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Induction of Cytochrome P450 Enzymes

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1. Introduction

1.1. Cytochrome P450 Enzymes and the Adaptive Response

Organisms are constantly exposed to an ever-changing spectrum of foreign chemicals or xenobiotics. In response to this challenge, adaptive mechanisms have evolved in higher eukaryotes that allow them to detect an insulting agent and accordingly increase its metabolism and clearance. Commonly, the front line in this metabolic defense is the cytochrome P450 monooxygenases (CYPs). These enzymes catalyze the first step in the metabolism of lipophilic xenobiotics to more water-soluble compounds that can be readily excreted. A common feature of the CYPs is that exposure to a xenobiotic substrate often results in increased expression of the CYP enzyme(s) capable of its metabolism. This adaptive response, known as induction, is a tightly regulated process that is controlled primarily at the level of transcription. Regulating the expression of CYPs in a manner that is sensitive to xenobiotic exposure allows the cell to increase the levels of the necessary CYP enzymes only as needed to facilitate elimination of a toxicant.

1.2. Overview of Nuclear Receptors

The adaptive response to xenobiotics is orchestrated in the cell by a subset of receptors that act primarily in the nucleus. For this chapter, we will employ a liberal definition of nuclear receptor (NR) that includes all signaling molecules that function as ligand-binding transcription factors that bind to specific DNA enhancer sequences and upregulate the transcription of CYP genes. Two classes of NRs will be discussed in this chapter. Members of the nuclear hormone receptor (NHR) superfamily to be reviewed include the constitutive androstane receptor (CAR), the pregnane X-receptor (PXR), and the peroxisome proliferator activated receptors (PPARs). A single member of the PAS superfamily, the aryl hydrocarbon receptor (AHR), will also be discussed.

The NHRs have a modular structure characterized by an N-terminal ligand-independent AF-1 transactivation domain (TAD), a highly conserved DNA binding domain (DBD) containing two zinc finger motifs, and a ligand binding domain (LBD) that contains a ligand-dependent AF-2 TAD in its C-terminal portion[1]. The CAR and the PXR play major roles in the induction of the CYP2 and CYP3

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genes, while PPARα mediates the upregulation of the CYP4 family[2]. The PXR, CAR, and PPARα are similar in that they form heterodimers with the retinoid X receptor (RXR) to bind response elements that contain two copies of the core sequence AG(G/T)TCA arranged as everted repeats (ER) or direct repeats (DR) separated by varying numbers of nucleotide spacers[2]. These response elements are commonly designated by the type of repeat, followed by the nucleotide spacer number; for example, a direct repeat separated by four nucleotides is termed "DR4." The binding specificity and the transactivation potential of different NHR/RXR heterodimers can be determined by the spacer number, the nucleotide sequence in the half-sites, and often by sequences 5′ of the half-sites[1].

The nuclear receptor known as the aryl hydrocarbon receptor (AHR) is a member of the PAS superfamily of proteins and regulates the CYP1 genes[3, 4]. The PAS domain was named for the first three proteins in which it was recognized: *P*ER, *A*RNT, and *S*IM[5]. Most members of the PAS superfamily, including the AHR, have an N-terminal basic helix-loop-helix (bHLH) domain adjacent to the PAS domain and a carboxy terminal domain that influences transcription[6]. The AHR binds to specific response elements upstream of the CYP1 genes containing the core sequence TNGCGTG to induce gene expression.

A common characteristic of NRs is that they interact with coactivators and corepressors. In general, in the absence of ligand or when bound by antagonist, NRs exist in a complex with corepressors, such as SMRT or NcoR, which inhibit transcriptional activity through recruitment of histone deacetylases or other mechanisms[7, 8]. Activation of the NR by agonist binding or phosphorylation causes a conformational change in the receptor that results in the dissociation of corepressors and the recruitment of coactivators, such as SRC-1 and CBP, which interact with NRs through conserved LXXLL motifs[7–9]. Coactivators can increase the rate of gene transcription through chromatin remodeling or by interacting with components of the basic transcriptional machinery to increase the number of functional basal promoter complexes[7, 8]. The final composition and activity of the recruited multiprotein transcriptional complex is dependent on both promoter and enhancer sequences, as well as on the specific ligand bound to the NR.

Our goal for this chapter is to provide an overview of the discoveries that have occurred over the last 10 years in the area of NR-mediated induction of CYP enzymes by xenobiotics. Our current understanding of the mechanism of signal transduction of each receptor will be presented, and we will highlight some areas where further investigation is needed. Along the way, we will also touch on emerging physiological roles of some NRs. While we hope to provide a basic working knowledge of NR-mediated signal transduction, the breadth of the topic prevents us from discussing in depth many of the more detailed aspects of NR signaling pathways. For those desiring more information on specific topics, readers are referred to relevant reviews.

2. The Pregnane X Receptor

2.1. Introduction

The CYP3A enzymes are the most abundant cytochrome P450s in human liver and are responsible for the metabolism of endogenous steroids and numerous xenobiotics[10]. The main isoform in humans, CYP3A4, is estimated to be responsible for the metabolism of more than 50% of the currently used drugs and is considered central in many clinically important drug interactions[11]. Due to the importance of the CYP3A enzymes, the mechanisms of CYP3A induction are of special interest and have been an area of intense research.

A series of discoveries over many years have led to our current understanding of CYP3A induction. Early studies demonstrated that the administration of certain steroids to rats caused greatly enhanced transcription of CYP3A genes in the liver and small intestine. Induction was seen after treatment with the potent glucocorticoid dexamethasone and, paradoxically, also with the synthetic antiglucocorticoid pregnenolone 16α-carbonitrile (PCN)[12–14]. The response of CYP3A to glucocorticoids was distinct from a classical glucocorticoid receptor (GR)-dependent response with respect to both the time course of induction and the dose of dexamethasone required, as well as the rank order of the potency of various steroids[15, 16]. Analysis of the promoter region of the CYP3A23 gene revealed conserved enhancer elements similar to those recognized by NRs;

however, further experimentation showed that the elements were not bound by the GR[17, 18]. It was postulated that the induction of CYP3A23 involved a novel NR acting by a mechanism distinct from that of the classical GR pathway.

2.2. The PXR

The long-standing paradox of CYP3A induction by both GR agonists and antagonists was explained after a novel orphan NR was cloned and characterized[19]. Initial experiments to identify ligands of the orphan receptor demonstrated that it could be activated by many compounds, including dexamethasone, 6,16α-dimethyl pregnenolone, and PCN. The unusual pharmacology of the orphan receptor, specifically its activation by glucocorticoids (dexamethasone) and antiglucocorticoids (RU486 and PCN), strongly suggested that it was the unknown mediator of CYP3A induction observed in earlier studies. Further experimentation proved this to be the case and the receptor was named the pregnane X receptor (PXR) because of its strong activation by natural and synthetic pregnanes.

After the identification of PXR in the mouse, orthologues of the receptor were identified in many other species including human, rabbit, and rat[20–22]. The human orthologue was named steroid and xenobiotic receptor (SXR) and also pregnane activated receptor (PAR)[20, 21]. For simplicity, we will use the name PXR to refer to all orthologues. A comparison of PXR amino acid sequences among different mammalian species shows that while the DBD is highly conserved (>90% identity), the LBD displays much more variability (~80% identity)[23]. In all species, PXR is highly expressed in the liver and to a lesser extent in the small intestine[19, 23]. The PXR expression profile matches that observed for induction of CYP3A enzymes and provides further evidence for the idea that PXR is the master regulator of CYP3A expression.

