., and Wolf, C. R. 1992. Molecular analysis of cytochrome P450s in the CYP2 gene family. *In* Progress in Drug Metabolism, Vol. 13. *Edited by* G. G. Gibson. Taylor & Francis, London, pp. 73-139.

Henry, E. C., Rucci, G., and Gasiewicz, T. A., 1989. Characterization of multiple forms of the Ah receptor: comparison of species and tissues. *Biochemistry* **28**: 6430-6440

Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**: 954-958.

Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y., Pray-Grant, M., Perdew, G. H., and Bradfield, C. A. 1997. Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signalling pathway. *J. Biol. Chem.* **272**: 8581-8593.

Holmes, J. L., and Pollenz, R. S. 1997. Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: development of quantitative western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates. *Mol Pharmacol* **52**: 202-211.

Hord, N. G., and Perdew, G. H. 1994. Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: Characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator. *Mol Pharmacol* **46**: 618-626.

Houser, W., Hines, R., and Bresnick, E. 1985. Implication of the "4S" polycyclic aromatic binding protein in the transregulation of rat cytochrome P-450c expression. *Biochemistry* **24**: 7839-7845.

Ihle, J. N. 1995. Cytokine receptor signalling. *Nature* **377**: 591-594.

Ihle, J. N. 1996. Signaling by the cytokine receptor superfamily in normal and transformed hematopoietic cells. *Adv Cancer Res* **68**: 21-65.

Jansson, J.-O., Eden, S., and Isaksson, O. 1985. Sexual dimorphism in the control of growth hormone secretion. *Endocrine Rev* **6**: 128-150.

Jellinck, P. H., Forkert, P. G., Riddick, D. S., Okey, A. B., Michnovicz, J. J., and Bradlow, H. L. 1993. Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. *Biochem Pharmacol* **45**: 1129-1136.

Jones, E. J., and Riddick, D. S. 1996. Regulation of constitutive rat hepatic cytochromes P450 by 3-methylcholanthrene. *Xenobiotica* **26**: 995-1012

Kato, R., and Yamazoe, Y. 1993. Hormonal regulation of cytochrome P450 in rat liver. *In* Cytochrome P450, Handbook of Experimental Pharmacology Vol. 105. *Edited by* J. B. Schenkman, and H. Greim. Springer-Verlag, Berlin, pp. 447-459.

Katzung, B. G. (Ed) 1995. Basic and Clinical Pharmacology (6th edition.). Appleton & Lange, Norwalk, CT.

Kocarek, T. A., Schuetz, E. G., and Guzelian, P. S. 1993. Expression of multiple forms of cytochrome P450 messenger RNAs in primary cultures of rat hepatocytes maintained on matrigel. *Mol Pharmacol* **43**: 328-334.

Kohler, G., and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495-497.

Krishnan, V., Porter, W., Santostefano, M., Wang, X. H., and Safe, S. 1995. Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells. *Mol Cell Biol* **15**: 6710-6719.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Landers, J. P., and Bunce, N. J. 1991. The Ah receptor and the mechanism of dioxin toxicity. *Biochem J* **276**: 273-287.

LeBlanc, G. A., and Waxman, D. J. 1988. Feminization of rat hepatic P-450 expression by cisplatin. Evidence for perturbations in the hormonal regulation of steroid-metabolizing enzymes. *J Biol Chem* **263**: 15732-15739.

LeBlanc, G. A., and Waxman, D. J. 1990. Mechanisms of cyclophosphamide action on hepatic P-450 expression. *Cancer Res* **50**: 5720-5726.

Legraverend, C., Mode, A., Westin, S., Strom, A., Eguchi, H., Zaphiropoulos, P. G., and Gustafsson, J. A. 1992. Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion. *Mol Endocrinol* **6**: 259-266.

Li, D., Schuetz, E. G., and Guzelian, P. S. 1991. Hepatocyte culture in study of P450 regulation. *Methods Enzymol* **206**: 335-344.

Liddle, C., Mode, A., Legraverend, C., and Gustafsson, J. A. 1992. Constitutive expression and hormonal regulation of male sexually differentiated cytochromes-P450 in primary cultured rat hepatocytes. *Arch Biochem Biophys* **298**: 159-166.

