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Topic 1.7

Biological function and mode of action of nuclear xenobiotic receptors*

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Abstract: Two related nuclear receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), act as xenobiotic sensors that protect the body from a multitude of foreign chemicals (xenobiotics) and play a central role in the metabolism and clearance of steroids and toxic endogenous lipids (endobiotics). A structurally diverse array of chemicals including pharmaceutical drugs, steroids, herbal extracts, and pesticides activate PXR or CAR. This activation results in induction of overlapping, but yet distinct drug clearance pathways consisting of cytochrome P450 enzymes, conjugating enzymes, drug transporters, and other related proteins. Similar pathways are also utilized to protect the body from toxic compounds of endogenous origin. Thus, the xenobiotic regulatory circuit contributes both to drug–drug and food–drug interactions as well as endocrine disruption. Consistent with the notion that xenobiotic receptors regulate drug clearance, single nucleotide polymorphisms (SNPs) in either the receptors themselves or receptor-binding sites in the regulatory region of genes encoding metabolic enzymes appear to contribute to the polymorphic expression of components of drug clearance pathways. Together, the xenobiotic receptors PXR and CAR confer metabolic immunity via the ability to control an integrated array of target genes.

XENOBIOTIC METABOLISM AND CLEARANCE: OVERVIEW

In the process of consuming foods, numerous foreign compounds enter our body that are neither used as dietary energy sources nor as building blocks for biological matrices. Uptake of such xenobiotics occurs mainly with food and water consumption, but also by inhalation or transdermally. In addition, normal metabolism and residing microbes, such as intestinal bacteria, can produce de novo toxins. Unless metabolized and eliminated, these substances may accumulate, giving rise to harmful effects by a variety of mechanisms including interaction with hormone receptors (endocrine active substances, i.e., EASs) or reaction with nucleic acids (genotoxic carcinogens) and membrane solubilization (cytoxic lipids). Thus, the entero-hepatic system has evolved to efficiently detoxify and eliminate unwanted chemicals. This system consists of microsomal cytochrome P450 enzymes (CYPs) and other oxidating and hydroxylating enzymes (phase I response), conjugation enzymes such as glucuronosyl- and sulfotransferases (phase II response) and membrane-bound drug pumps such as MDR1 (phase III), that function in a concerted fashion to inactivate and clear chemical compounds (reviewed in ref. [1]). The same system is also utilized to metabolize endogenous compounds such as steroids, bile acids, thyroid hormone, retinoids, cytokines, and fatty acids. To cover the potential diversity of molecular structure that is present within a given ecosystem, metabolic enzymes and drug transporters must have broad speci-

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ficities. Furthermore, each class and subclass of enzymes forms a large family with overlapping, yet distinct substrate specificity.

One characteristic of xenobiotic metabolizing enzymes and transporters is their inducibility by their substrates. This allows enhanced production of these proteins only as needed. For example, the antibiotic rifampicin induces CYP3A isozymes, whereas the anti-epileptic drug phenobarbital (PB) or the planar hydrocarbon 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) induces CYP2B isozymes. These compounds also induce a variety of other metabolic enzymes and transporters. While the aryl hydrocarbon receptor (AhR) mediates induction of CYP1A, CYP1B, and glutathione-*S*-transferase (GST) by dioxin and other structurally similar polycyclic aromatic hydrocarbons, the molecular basis through which the vast majority of drugs and toxins induce the xenobiotic response was only recently uncovered.

Orphan nuclear receptors are structurally related to known nuclear hormone receptors, but lack previously identified physiological ligands or activators. In the last decade, the biological role of several of these orphans, in particular those which act as heterodimers with the retinoid X receptor (RXR), have been revealed through the isolation of relevant endogenous ligands as well as by generation of knock-out mouse models that lack the functional receptors. This work has led to the realization that many orphans act as sensors for dietary lipids as opposed to high affinity endogenous hormones. For example, liver X receptor (LXR), peroxisome proliferator activated receptor (PPAR), and farnesoid X receptor (FXR) have been identified as sensors for cholesterol, fatty acids, and bile acids, respectively, to cooperatively regulate lipid homeostasis (reviewed in ref. [2]). In addition, two closely related receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), have emerged as xenobiotic sensors that mediate induction of drug clearance pathways to ensure rapid detoxification of potentially harmful substances. In this chapter, we will focus on PXR and CAR, their role in drug clearance, molecular mechanisms of their action, and the implication of xenobiotic regulation in pathophysiological conditions and endocrine disruption.