2.3. PXR Ligands and Species Differences

The PXR is a very promiscuous, low affinity receptor that is activated by a wide array of structurally diverse compounds[22]. Crystal structures of the human PXR (hPXR) LBD, both with and without agonist, have been useful in understanding the receptor's ability to accommodate ligands of various structures and sizes[24]. The crystal structure of the hPXR LBD in the absence of ligand revealed a hydrophobic ligand binding pocket that is larger than that of most NHRs; furthermore, a unique flexible loop found adjacent to the ligand-binding cavity likely contributes to the ability of the PXR to bind both small and large ligands[24]. When the LBD was cocrystallized with a hPXR ligand, SR12813, it was discovered that the ligand could dock into the ligand binding pocket in three different orientations, each with a distinct pattern of hydrogen bonding and van der Waals contacts[24]. Thus, unlike many NHRs, the PXR can bind a variety of hydrophobic ligands in multiple binding orientations.

The ligand-binding specificity of the PXR is markedly different among species[22]. For example, PCN is a strong activator of rat and mouse PXR, but has little effect on rabbit or human PXR. Conversely, rifampicin, phenobarbital, and SR12813 activate both rabbit and human PXR, but have little effect on rodent PXR. These species differences are due to differences in the amino acid sequence of the LBD of the receptor. Using the crystal structure of SR12813 bound to hPXR, four polar residues in the LBD that interacted with SR12813 were identified that were different from the corresponding amino acids in the mouse PXR (mPXR)[24]. When the residues in the mPXR were mutated to the amino acids found in the human receptor, the mutant mPXR was no longer responsive to the rodent-specific inducer PCN but rather to the human-specific agonist SR12813[24]. The dependence of specific ligand binding on PXR amino acid sequence has also been demonstrated *in vivo*. A PXR-null mouse that has been "humanized" by integrating an albumin-SXR (hPXR) transgene is responsive to the PXR ligands rifampicin and PB, but is no longer responsive to PCN[25].

2.4. Activation of Transcription

Analyses of PXR target gene promoters revealed that the receptor can upregulate transcription by binding as a heterodimer with RXR to several different motifs, including DR3, DR4, and ER6 elements (Figure 8.1)[10]. The human CYP3A4

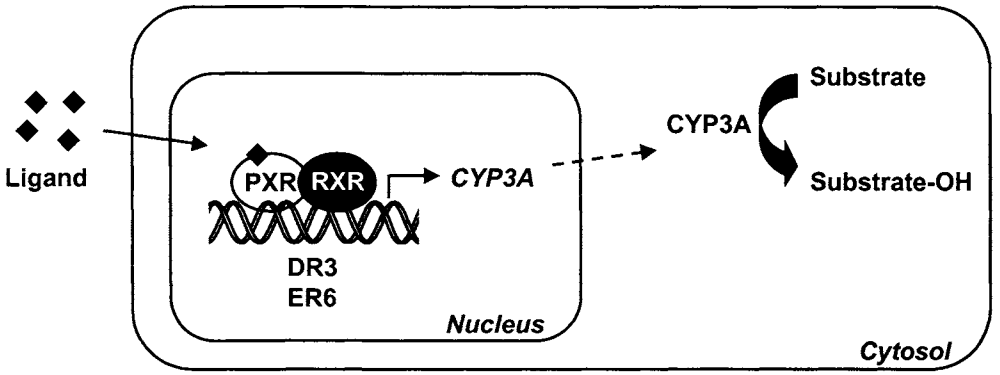

Figure 8.1. A model of the transcriptional regulation of CYP3A expression by PXR. The PXR binds as a heterodimer with RXR to response elements in the promoter of CYP3A and other target genes. Binding of ligand to the PXR results in increased CYP3A enzyme activity, which in turn increases the hydroxylation of substrates such as steroids, bile acids, and drugs.

gene contains a proximal ER6 response element and a distal xenobiotic response element module (referred to as XREM) consisting of both an imperfect DR3 and an ER6 element[26]. Although PXR-mediated transactivation can be conferred by the proximal ER6 element alone, maximal induction of the CYP3A4 gene requires both the ER6 and XREM motifs[26]. The PXR can also activate the transcription of a number of CYP2B genes, which are classically thought of as target genes for the nuclear receptor, CAR[27–29]. Interestingly, the PXR has been demonstrated to upregulate CYP2B expression by binding to the same DR4 elements upstream of the CYP2B gene to which CAR binds[27–30]. The reciprocal is also true in that CAR can bind to response elements in the CYP3A genes to induce gene expression[27, 28, 31]. These findings and others have made it increasingly clear that CAR and PXR serve broad and often overlapping functions[32, 33].

In addition to PXR and CAR, other NRs are also involved in the regulation of CYP3A expression. For example, activation of the GR by dexamethasone increases the expression of both PXR and CAR through glucocorticoid response elements (GREs) in their promoters, and this can increase the expression of CYP3A[34–36]. The transcription factor hepatocyte nuclear factor-4 (HNF-4) seems to play an important role in CYP3A expression as well. It has been shown that binding of HNF-4 to the promoter of CYP3A23 is necessary to maintain both its constitutive expression and its induction by dexamethasone[37]. Similarly, binding of HNF-4α to a specific *cis*-acting element in the CYP3A4 gene promoter was found to be necessary for transactivation of gene expression by PXR or CAR[38]. Moreover, HNF-4α-null mice express neither PXR nor CAR, indicating that expression of these receptors is regulated by HNF-4α[39]. Given the complexity of emerging cross talk pathways among receptors, it is likely that other NRs may be implicated in CYP3A regulation in the future.

2.5. Mouse Models

An important advancement in the PXR field came with the generation of a PXR-null mouse model[25, 40]. Data obtained using these mice have confirmed that the PXR plays a major role in regulating CYP3A gene expression and in xenobiotic metabolism. Mice that lack PXR do not induce CYP3A in response to PCN or other PXR-specific ligands and exhibit altered metabolism of xenobiotics that are CYP3A substrates[25, 40]. The exact role of the PXR in maintaining the constitutive expression of CYP3A remains unclear as the two independently derived PXR-null models display either unchanged or increased levels of basal CYP3A[25, 40].

Mouse models have also been used to demonstrate a role for PXR in regulating the levels of toxic bile acids. It had been previously established

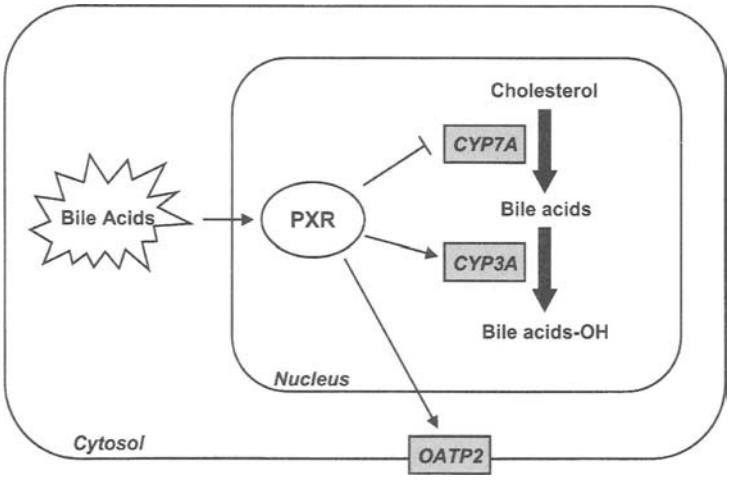

Figure 8.2. An overview of the PXR's involvement in regulation of bile acid metabolism. Bile acids, such as LCA can bind and activate the PXR to regulate hepatic gene expression. The PXR negatively regulates the expression of CYP7A, which catalyzes the rate-limiting step in the conversion of cholesterol to bile acids. Conversely, the PXR upregulates the expression of CYP3A and OATP2, which are involved in the metabolism and transport of bile acids, respectively. This coordinate regulation of genes results in the increased clearance of toxic bile acids from the hepatocyte.