Lin, F. H., Clark, G., Bimba, L. S., Lucier, G. W., and Goldstein, J. A. 1991a. Influence of the Ah locus on the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the hepatic epidermal growth factor receptor. *Mol Pharmacol* **39**: 307-313.

Lin, F. H., Stohs, S. J., Bimba, L. S., Clark, G., Lucier, G. W., and Goldstein, J. A. 1991b. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the hepatic estrogen and glucocorticoid receptors in congenic strains of Ah responsive and Ah nonresponsive C57BL/6J mice. *Toxicol Appl Pharmacol* **108**: 129-139.

Lu, A. Y., and West, S. 1980. Multiplicity of mammalian microsomal cytochrome P450. *Pharmacol Rev* **31**: 277-295.

Luska, A., Shen, E., and Whitlock, J. P. 1993. Protein-DNA interactions at a dioxin-responsive enhancer - Analysis of 6 bona-fide DNA-binding sites for the liganded Ah receptor. *J Biol Chem* **268**: 6575-6580.

Ma, Q., and Whitlock, J. P. 1997. A novel cytoplasmic protein that interacts with the AH receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Biol Chem* **272**: 8878-8884.

Mason, G. G. F., Witte, A. M., Whitelaw, M. L., Antonsson, C., McGuire, J., Wilhelmsson, A., Poellinger, L., and Gustafsson, J. A. 1994. Purification of the DNA binding form of dioxin receptor - role of the arnt cofactor in regulation of dioxin receptor function. *J Biol Chem* **269**: 4438-4449.

Merchant, M., Arellano, L., and Safe, S. 1990. The mechanism of action of α -naphthoflavone as an inhibitor of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 gene expression. *Arch Biochem Biophys* **281**: 84-89.

Morgan, E. T. 1989. Suppression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. *Mol Pharmacol* **36**: 699-707.

Morgan, E. T. 1993. Down-regulation of multiple cytochrome-P450 gene products by inflammatory mediators in vivo - Independence from the hypothalamo-pituitary axis. *Biochem Pharmacol* **45**: 415-419.

Morgan, E. T., Thomas, K. B., Swanson, R., Vales, T., Hwang, J., and Wright, K. 1994. Selective suppression of cytochrome P-450 gene expression by interleukins 1 and 6 in rat liver. *Biochem Biophys Acta* **1219**: 475-483.

Morishima, N., Yoshioka, H., Higashi, Y., Sogawa, K., and Fujii-Kuriyama, Y. 1987. Gene structure of cytochrome P-450(M-1) specifically expressed in male rat liver. *Biochemistry* **26**: 8279-8285.

Murray, M., Cantrill, E., Mehta, I., and Farrell, G. C. 1992. Impaired expression of microsomal cytochrome-P450-2C11 in choline-deficient rat liver during the development of cirrhosis. *J Pharmacol Exp Ther* **261**: 373-380.

Nadin, L., Butler, A. M., Farrell, G. C., and Murray, M. 1995. Pretranslational down-regulation of cytochromes P450 2C11 and 3A2 in male rat liver by tumor necrosis factor alpha. *Gastroenterology* **109**: 198-205.

Nebert, D. W. 1989. The *Ah* locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol* **20**: 137-152.

Nebert, D. W., and Gonzalez, F. J. 1987. P450 genes: structure, evolution, and regulation. *Annu Rev Biochem* **56**: 945-993.

Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. 1993. The P450 superfamily - Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**: 1-51.

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**: 1-42.

Nisbet, I. C. T., and Lagoy, P. K. 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). *Regul Toxicol Pharmacol* **16**: 290-300.

Okey, A. B. 1990. Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther* **45**: 241-298.

Okey, A. B., Bondy, G. P., Mason, M. E., Nebert, D. W., Forster-Gibson, C. J., Muncan, J., and Dufresne, M. J. 1980. Temperature-dependent cytosol-to-nucleus translocation of the *Ah* receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in continuous cell culture lines. *J Biol Chem* **255**: 11415-11422.

Okey, A. B., Denison, M. S., and Harper, P. A. 1986. Induction of drug metabolising enzymes: Mechanism and consequences. *Clin. Biochem.* **19**: 132-141.

Okey, A. B., Dube, A. W., and Vella, L. M. 1984. Binding of benzo(a)pyrene and dibenz(a,h)anthracene to the *Ah* receptor in mouse and rat hepatic cytosols. *Cancer Res* **44**: 1426-1432.

Okey, A. B., Riddick, D. S., and Harper, P. A. 1994a. The *Ah* receptor - mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol Lett* **70**: 1-22.

Okey, A. B., Riddick, D. S., and Harper, P. A. 1994b. Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends Pharmacol Sci* **15**: 226-232.

Okey, A. B., Riddick, D. S., and Harper, P. A. 1995. *Ah* receptor role in TCDD toxicity: Still some mysteries but no myth - A reply to the commentary by Dr L.W.D. Weber and Dr B.U. Stahl. *Toxicol Lett* **75**: 249-254.

Okey, A. B., and Vella, L. M. 1982. Binding of 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to a common *Ah* receptor site in mouse and rat hepatic cytosols. *Eur J Biochem* **127**: 39-47.

Okey, A. B., Vella, L. M., and Harper, P. A. 1989. Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P₁-450 by 3-methylcholanthrene. *Mol Pharmacol* **35**: 823-830.

Okita, R. T., and Masters, B. S. S. 1992. Biotransformations - The cytochromes P450. *In* Textbook of biochemistry with clinical correlations (3rd edition) *Edited by* T. M. Devlin. Wiley-Liss, New York, pp. 981-999.

Omura, T., and Sato, R. 1964. The carbon monoxide-binding pigment of liver microsomes. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370-2378.

Ortiz de Montellano, P. R. (Ed.). 1995. Cytochrome P450: structure, mechanism, and biochemistry. (2nd edition). Plenum Press, New York.

Paine, A. J. 1990. The maintenance of cytochrome P-450 in rat hepatocyte culture: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P-450 system. *Chem-Biol Interact* **74**: 1-31.

Park, S. S., Fujino, T., West, D., Guengerich, F. P., and Gelboin, H. V. 1982. Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. *Cancer Res* **42**: 1798-1808.

Perdew, G. H. 1988. Association of the Ah receptor with the 90-kDa heat shock protein. *J Biol Chem* **263**: 13802-13805.

Perdew, G. H. 1991. Comparison of the nuclear and cytosolic forms of the Ah receptor from Hepa-1c1c7 cells - Charge heterogeneity and ATP binding properties. *Arch Biochem Biophys* **291**: 284-290.

Perdew, G. H. 1992. Chemical cross-linking of the cytosolic and nuclear forms of the Ah receptor in hepatoma cell line 1c1c7. *Biochem Biophys Res Commun* **182**: 55-62.

Piskorska-Pliszczyńska, J., Astroff, B., Zacharewski, T., Harris, M., Rosengren, R., Morrison, V., Safe, L., and Safe, S. 1991. Mechanism of action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonists - Characterization of 6-[I-125]methyl-8-iodo-1,3-dichlorodibenzofuran-Ah receptor complexes. *Arch Biochem Biophys* **284**: 193-200.

Piskorska-Pliszczynska, J., Keys, B., Safe, S., and Newman, M. S. 1986. The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. *Toxicol Lett* **34**: 67-74.

Poland, A., and Bradfield, C. 1992. A brief review of the Ah locus. *Tohoku J Exp Med* **168**: 83-87.

Poland, A., Glover, E., and Bradfield, C. A. 1991. Characterization of polyclonal antibodies to the Ah receptor prepared by immunization with a synthetic peptide hapten. *Mol Pharmacol* **39**: 20-26.

Poland, A., Glover, E., and Kende, A. S. 1976. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J Biol Chem* **251**: 4936-4946.

Poland, A., and Knutson, J. C. 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**: 517-554.

Pollenz, R. S., Sattler, C. A., and Poland, A. 1994. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. *Mol Pharmacol* **45**: 428-438.

Pongratz, I., Mason, G. G. F., and Poellinger, L. 1992. Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor - Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. *J Biol Chem* **267**: 13728-13734.

Porter, T. D., and Coon, M. J. 1991. Cytochrome-P-450 - Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266**: 13469-13472.

Rane, A., and Ask, B. 1992. A conspicuous down-regulating effect of morphine on essential steroid hydroxylation reactions and certain drug N-demethylations. *J Steroid Biochem Mol Biol* **41**: 91-98.