that treatment of rats with PCN decreased the expression of CYP7A1, the enzyme that catalyzes the rate limiting step in the synthesis of bile acids from cholesterol[41]. To examine whether the PXR played a role in the repression of CYP7A1, a PXR-null mouse model was utilized[40]. It was demonstrated that the PXR mediated not only the repression of CYP7A1 by PCN, but also its basal expression. Additionally, the organic anion transporter polypeptide 2 (OATP2), a bile acid transporter, was found to be induced by PCN in wild-type animals but not in PXR-null mice. When bile acids were examined for their ability to activate the PXR, it was found that a secondary bile acid, lithocholic acid (LCA), was an efficacious activator of both mouse and human PXR[40]. In a parallel study utilizing the humanized PXR mouse model, bile acids such as LCA were identified as PXR ligands that could induce CYP3A expression and it was shown that CYP3A catalyzed the hydroxylation and detoxification of bile acids[42]. Administration of LCA to mice results in severe hepatotoxicity. Both studies demonstrated that PXR-null mice were resistant to LCA toxicity and furthermore, that sustained activation of the PXR protected against LCA-induced hepatotoxicity in wild-type mice[40, 42]. Collectively, these findings demonstrate that a regulatory loop exists by which elevated concentrations of bile acids activate the PXR to block new bile acid biosynthesis and to induce the metabolism and excretion of existing bile acids (Figure 8.2).

2.6. Future Research

The finding that CAR binds to many of the same response elements as the PXR and that these two receptors share many ligands and target genes has made it clear that the net effect of a xenobiotic on CYP3A gene expression will often depend on more than one receptor pathway. Identifying all of the NRs involved in the regulation of CYP3A will be necessary in the future. In addition, the promoters of many suspected PXR target genes, including CYP7A and OATP2, have not yet been characterized. Since the expression of CYP3A is coordinately regulated by PXR, CAR, and other NRs, it seems likely that other PXR target genes are regulated in a similar fashion. The analyses of regulatory regions in novel genes may provide additional insight as to how the PXR interacts with other NRs at response elements to regulate gene expression. In the future, continued analyses

of the cross talk that occurs among the PXR and other NRs will be an exciting area of research that will eventually provide the details necessary to understand how the PXR works with other receptors to form a master regulatory circuit that controls CYP3A expression.

3. The Constitutive Androstane Receptor

3.1. Introduction

In early studies it was observed that treatment of rats with phenobarbital (PB) caused a marked proliferation of the liver and endoplasmic reticulum, an increase in DNA synthesis, and increased activities of drug- and steroid hormone-metabolizing enzymes[43, 44]. PB is now considered the prototype for a large group of structurally diverse, lipophilic chemicals that induce a similar spectrum of effects. PB and PB-type chemicals induce the expression of numerous cytochrome P450 genes, including genes in the CYP1A, CYP2B, CYP2C, and CYP3A subfamilies[45, 46]. Of these, the CYP2B subfamily is most effectively induced and will be discussed here as a paradigm.

The coordinate induction of hepatic enzymes by PB has long been recognized to require direct activation of transcription[47]. While evidence was suggestive of a receptor-mediated process, studies aimed at identifying a PB-binding receptor were hindered for years by lack of an appropriate model system. A significant advance in understanding PB-induced gene expression came with the characterization of a regulatory element in the CYP2B genes. Using transgenic mice containing rat CYP2B2 promoter constructs of different lengths, it was determined that PB-responsiveness was due to regulatory regions at least ~1 kb upstream of the CYP2B2 core promoter region[48]. Experiments in primary cultures of rat hepatocytes identified a 163 bp fragment ~2.3 kb upstream of the CYP2B2 gene that conferred PB-responsive activity and this enhancer was termed the PB response element (PBRE)[49]. The responsiveness to PB conferred by the PBRE was eventually refined to a core 50 bp element that contained three distinct DNA-binding motifs[50]. Later, a similar 51 bp enhancer was characterized in the mouse CYP2B10 gene and was termed the PB responsive enhancer module (PBREM)[51, 52]. Sequence analysis revealed that the PBREM contained two DR4 motifs, commonly referred to as NR-binding sites one and two (NR1 and NR2), which flanked a nuclear factor 1 (NF1)-binding site[31, 52].

3.2. The Nuclear Receptor CAR

A search for the receptors capable of binding to PBREM ensued in the hopes of identifying the elusive "PB receptor." Findings from two laboratories were incorporated to eventually identify the NR that could bind PBREM in response to PB. In one experimental approach, proteins that could bind the NR1 sequence of the PBREM were isolated from PB-treated mouse liver nuclear extracts using DNA affinity chromatography[53]. When the proteins were analyzed using electromobility shift assays with the NR1 element and various NR antibodies, it was found that the NR1-nuclear protein complex contained RXRα. A search of the literature revealed that a separate laboratory had previously identified a liver-enriched orphan NR that could function as a heterodimer with RXRα to bind a retinoic acid receptor element (RARE), which contains a DR5[54, 55]. The orphan receptor had originally been identified as a "constitutively active receptor," or CAR, based on findings that the receptor could activate transcription from a RARE without the addition of exogenous ligand[55]. Based on these earlier findings, the unidentified NR binding to NR1 of the PBREM was postulated to be CAR. Further experimentation using primary mouse hepatocytes and whole animals proved this to be the case and, furthermore, suggested that CAR could mediate the induction of CYP2B by PB[31, 52, 53, 56]. Contrary to findings in early studies that used transfected cell lines, it was later demonstrated in primary hepatocytes and *in vivo* that CAR is sequestered in the cytosol in untreated cells and that its nuclear translocation is dependent on treatment with PB or PB-type chemicals[56, 57].

3.3. Mediators of CAR Activity

Although PB treatment induces the nuclear translocation and transcriptional activity of CAR, results from ligand-binding assays have indicated that neither PB nor known PB metabolites are

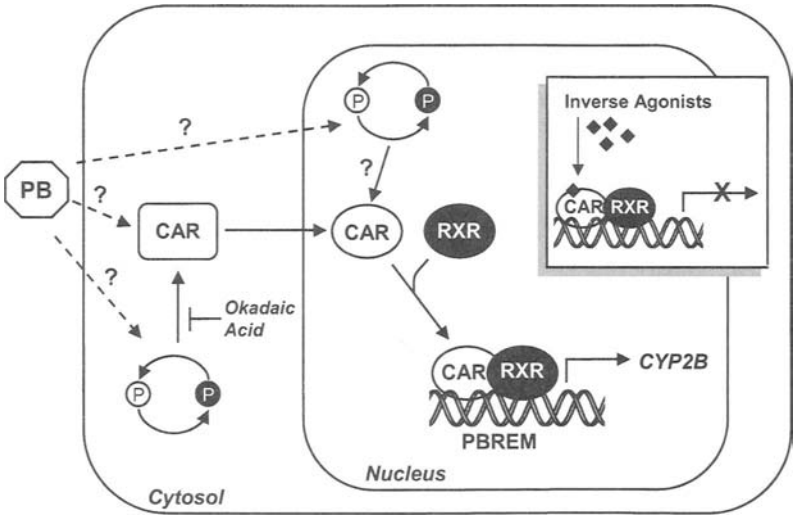

Figure 8.3. A model of CAR-mediated induction of CYP2B expression by PB. Inactive CAR normally resides in the cytoplasm. PB acts on unknown cellular targets to induce the nuclear accumulation of CAR. In the nucleus, activated CAR heterodimerizes with RXR to bind response elements in the promoter of CYP2B and other target genes to induce gene expression. Phosphorylation events are thought to be important in regulating the CAR signaling pathway. *Inset:* In the absence of PB and other CAR activators, inverse agonists can bind CAR and repress its transcriptional activity.

bona fide CAR ligands. So how might PB function to activate CAR? Because of CAR's apparent constitutive activity, it has been postulated that subcellular localization may be a major determinant of receptor activity. In this regard, treatment with okadaic acid, a phosphatase inhibitor, has been shown to block the PB-induced nuclear translocation of CAR[56]. These findings suggest that the localization of CAR in the cell may be regulated by phosphorylation events. Thus, PB may activate CAR-mediated gene transcription by altering the phosphorylation status of the receptor or related cellular targets, resulting in CAR's translocation to the nuclear compartment (Figure 8.3). Additional phosphorylation events in the nucleus have also been postulated to be important[58].

Ligands that bind directly to CAR have also been identified. Initially, in a search for CAR activators, it was discovered that the constitutive activity of CAR could be repressed by androstanes, which are testosterone metabolites[59]. As a result, CAR is now known as the "constitutive androstane receptor." Androstanes bind CAR directly to repress its transcriptional activity and have been termed "inverse agonists"[32, 59]. While androstanes are effective mouse CAR (mCAR) inverse agonists, they have little effect on human CAR (hCAR). Also, it is important to note that supraphysiological concentrations of androstanes are required to repress mCAR-mediated gene expression; thus, androstanes are not likely the physiological ligand of CAR. Interestingly, pharmacological concentrations of several endogenous steroids have also been demonstrated to activate (estrogens) or repress (androgens, progesterone) mCAR activity while having little effect on hCAR activity[58]. Thus far, the only identified steroidal compound that exerts activity toward hCAR is the progesterone metabolite 5β-pregnane-3,20-dione, which at pharmacological concentrations can directly bind the receptor and increase its activity above the constitutive level[32]. Collectively, these findings raise the intriguing possibility that a yet unidentified physiological steroid may function as an endogenous CAR ligand to either activate or repress activity.

Direct-binding xenobiotic ligands of CAR have also been identified and many of these show marked species specificity as well. For example, the planar hydrocarbon 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene binds directly to mCAR and is

the strongest agonist identified to date, but it apparently lacks activity toward hCAR[60]. Conversely, an agonist selective for hCAR, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime, has recently been identified[61]. Moreover, the antifungal agent clotrimazole is a potent inverse agonist of hCAR while it has little or no activity toward mCAR[32]. Other CAR xenobiotic activators that have been reported include PCBs, chlorinated pesticides such as DDT, and methoxychlor[31, 32].

3.4. Activation of Transcription

The fact that both PB and direct-binding ligands can regulate CAR suggests that there are multiple mechanisms for the regulation of CAR activity. While important differences likely exist in the cellular targets affected by receptor agonists compared to PB, agonists of CAR are similar to PB in that they induce the nuclear translocation and binding of CAR to DNA. A model of how PB may induce CYP2B expression through the CAR pathway is shown in Figure 8.3. PB interacts with unknown cellular targets to likely alter the phosphorylation status of CAR and induce its translocation to the nucleus. The receptor may undergo further modifications before binding as a heterodimer with RXR to PBREM to induce CYP2B expression. The PBREM is highly conserved in rat, mouse, and human CYP2B genes. The NR1 site seems to serve as the major CAR-binding site and is critical for CAR transactivation of CYP2B genes[53]. Once bound to PBREM, the final effect of CAR regulators on gene expression seems to be determined by the ability of CAR to recruit coactivators to the transcriptional complex. In this regard, it has been demonstrated that CAR can interact with a number of coregulators, including SMRT, SRC-1, and GRIP-1[59, 62, 63].

In addition to the DR4 elements in PBREM, CAR can bind to a variety of DNA motifs including DR3 elements, DR5 motifs (e.g., those found in RARE), and ER6 motifs[31, 54, 60]. These response elements are the same as those recognized by PXR and, not surprisingly, CAR and the PXR share many overlapping target genes[46]. Indeed, it has been demonstrated that CAR transactivates the CYP3A genes by binding to the same response element that serves as the PXR-binding site[27, 28, 31].

Aside from the PXR, other NRs are also important in CYP2B expression. As mentioned earlier, HNF-4α is critical for CAR expression, as HNF-4α-null mice express neither PXR nor CAR[39]. Both the GR and HNF4-α can bind to elements in the CAR promoter to regulate the level of CAR expression, which in turn can influence the expression of CYP2B and likely other CAR target genes[36, 38]. The study of interactions of NRs with the CAR pathway is a relatively new area of investigation and roles for other NRs in CAR-mediated CYP expression are likely to be identified in the future.

3.5. Mouse Models

The generation of mice null at the CAR locus has recently been reported[64]. Mice lacking CAR are resistant to many of the toxic effects of PB, including hepatomegaly and increased DNA synthesis, confirming that CAR mediates these toxic phenotypes[64]. In addition, studies using this model have confirmed that CAR is essential in mice for the induction of the CYP2B genes by PB[64]. The CAR-null model has been invaluable in identifying novel PB-inducible genes that are regulated by CAR. The analysis of over 8,500 genes using DNA microarray technology was recently performed to examine PB-induced hepatic gene expression in CAR-null mice compared to wild-type mice[65]. Findings from this study demonstrate that CAR mediates the PB-inducible expression of numerous hepatic genes, both negatively and positively. After PB treatment, the expression of more than 70 genes was found to be dependent on CAR, while 60 genes were regulated in a CAR-independent manner. About half of the CAR-dependent genes encoded xenobiotic metabolizing enzymes (XMEs), highlighting the importance of this receptor in protecting organisms against xenobiotic exposure. Interestingly, some CAR-dependent genes were downregulated in response to PB and were found to encode proteins that play roles in basic liver function, fatty acid metabolism, and signal transduction. These findings provide evidence for the idea that CAR is not only important in regulating the expression of XMES, but also that it plays an important physiological role as well.

Using a combination of both PXR- and CAR-null mice, the ability of CAR and PXR to share

response elements and induce the same target genes has been demonstrated *in vivo*. For example, treatment of PXR-null mice with PB results in the induction of CYP3A and this has been shown to occur through CAR binding to the CYP3A promoter[31, 45]. Similarly, treatment of CAR-null mice with the mPXR ligand dexamethasone results in CYP2B induction through the binding of PXR to PBREM[46]. Through studies such as these, the relative contribution of each of these receptors on CYP gene expression has begun to be explored.

A "humanized" mouse model that expresses hCAR rather than mCAR in the liver has recently been engineered[66]. Given the fact that the effect of xenobiotics on CAR activity differs significantly among species, this model should prove useful in evaluating the relevance of toxic responses. For example, once the toxicity of an agent is determined to be dependent on mCAR using the CAR-null mouse model, the humanized mice can be used to evaluate whether the toxic response can also be mediated by hCAR. Recent studies employing this approach have implicated CAR in the hepatotoxicity of acetaminophen in humans[66]. It was found that acetaminophen at high doses activates hCAR and induces the expression of CYP1A2 and CYP3A, which are the enzymes that catalyze the rate-limiting step in the formation of toxic acetaminophen metabolites. These findings have identified CAR as a possible therapeutic target in cases of acetaminophen overdose[66]. In addition, a separate study using humanized mice and CAR-null mice demonstrated that CAR plays a role in protecting the body from elevated bilirubin levels by inducing the expression of enzymes involved in bilirubin clearance[67].