Reik, L. M., Maines, S. L., Ryan, D. E., Levin, W., Bandiera, S., and Thomas, P. E. 1987. A simple, non-chromatographic purification procedure for monoclonal antibodies. *J Immunol Methods* **100**: 123-130.

Reisz-Porszasz, S., Probst, M. R., Fukunaga, B. N., and Hankinson, O. 1994. Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol Cell Biol* **14**: 6075-6086.

Reyes, H., Reisz-Porszasz, S., and Hankinson, O. 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256**: 1193-1195.

Riddick, D. S. 1998. Drug Biotransformation. *In Principles of Medical Pharmacology*, (6th edition). *Edited by* H. Kalant, and W. H. E. Roschlau. Oxford University Press, New York, pp. 38-54

Riddick, D. S., Harper, P. A., and Okey, A. B. 1994a. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin versus 3-methylcholanthrene. Ah receptor binding, transformation and induction of CYP1A1. *In Cytochrome P450*, 8th International conference, *Edited by* M. C. Lechner. John Libbey Eurotext Ltd, Paris, pp. 551-554.

Riddick, D. S., Huang, Y., Harper, P. A., and Okey, A. B. 1994b. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin versus 3-methylcholanthrene: comparative studies of Ah receptor binding, transformation, and induction of CYP1A1. *J Biol Chem* **269**: 12118-12128.

Ronis, M. J. J., Lumpkin, C. K., Thomas, P. E., Ingelman-Sundberg, M., and Badger, T. M. 1992. The microsomal monooxygenase system of regenerating liver - An examination of the role of estradiol in the demasculinization of drug metabolism produced by 2/3 partial hepatectomy. *Biochem Pharmacol* **43**: 567-573.

Rosen, J., Day, A., Jones, T. K., Jones, E. T. T., Nadzan, A. M., and Stein, R. B. 1995. Intracellular receptors and signal transducers and activators of transcription superfamilies: novel targets for small-molecule drug discovery. *J Med Chem* **38**: 4855-4874.

Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Lieber, C. S., and Levin, W. 1984. Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239-1250

Ryan, D. E., and Levin, W. 1990. Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol Ther* **45**: 153-239.

Ryan, D. E., and Levin, W. 1993. Age- and gender-related expression of rat liver cytochrome P450. *In* Cytochrome P450, Handbook of Experimental Pharmacology Vol 105. Edited by J. B. Schenkman, and H. Greim. Springer-Verlag, Berlin, pp. 461-476.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **21**: 51-88.

Safe, S. H. 1986. Comparative toxicology and mechanism of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu Rev Pharmacol Toxicol* **26**: 371-399.

Sakai, H., Okamoto, T., Yamamoto, R., Sindhu, R. K., and Kikkawa, Y. 1992. Suppressive effect of interleukin-1 on pulmonary cytochrome-P450 and superoxide anion production. *Biochem Biophys Res Commun* **185**: 1083-1090.

Santostefano, M., Piskorska-Pliszczyńska, J., Morrison, V., and Safe, S. 1992. Effects of ligand structure on the in vitro transformation of the rat cytosolic aryl hydrocarbon receptor. *Arch Biochem Biophys* **297**: 73-79.

Schafer, M. W., Madhukar, B. V., Swanson, H. I., Tullis, K., and Denison, M. S. 1993. Protein kinase-C is not involved in Ah receptor transformation and DNA binding. *Arch Biochem Biophys* **307**: 267-271.

Schenkman, J. B. 1992. Steroid metabolism by constitutive cytochrome-P450. *J Steroid Biochem Mol Biol* **43**: 1023-1030.

Schmidt, J. V., Carver, L. A., and Bradfield, C. A. 1993. Molecular characterization of the murine Ahr gene - organization, promoter analysis, and chromosomal assignment. *J Biol Chem* **268**: 22203-22209.

Sewall, C. H., Lucier, G. W., Tritscher, A. M., and Clark, G. C. 1993. TCDD-mediated changes in hepatic epidermal growth factor receptor may be a critical event in the hepatocarcinogenic action of TCDD. *Carcinogenesis* **14**: 1885-1893.

Shapiro, B. H., Agrawal, A. K., and Pampori, N. A. 1995. Gender differences in drug metabolism regulated by growth hormone. *Int J Biochem Cell Biol* **27**: 9-20.