3.6. Future Directions

The identification of PBREM and CAR, have led to major advances in understanding how PB and PB-like chemicals regulate gene expression However, many unanswered questions remain. Exactly how PB interacts with the CAR signaling pathway to induce gene expression is still unclear. Moreover, it is not known if CAR agonists mediate gene expression by mechanisms similar to or distinct from those of PB. In addition, further investigation into how phosphorylation is involved in regulating the CAR pathway is important. The dependence of CAR signaling on phosphorylation may represent a model of activation that could be applicable to the other xenobiotic receptors. It is not known whether physiologically relevant endogenous agonists and inverse agonists exist. If identified, these endogenous CAR ligands will offer clues as to what role CAR plays in normal physiology. Compared to the PXR, CAR seems to bind to a more limited spectrum of steroidal compounds and xenobiotics. Solving the crystal structure of CAR's LBD will allow for the investigation of how ligand specificity is determined between these two receptors and may provide information useful in the evaluation of their separate but overlapping roles in regulating gene expression. Finally, the most challenging area of research for the future will be in understanding how CAR, the PXR, and other NRs interact to regulate CYP gene expression.

4. The Peroxisome Proliferator Activated Receptor α

4.1. Introduction

Peroxisome proliferators (PPs) are a group of structurally dissimilar chemicals that cause a similar spectrum of effects including a proliferation of peroxisomes in the hepatocyte, liver hyperplasia, and an increase in the expression of numerous enzymes involved in fatty acid oxidation[68]. The enzymes upregulated by PPs include a large number of enzymes involved in the β-oxidation of fatty acids and the CYP4A enzymes, which are important in the ω-oxidation of many medium and long-chain fatty acids[68, 69]. In the body, fatty acids are oxidized to produce energy when other substrates are not available, such as during times of fasting or starvation[70].

The ability of PPs to cause the rapid, coordinate transcriptional upregulation of gene expression in a tissue-specific manner suggested that PPs acted through a NR-mediated mechanism[71]. This proved to be the case when a screen for novel NHRs identified a mouse cDNA encoding an orphan receptor that could be activated by known PPs, such as the drug clofibrate[72]. The receptor was named the peroxisome proliferator activated receptor, or PPAR[72]. In later studies, the rat homologue of the PPAR

was identified and it was found that the receptor could not only be activated by PPs, but also by endogenous fatty acids[73].

4.2. PPAR Isoforms

The PPAR that was originally cloned from mouse is now known as the alpha isoform, or PPARα. This designation arose after the identification of two additional distinct PPAR isoforms, termed PPARβ (also referred to as δ) and PPARγ The three PPAR isoforms are encoded by three separate genes and have been identified in many species including human, rat, and rabbit[68]. The three PPAR isoforms play distinct roles and display tissue specific expression patterns[74]. The PPARα is highly expressed in the liver and kidney and plays a major role in regulating the catabolism of fatty acids. Not surprisingly, the CYP4A enzymes are coexpressed with PPARα in these tissues[75]. The PPARγ gene actually gives rise to two gene products, PPARγ1 and PPARγ2, through differential promoter usage. The PPARγ2 isoform is highly expressed in adipose tissue and mediates adipogenesis and lipid storage; however, PPARγ1, which is expressed more broadly and at lower levels, can also induce adipogenesis[76]. The PPARβ is ubiquitously expressed and while the exact physiological function of this isoform is still unclear, recent findings have suggested that this isoform modulates the activity of both PPARα and PPARγ[77]. Since PPARβ and PPARγ do not seem to regulate the expression of CYP4A or any other P450 enzyme, these isoforms will not be discussed to any great extent.

4.3. PPARα Ligands

While the quantitative effects of agonist binding on the activity of PPARα seem to be species-specific, the spectrum of ligands that can activate the PPARα across species is similar. Clofibrate, originally recognized for its ability to increase both the number and size of peroxisomes when administered to rats, is considered the prototype for a class of drugs called fibrates, which are all potent PPARα ligands[78, 79]. The fibrate drugs are widely used today as lipid lowering agents in humans. Other synthetic ligands of the PPARα include the industrial plastisizer mono (2-ethylhexyl) phthalate, trichloroacetic acid, and the pesticide DTT[80]. Interestingly, these xenobiotics induce the expression of CYP4A even though this enzyme does not seem to play a role in their metabolism.

Many of the endogenous fatty acids that are metabolized by CYP4A are also PPARα ligands. These include an array of saturated and unsaturated very long-chain fatty acids, such as linoleic acid, palmitic acid, and arachidonic acid[73, 81]. Moreover, findings using acyl-CoA oxidase (AOX)-null mice suggest that the acyl-CoA derivatives of very long-chain fatty acids are most likely endogenous PPARα ligands. In mice with a disrupted AOX gene, acyl-CoA derivatives accumulate to high levels and the animals display a phenotype similar to that seen after treatment of rodents with synthetic PPs[82]. Some eicosanoids and eicosanoid metabolites that are important mediators of inflammation, including leukotriene B_4 and prostaglandins, are PPARα ligands[83, 84]. These arachidonic acid derivatives can be metabolized by CYP4A to compounds that are inactive in terms of mediating the inflammatory response[75]. In light of the role that PPARα plays in the induction of CYP4A, it is not surprising that mice lacking PPARα have been demonstrated to display a prolonged inflammatory response[85].

4.4. Activation of Transcription

The experimental drug Wy14643, an acetic acid derivative of clofibrate, is a potent PPARα agonist and was instrumental in elucidating the signaling pathway of PPARα. The PPARα binds as a heterodimer with RXR to DNA motifs termed peroxisome proliferator response elements (PPREs) (Figure 8.4)[86]. The core PPRE sequence was initially identified as an imperfect DR1 motif by analyzing the promoter of the AOX gene[87]. Unlike the PXR and CAR, PPARα can form heterodimers with either ligand-free or 9-*cis* retinoic acid-bound RXR, and ligand binding to either RXR or PPARα can activate gene expression through PPREs[88, 89]. Other NRs can also bind to PPREs and competition for binding has been observed among the three PPAR isoforms as well as HNF-1, thyroid receptor, and RXR/RXR dimers. Depending on the NR complex bound to the PPRE, the transcription of a target gene can be either activated or repressed. Studies have shown

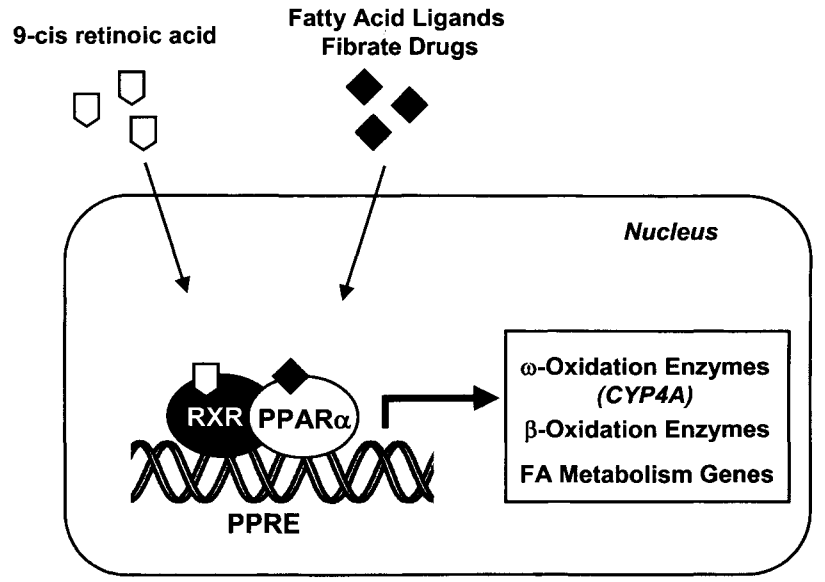

Figure 8.4. A model of the transcriptional regulation of gene expression by PPARα. The PPARα binds as a heterodimer with RXR to PPREs upstream of target genes. Ligand binding to either the RXR or PPAR activates the transcriptional complex resulting in the induction of numerous genes involved in fatty acid oxidation and metabolism, such as CYP4A.

that the specificity of a PPRE for NR binding is determined not only by the sequence of the DR1 element, but also by the sequence immediately 5′ of the PPRE[90, 91].

As with other NRs, transcriptional regulation by the liganded PPAR involves the interaction of many cofactors that form distinctive multiprotein complexes. In addition to several common coregulators that have been shown to interact with the PPAR (e.g., SRC-1, CBP, SMRT), coactivators that seem to be PPAR-specific have also been identified including PPAR binding protein (PBP) and PPAR interacting protein (PRIP). Interestingly, a transcriptionally active PPARα-interacting cofactor (PRIC) complex from rat liver nuclear extracts was recently isolated and was found to contain over 25 different proteins, including PBP, PRIP, CBP, and a novel coactivator PRIC285[92]. The composition of this PRIC complex may provide an insight into the basis for differences in tissue and species sensitivity to PPs.

The crystal structures of the PPARα LBD bound to an agonist with a coactivator motif from SRC-1 and, alternatively, bound to an antagonist with a corepressor motif from SMRT have recently been resolved[93, 94]. It was found that agonists cause the recruitment of coactivators by interacting with the ligand-dependent activation helix (AF-2) to maintain it in an active conformation. In this active conformation, the AF-2 helix can bind tightly to LXXLL motifs in coactivators and stabilize coactivator binding into a hydrophobic cleft that is formed in the receptor[93]. In the unliganded state, the PPARα is preferentially bound to corepressors. The crystal structures reveal that corepressor motifs bind to a hydrophobic groove in the receptor and prevent the AF-2 helix from interacting with coactivators. The binding of antagonists further stabilizes the inactive conformation of the receptor by altering the position of a residue in the AF-2 helix that is critical to agonist binding. These studies have demonstrated how ligands can promote basic structural changes in the PPARα to mediate its interaction with coregulators, and hence, its transcriptional activity. Results of these studies have also allowed for the realization that NRs can distinguish coactivators from corepressors by the length of their conserved interaction motifs. Importantly, these findings have also resulted in a model of

receptor repression that can likely be applied to many NRs[94].

4.5. Species Differences

Quantitatively, the response of humans and rodents to PPs has been found to differ dramatically. Humans are indeed responsive to PPs in regard to their ability to reduce serum lipids *in vivo*, a response known to be mediated by PPARα in rodents[95]. However, chronic exposure of rats and mice to PPs causes a dramatic peroxisome proliferation response in the liver and eventually leads to liver tumors. These PP-induced toxicities have not been observed in humans even though the fibrate drugs have long been used at high doses in humans to lower triglyceride and cholesterol levels[71, 96]. Several mechanisms have been postulated to play a role in the seemingly refractory nature of humans to PP toxicities. Different expression levels of PPARα and the existence of a splice variant of PPARα in humans that may negatively regulate PPARα have been suggested to play a role[97, 98]. Recently, the human PPARα transgene was introduced by an adenoviral approach into PPARα-null mice and was found to be as effective as the mouse PPARα in transcriptionally activating PPARα target genes under *in vivo* conditions[99]. The findings of this study demonstrate that the human PPARα is fully competent to induce PP-induced pleiotropic responses in the context of mouse liver[99]. Thus, other factors in the human liver environment are likely important in PPARα function and in determining the PP response in humans. Competition between PPARα and other NRs for binding to RXR or coactivators has been postulated to play a role in species differences[80]. Moreover, differences in the sequence of PPREs and surrounding sequences in target genes exist between humans and rodents, and it is not known exactly how this affects PPARα transactivation potential *in vivo*. The analysis of changes in global gene expression in wild-type and null animals in response to PPs has been performed using DNA microarrays and this approach may eventually allow for a better understanding of how the PPARα mediates the toxic response to PPs in rodents[100].

4.6. Mouse Models

A PPARα-null mouse model has been generated and the animals are viable and fertile[101]. While exhibiting no detectable gross phenotype in the fed state, experiments using null mice have demonstrated that the PPARα plays an important role in the hepatic response to fasting. Unlike wild-type mice, fasted PPARα null animals do not upregulate the expression of fatty acid oxidation enzymes, including CYP4A, and they exhibit hypoglycemia, hypoketonemia, and elevated plasma levels of free fatty acids[102]. These findings and others have demonstrated that PPARα plays a central role in maintaining lipid homeostasis.

The PPARα-null mice do not display the typical toxic responses after exposure to PPs. Studies have shown that treatment of null mice with PPs does not induce liver hyperplasia, peroxisome proliferation, or hepatocarcinogenesis, confirming that PPARα is the mediator of these PP-induced toxic responses[80, 101]. Moreover, these findings suggest that the PPARβ and PPARγ isoforms do not play a critical role in these PP-induced toxicities. The induction of CYP4A and many other fatty acid oxidation enzymes in response to PPs is also absent in mice lacking PPARα confirming that it mediates the induction of these enzymes. Interestingly, while PPARα-null mice have lost the CYP4A induction response, basal levels of CYP4A are not affected, indicating that other NRs control the constitutive expression of CYP4A[102].

Studies using rodents have also demonstrated that a normal AOX gene is necessary for proper physiological regulation of the PPARα[82]. The AOX gene encodes an enzyme critical in the β-oxidation of certain very long-chain fatty acid acyl-CoA metabolites[68]. Targeted disruption of the AOX gene in mice results in sustained PPARα activation, leading to profound peroxisome proliferation and increased levels of PPARα target genes, such as the CYP4A genes[82]. These findings suggest that acyl-CoA metabolites, and possibly other unmetabolized oxidase substrates, are endogenous ligands of the PPARα and that AOX is critical in metabolizing these ligands *in vivo*.

4.7. Future Directions

Over the last 10 years, great strides have been made in understanding the biology of the PPARα. The synthesis of specific and potent PPARα agonists have made it possible to examine the mechanism of signal transduction of the PPARα. Furthermore, the resolution of the crystal structures

of PPARα bound to ligands and coregulators has resulted in a model of how agonists and antagonists alter the conformation of NRs to mediate coregulator binding. In the future, a more complete understanding at the molecular level is needed as to how PPAR/coregulator complexes interact with other proteins to modulate gene expression in a species- and tissue-specific fashion.

The generation of a PPARα-null mouse has been critical in establishing a major role for PPARα in lipid homeostasis and has confirmed the role of PPARα in PP-induced toxicity in rodents. However, many questions remain concerning differences between mice and humans in regard to the PPARα pathway. The basis for species differences in the response to PPs is unclear and it is not known what role differences in the expression of CYP4A and other PPARα target genes play in mediating this response. In the future, generation of a "humanized" PPARα mouse, such as those available for the NRs, PXR and CAR, will be useful for long-term studies to investigate species differences and to allow for the more accurate extrapolation of findings to human risk assessment when evaluating PP-induced toxicities.

5. The Aryl Hydrocarbon Receptor

5.1. Introduction

Nearly 50 years ago, it was noted that rats exposed to 3-methylcholanthrene (3-MC) displayed a marked increase in metabolic capacity toward that substrate and other polycyclic aromatic hydrocarbons (PAHs)[103]. This enhanced metabolic activity was referred to as "aryl hydrocarbon hydroxylase" (AHH) based on the ability of these enzymes to efficiently hydroxylate aromatic hydrocarbons[104]. It is now known that AHH activity is the collective activities of the CYP1A1, CYP1A2, and CYP1B1 enzymes.

Over the next 30 years, two lines of evidence led to the identification of the AHR, the protein that functioned as the PAH sensor and regulated AHH activity. The first indications that such a receptor existed came from genetic studies of inbred mouse strains. Early studies demonstrated that C57BL/6 mice were much more responsive than DBA mice to the PAH-induced upregulation of AHH activity[105]. Using classical genetic approaches, the locus responsible for the AHH inducibility phenotype was shown to segregate in a simple autosomal dominant fashion. This locus was termed the "*Ah*" locus because of its ability to mediate responsiveness to *a*ryl *h*ydrocarbons[106, 107]. The allele found in the more responsive C57BL/6 strain was designated as Ah^b while the allele that conferred decreased responsiveness in DBA mice was termed Ah^d[108].