Shimada, M., Murayama, N., Yamauchi, K., Yamazoe, Y., and Kato, R. 1989. Suppression in the expression of a male-specific cytochrome P450, P450-male: difference in the effect of chemical inducers on P450-male mRNA and protein in rat livers. *Arch Biochem Biophys* **270**: 578-587.

Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. 1995. Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. *Proc Natl Acad Sci USA* **92**: 1936-1940.

Strom, A., Eguchi, H., Mode, A., Legraverend, C., Tollet, P., Stromstedt, P. E., and Gustafsson, J. A. 1994. Characterization of the proximal promoter and two silencer elements in the CYP2C11 gene expressed in rat liver. *DNA Cell Biol* **13**: 805-819.

Strom, A., Westin, S., Eguchi, H., Gustafsson, J. A., and Mode, A. 1995. Characterization of orphan nuclear receptor binding elements in sex-differentiated members of the CYP2C gene family expressed in rat liver. *J Biol Chem* **270**: 11276-11281.

Stupans, I., Richards, D. A., and McClure, M. T. 1995. Effects of 5-fluorouracil treatment on rat liver microsomal enzymes. *Xenobiotica* **25**: 1-8.

Sundseth, S. S., Alberta, J. A., and Waxman, D. J. 1992. Sex-specific, growth hormone-regulated transcription of the cytochrome-P450 2C11 and 2C12 genes. *J Biol Chem* **267**: 3907-3914.

Sutter, T. R., and Greenlee, W. F. 1992. Classification of members of the Ah gene battery. *Chemosphere* **25**: 223-226.

Swanson, H. I., and Bradfield, C. A. 1993. The AH-receptor - genetics, structure and function. *Pharmacogenetics* **3**: 213-230.

Swanson, H. I., Chan, W. K., and Bradfield, C. A. 1995. DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J Biol Chem* **270**: 26292-26302.

Swinney, D. C., Ryan, D. E., Thomas, P. E., and Levin, W. 1987. Regioselective progesterone hydroxylation catalyzed by eleven rat hepatic cytochrome P-450 isozymes. *Biochemistry* **26**: 7073-7083.

Thal, C., Elkahwaji, J., Loeper, J., Tinel, M., Doostzadeh, J., Labbe, G., Leclaire, J., Beaune, P., and Pessayre, D. 1994. Administration of high doses of human recombinant interleukin-2 decreases the expression of several cytochromes P-450 in the rat. *J Pharmacol Exp Ther* **268**: 515-521.

Thummel, K. E., and Schenkman, J. B. 1990. Effects of testosterone and growth hormone treatment on hepatic microsomal P450 expression in the diabetic rat. *Mol Pharmacol* **37**: 119-129.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350-4354.

Tsui, H. W., and Okey, A. B. 1981. Rapid vertical tube rotor gradient assay for binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor. *Can J Physiol Pharmacol* **59**: 927-931.

Venepally, P., Chen, D., and Kemper, B. 1992. Transcriptional regulatory elements for basal expression of cytochrome P450IIC genes. *J Biol Chem* **267**: 17333-17338.

Waxman, D. J. 1984. Rat hepatic cytochrome P-450 isozyme 2c. Identification as a male-specific, developmentally induced steroid 16 α -hydroxylase and comparison to a female-specific cytochrome P-450 isoenzyme. *J Biol Chem* **259**: 15481-15490.

Waxman, D. J. 1988. Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71-84.

Waxman, D. J., and Chang, T. K. H. 1995. Hormonal regulation of liver cytochrome P450 enzymes. *In* Cytochrome P450: structure, mechanism, and biochemistry (2nd edition). *Edited by* P. R. Ortiz de Montellano. Plenum Press, New York, pp. 391-417.

Waxman, D. J., Dannan, G. A., and Guengerich, F. P. 1985. Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24**: 4409-4417.

Waxman, D. J., Ram, P. A., Park, S. H., and Choi, H. K. 1995. Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, STAT 5-related DNA binding protein - proposed role as an intracellular regulator of male-specific liver gene transcription. *J Biol Chem* **270**: 13262-13270.

White, T. E. K., and Gasiewicz, T. A. 1993. The human estrogen receptor structural gene contains a DNA sequence that binds activated mouse and human Ah receptors - A possible mechanism of estrogen receptor regulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem Biophys Res Commun* **193**: 956-962.