The second line of evidence came from pharmacological studies using an extremely potent inducer of AHH activity, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or "dioxin")[109]. Using radiolabeled TCDD, a receptor in mouse liver cytosol was identified that bound this ligand with high affinity and in a saturable and reversible manner[110, 111]. The proof that this TCDD-binding site was in fact the AHR was 3-fold. First, it was found that receptor isolated from mice harboring the responsive Ah^b allele bound ligands with higher affinity than did receptor isolated from mice harboring the less responsive Ah^d allele[105, 106, 112, 113]. Second, competitive binding studies with various dioxin congeners revealed that binding affinities correlated with their potency as inducers of AHH activity[114–116]. The last line of evidence was biochemical in nature. In the absence of ligand, the receptor was found in the cytosolic fraction of cell extracts; however, the binding site/receptor was found in the nuclear fraction after exposure to ligand[117]. Thus, genetic, biochemical, and pharmacological evidence demonstrated that the *Ah* locus encoded the AHR and this protein was the mediator of AHH induction.

5.2. The AHR

It was many years before the AHR was cloned and characterized. Attempts to purify the receptor were initially hampered by its low cellular concentration and relative instability. The development of a photoaffinity ligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin, was the essential step that allowed the eventual purification of the AHR[118, 119]. Once the receptor was purified, a partial amino acid sequence was obtained and this lead to the cloning of the AHR cDNA from mouse liver[120, 121]. The deduced amino acid sequence revealed that the AHR was

as a member of the PAS superfamily of proteins[120, 121]. The AHR was found to be most similar in amino acid sequence to the AHR nuclear translocator (ARNT). Interestingly, ARNT had been cloned only a year before in a screen to identify gene products that were important in AHR signaling in a mouse hepatoma cell line[122]. One mutant cell line that was deficient in signaling expressed normal amounts of AHR, but the cells did not upregulate AHH activity after agonist exposure. A human genomic DNA fragment that rescued the mutant phenotype was found to contain the ARNT gene product. Further experimentation demonstrated that the corresponding ARNT protein was required to direct the activated AHR to specific regulatory elements upstream of target genes such as CYP1A1[123]. The structural similarities of the AHR and ARNT were recognized and it was postulated that the proteins might be dimerization partners. This proved to be the case, making AHR and ARNT the first PAS protein heterodimer to be shown to have physiological relevance[123, 124].

The overall structural organization of the AHR is typical of most members of the PAS superfamily of proteins. The N-terminus of the AHR contains a bHLH domain that is important in dimerization and subsequent positioning of the basic regions of the proteins such that they can bind to specific DNA enhancer motifs[125–127]. As in most PAS proteins, the bHLH region is found immediately N-terminal to the PAS domain. The 250–300 amino acids comprising the PAS domain contain two highly degenerate repeats, termed "A" and "B" repeats[6]. The PAS domain of the AHR harbors the LBD, a dimerization surface for binding to ARNT, and an interaction surface for chaperones such as Hsp90 and ARA9 (also called AIP, or XAP2)[6, 128, 129]. The region of the AHR important in ligand binding and chaperone binding overlaps the PAS B repeat[125, 130]. The C-terminus of the AHR encodes a hypervariable TAD[125].

The AHR is expressed in many cell types and tissues with high levels of expression found in placenta, lung, thymus, and liver. The expression profile of the AHR is in good agreement with the expression of PAH-target genes. However, the expression of CYP1 genes is fairly tissue specific, with CYP1A2 primarily found in the liver, CYP1A1 highly expressed in epithelial cells throughout the body, and CYP1B1 found in mesenchymal cells[131, 132]. This indicates that factors other than AHR expression level are involved in the tissue specific expression of these CYP1 genes.

5.3. AHR Ligands

Putative orthologues of the AHR have been identified in numerous higher eukaryotes, including nematodes, insects, fish, birds, and mammals. Striking differences in molecular weight of the AHR are observed among various species, and even in different strains of laboratory mice. This difference is mostly due to differences in the length of the C-terminus and results from different stop codon usage. Despite differences in receptor size, the vertebrate AHR signaling pathway is highly conserved across species and the induction of CYP1 gene expression is observed in all species[133, 134]. Importantly, significant species and strain differences have been observed in ligand-binding affinities. It seems that changes in specific amino acid residues in the LBD may be responsible for these differences. For example, the *Ah* alleles found in C57BL/6 and DBA mice exhibit a 10-fold difference in ligand binding and this arises, at least in part, from an alanine to valine substitution at amino acid 375[135, 136]. Moreover, the human AHR has the same mutation at the corresponding amino acid and is similar to the Ah^d allele in that it binds the ligand with 10-fold less affinity compared with the Ah^b allele[124]. Since the crystal structure of the AHR has not been solved, the identification of amino acids important in ligand binding has relied upon the examination of ligand-binding affinities of AHRs with different amino acid mutations.

The most extensively studied agonists are the halogenated aromatic hydrocarbons such as TCDD, polychlorinated biphenyls, and polychlorinated dibenzofurans as well as PAHs such as benzo[a]pyrene and 3-MC[3]. One of the highest affinity ligands of the AHR and the most potent inducer of CYP1A1 expression is TCDD. As the result of this ligand–receptor interaction, exposure to TCDD produces a wide variety of toxic effects that are species- and tissue-specific[3]. The response to TCDD is due to the fact that TCDD has a remarkably high affinity for the AHR (on the order of 10^{-12} M, K_D) and that this ligand is

resistant to metabolism. The toxic endpoints are dependent on the AHR and are thought to arise from long-term alterations in AHR-mediated gene expression, but it is still unclear if TCDD toxicity involves the transcriptional upregulation of CYP1A genes. The discussion of TCDD here will focus primarily on its use as a prototype agonist of the AHR and the mechanism by which it acts as an inducer of the CYP1 genes.

Apart from xenobiotics, it is assumed the AHR recognizes some endogenous ligand. While some endogenous compounds, such as heme degradation products, have been shown to bind and activate the AHR, no compound has been convincingly demonstrated to be the bona fide "endogenous AHR ligand"[137]. Naturally occurring AHR ligands have been found in teas, fruits, vegetables, and herbal supplements and include polyphenolic compounds such as flavonoids, indoles, and various carotinoids. The continued identification and analysis of these naturally occurring ligands may provide insight that could lead to the identification of an endogenous AHR ligand in the future, or to the identity of the environmental stresses that have led to the evolutionary conservation of the receptor system.

5.4. Activation of Transcription

While it has long been recognized that the expression of CYP1 genes is regulated at the level of transcription, it took many years to develop our current understanding of how the AHR mediates upregulation of gene transcription in response to xenobiotics. An overview of the mechanism of AHR-mediated gene expression is shown in Figure 8.5. In the absence of ligand, the AHR is found in a cytosolic complex with two molecules of Hsp90, an immunophilin-like chaperone protein known as ARA9 and the chaperone p23[138–141]. The Hsp90 chaperone is a necessary component of the AHR pathway and seems to anchor the receptor in the cytosol as well as hold the protein in a high affinity ligand-binding conformation[142–145]. The ARA9 protein has been shown to increase the amount of properly folded AHR in the cytoplasm, while the chaperone p23 has been suggested to play a role in regulating ligand responsiveness and receptor translocation[145, 146].