Whitelaw, M., Pongratz, I., Wilhelmsson, A., Gustafsson, J. A., and Poellinger, L. 1993. Ligand-dependent recruitment of the ARNT coregulator determines DNA recognition by the dioxin receptor. *Mol Cell Biol* **13**: 2504-2514.

Whitlock, J.P. 1990. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol* **30**: 251-277.

Wood, T. J. J., Sliva, D., Lobie, P. E., Pircher, T. J., Gouilleux, F., Wakao, H., Gustafsson, J. A., Groner, B., Norstedt, G., and Haldosen, L. A. 1995. Mediation of growth hormone-dependent transcriptional activation by mammary gland factor Stat 5. *J Biol Chem* **270**: 9448-9453.

Wortelboer, H. M., Dekruif, C. A., Vaniersel, A. A. J., Falke, H. E., Noordhoek, J., and Blaauboer, B. J. 1992. Acid reaction products of indole-3-carbinol and their effects on cytochrome P450 and phase-II enzymes in rat and monkey hepatocytes. *Biochem Pharmacol* **43**: 1439-1447.

Wrighton, S. A., and Stevens, J. C. 1992. The human hepatic cytochromes-P450 involved in drug metabolism. *Crit Rev Toxicol* **22**: 1-21.

Wu, L., and Whitlock, J. P. 1992. Mechanism of dioxin action - Ah receptor-mediated increase in promoter accessibility in vivo. *Proc Natl Acad Sci USA* **89**: 4811-4815.

Wynder, E. L., and Hoffmann, D. 1994. Smoking and lung cancer: Scientific challenges and opportunities. *Cancer Res* **54**: 5284-5295.

Yao, E. F., and Denison, M. S. 1992. DNA sequence determinants for binding of transformed Ah-receptor to a dioxin-responsive enhancer. *Biochemistry* **31**: 5060-5067.

Yeowell, H. N., Waxman, D. J., LeBlanc, G. A., Linko, P., and Goldstein, J. A. 1989. Suppression of male-specific cytochrome P450 2c and its mRNA by 3,4,5,3',4',5'-hexachlorobiphenyl in rat liver is not causally related to changes in serum testosterone. *Arch Biochem Biophys* **271**: 508-514.

Yeowell, H. N., Waxman, D. J., Wadhera, A., and Goldstein, J. A. 1987. Suppression of the constitutive, male-specific rat hepatic cytochrome P-450 2c and its mRNA by 3,4,5,3',4',5'-hexachlorobiphenyl and 3-methylcholanthrene. *Mol Pharmacol* **32**: 340-347.

Yoshihara, S., Nagata, K., Wada, I., Yoshimura, H., Kuroki, H., and Masuda, Y. 1982. A unique change of steroid metabolism in rat liver microsomes induced with highly toxic polychlorinated biphenyl (PCB) and polychlorinated dibenzofuran (PCDF). *J Pharm Dyn* **5**: 994-1004.

Yoshioka, H., Morohashi, K.-I., Sogawa, K., Miyata, T., Kawajiri, K., Hirose, T., Inayama, S., Fujii-Kuriyama, Y., and Omura, T. 1987. Structural analysis and specific expression of microsomal cytochrome P-450(M-1) mRNA in male rat livers. *J Biol Chem* **262**: 1706-1711.

Yuan, W., Sequeira, D. J., and Backes, W. L. 1992. Modulation of rat hepatic cytochrome P450 by ethylbenzene treatment. *The Pharmacologist* **34**: 183.

Zacharewski, T., Harris, M., and Safe, S. 1991. Evidence for the mechanism of action of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated decrease of nuclear estrogen receptor levels in wild-type and mutant mouse Hepa-1c1c7 Cells. *Biochem Pharmacol* **41**: 1931-1939.

Zacharewski, T. R., Bondy, K. L., McDonell, P., and Wu, Z. F. 1994. Antiestrogenic effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on 17 β -estradiol-induced pS2 expression. *Cancer Res* **54**: 2707-2713.

Zaphiropoulos, P. G., Mode, A., Norstedt, G., and Gustafsson, J.-A. 1989. Regulation of sexual differentiation in drug and steroid metabolism. *Trends Pharmacol Sci* **10**: 149-153.

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