The signal transduction pathway of the AHR is well characterized and analogous to that of many NHRs, described above. Ligand binding to the

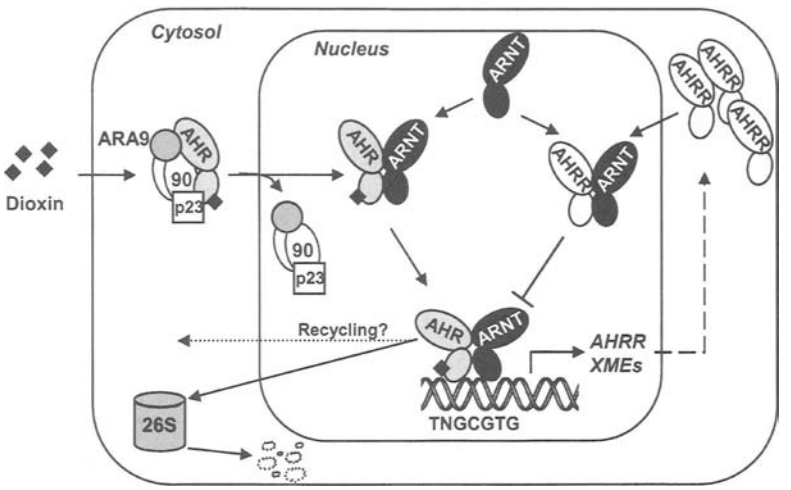

Figure 8.5. A model of AHR signal transduction. The AHR normally resides in the cytoplasm with the chaperones Hsp90, ARA9, and p23. Upon ligand binding, the AHR translocates to the nucleus where it exchanges its chaperones for ARNT. The AHR/ARNT heterodimer binds to dioxin response elements (DREs) to activate the transcription of downstream target genes, including the AHRR and XMEs, such as CYP1A. The ligand-activated AHR is exported from the nucleus and degraded through a proteosome pathway, or may undergo recycling within the cytoplasm. The AHRR protein, a negative regulator, can compete with the AHR for dimerization with ARNT resulting in inhibition of AHR-mediated gene expression.

cytosolic AHR induces a conformational change and nuclear translocation of the receptor. In the nucleus, the AHR sheds some of its associated chaperones and binds to its partner ARNT[147–150]. The resulting AHR/ARNT heterodimers bind to specific enhancers in DNA to alter DNA conformation and increase the transcription of target genes[151]. The enhancers, made up of the consensus sequence 5′-TNGCGTG-3′, were first characterized in the mouse CYP1A1 gene and have been called "dioxin responsive elements" (DREs), "xenobiotic responsive elements" (XREs), or "AH-responsive elements" (AHREs)[152–155]. For the remainder of this chapter, we shall refer to the enhancers as DREs. In addition to nucleotides in the core DRE, sequences outside of the DRE can modulate the binding affinity of AHR/ARNT to DNA and appear to be important determinants of AHR-mediated gene expression[155–157].

Functional DREs have been identified upstream of numerous AHR-inducible genes, many of which encode XMEs. These genes are collectively referred to as the *Ah* gene battery and include CYP1A1, CYP1A2, CYP1B1, NQO1 (NADPH : quinone oxidoreductase), ALDH3A1 (an aldehyde dehydrogenase), UGT1A6 (a UDP glucuronosyl transferase), and GSTYa (a glutathione *S*-transferase)[158]. The coordinate upregulation of these enzymes results in the enhanced metabolism of most inducers to hydrophilic compounds that can be more easily excreted from the body. Thus, the AHR plays an integral role in mediating the adaptive response to PAHs and related environmental chemicals.

Another interesting aspect of the AHR pathway is that prolonged agonist exposure results in the attenuation of signaling through the AHR. One mechanism by which this occurs is mediated through the AHR repressor protein (AHRR)[159, 160]. The AHRR is structurally similar to the AHR, except it lacks the PAS B-domain and its C-terminus functions as a transcriptional repressor. Because of these features, the AHRR can dimerize with ARNT in a manner that is independent of agonist. This heterodimer can bind to DREs to repress target gene transcription[159, 161]. The expression of the AHRR gene is controlled by a DRE and its transcription is upregulated upon exposure of the cell to AHR ligands. Another way the cell attenuates agonist-induced AHR signaling is by targeting ligand-bound AHR for degradation through the ubiquitin/proteosome pathway[162, 163].

The ARNT protein serves as a dimerization partner not only for the AHR, but also for other PAS proteins, such as the hypoxia inducible factors (HIF1α, HIF2α, HIF3α). When in a complex with ARNT, these various heterodimers mediate the upregulation of various genes important in dealing with cellular hypoxia[164]. It has been postulated that competition among PAS proteins for the limited pool of ARNT could be an important mechanism of transcriptional regulation. Some studies have found that activation of the HIF1α pathway can interfere with AHR-mediated induction of CYP1A1[165–167]. However, others have reported that simultaneous activation of both the HIF1α and the AHR pathways caused no changes in the expression level of any AHR or HIF1α target genes, suggesting ARNT is not a limiting factor[168]. These conflicting results may be due to differences in cell type and/or experimental conditions. Although cross talk between the AHR and HIF1α pathways seems to occur under certain conditions *in vitro*, it remains to be proven that competition for ARNT occurs *in vivo* and what, if any, effect this has on CYP1 gene expression or TCDD toxicity.

5.5. Mouse Models

Targeted disruption of the *Ah* locus in mice has been achieved by a number of laboratories. As expected, AHR-null mice fail to upregulate CYP1A1, CYP1A2, and other members of the *Ah* battery in response to AHR agonists[169, 170]. Furthermore, AHR-null mice are resistant to TCDD- and PAH-induced toxicity, confirming that the AHR is the mediator of dioxin toxicity[171, 172]. The AHR-null mouse models have also provided evidence for a physiological role for this receptor. These mice have defects in vascular development, display decreased fertility, and have overall decreased body weight compared to wild-type mice. Thus, in addition to mediating TCDD toxicity and the adaptive response to PAHs and other chemicals, the AHR clearly plays an important role in development. Such an observation supports the hypothesis that the AHR has an unknown endogenous ligand.

5.6. Future Directions

Significant advances have been made in many areas of AHR biology, especially in understanding

the AHR signal transduction mechanism in response to xenobiotics. However, the lack of a three-dimensional structure for the receptor has hindered the investigation of mechanisms underlying species-specific responses to certain ligands, such as TCDD. Also, it is not understood how ligand binding to the AHR alters its conformation to induce nuclear translocation. Determination of the crystal structure of the AHR will greatly facilitate the investigation of these and other aspects of AHR research. In spite of the questions remaining, the AHR signaling pathway has been a useful model to provide a broad understanding of the biological roles of PAS proteins. Yet, how the PAS domain mediates protein–protein interactions is still not fully understood. More in depth examination of the interactions between AHR and ARNT in the future should prove helpful in the identification and characterization of PAS domain function. Finally, although we know the AHR plays an important physiological role in development, the mechanism by which the AHR mediates these processes is not clear. For example, we do not know if AHR signaling during development is similar or different from the pathway by which AHR regulates xenobiotic metabolism. The ultimate identification of an endogenous AHR ligand will shed light on the physiological role of the AHR.

6. Conclusions

Over the last decade, great strides have been made in understanding the roles that the nuclear receptors PXR, CAR, PPARα, and AHR play in the induction of CYP genes. The ability of xenobiotics to bind and activate NRs to induce the expression of the CYP enzymes involved in their metabolism provides a mechanism by which an organism can mount an adaptive response to its changing chemical environment. The identification of endogenous ligands for some NRs indicates that these receptors play important roles in regulating CYP levels during physiological processes as well. It has become clear that the expression of many CYP genes is dependent on more than one NR. Recent studies have demonstrated that NRs often share xenobiotic ligands, response elements, and target CYP genes. The existence of multiple xenobiotic receptors with broad and sometimes overlapping functions likely increases the ability of an organism to detect and respond to a wide range of chemicals. The challenge for the future will be to understand how the NRs participate in a complex network to regulate CYP gene expression and to mediate the physiological response to xenobiotics.

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