PXR, A MEDIATOR OF CYP3A INDUCTION

The rodent receptor PXR was originally identified as candidate xenobiotic receptor that mediates induction of CYP3A by known chemical inducers [3] (for review, see ref. [4]). The human homolog was first isolated as the steroid and xenobiotic receptor (SXR) [5] and the pregnane-activated receptor (PAR) [6]. Several lines of evidence indicate that mammalian PXRs mediate CYP3A regulation by xenobiotics. First, both PXR and CYP3A are expressed most abundantly in the liver and the intestine, the tissues most involved in drug metabolism. Second, PXR, together with its obligatory partner RXR, binds to the sites in the CYP3A promoter that are known to mediate drug-inducibility and transactivates gene expression. These sites form either DR3 (direct repeats of AGGTCA or closely related sequence with the spacing of three nucleotides) or ER6 (everted repeats with six nucleotides spacing) type elements depending on the species and isoforms. Third, PXR is activated by numerous structurally unrelated drugs including those known to induce CYP3A expression. The activation, in most cases, involves direct binding of inducing chemicals to the ligand-binding domain (LBD), resulting in dissociation of corepressor molecules such as the silencing mediator for retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (NcoR) and simultaneous recruitment of coactivator molecules, including members of the p160 family (SRC-1, GRIP, and ACTR), RIP140, and PBP (DRIP205 or TRAP220) [7]. Together, these observations suggest PXR may be the central factor contributing to CYP3A regulation. This idea is furthered by the species specificity of the CYP response. Notably, the PXR LBD from different species is considerably divergent. Within the LBD, amino acid identity of human PXR and mouse PXR is only 76 %, whereas the DNA-binding domain is highly conserved (96 % identity). Accordingly, PXRs from different species display different ligand-binding specificities. For example, the rodent orthologue can be activated by the glucocorticoid antagonist, pregnenolone-

16α-carbonitrile (PCN), while the human receptor cannot. In contrast, the human receptor is activated by the antibiotic rifampicin, a potent drug interactor, while the rodent ortholog is not. This is consistent with the long-known species differences in drug inducibility of CYP3A and suggests that the receptor the organism employs rather than the CYP3A gene promoter determines the response profile. Genetic evidence for this idea came from creation of loss of function and gain of function mice [8,9]. PXR null mice are both viable and fertile, indicating that in the absence of toxic insults the xenobiotic response is dispensable. However, PXR null mice completely lack inducibility of CYP3A by PCN or PCN-mediated induction of resistance against multiple toxins [8–11]. Furthermore, a transgenic mouse strain in which PXR was replaced by its human ortholog in the liver shows a "humanized" response to species-specific inducers [8]. These results together unequivocally establish PXR as the central mediator of CYP3A induction.

The creation of mouse models with humanized xenobiotic responses offers a standardized in vivo system for predicting potential human drug–drug interactions and may thus aid pharmaceutical development. Historically, drug-induction of CYP3A has been considered an unexplained adverse side effect associated with drug–drug interactions. For decades, rodent models have been standard components in the assessment of potential toxicities in the development of candidate human drugs. However, the reliability of rodents as predictors of the human xenobiotic response is compromised due to species-variation. Cultured human primary hepatocytes are valuable alternative tools, but are compromised by inter-individual variability, limited and unpredictable availability as well as high cost. Thus, the generation of the transgenic mice expressing human PXR and deficient in rodent PXR represents a major step toward generating a standardized humanized toxicological model.

CAR, A MEDIATOR OF CYP2B INDUCTION

The nuclear receptor CAR was identified as a candidate xenobiotic receptor that mediates induction of CYP2B by PB-type inducers [12] (for review, see ref. [13]). Expressed predominantly in the liver, CAR, together with its heterodimeric partner RXR, binds to the DR4 element in the CYP2B promoter that is known to mediate inducibility by PB. In most transiently and stably transfected cells, CAR shows constitutive activity and induces expression of endogenous CYP2B. Its constitutive activity is repressed by antagonistic steroids such as androstanol and androstenol [14], whereas treatment with PB and PB-type compounds reactivates CAR [15]. The role of CAR in mediating CYP2B induction by PB-type inducers was confirmed by generating a knock-out mouse strain which lacks the CAR gene locus [16]. Like PXR null mice, CAR null mice are viable and fertile, indicating the CAR function is also dispensable in the absence of toxic insult. As expected, CAR null mice completely lack inducibility of CYP2B by PB and a PB-type inducer TCPOBOP as well as PB-mediated resistance to zoxazolamine or sensitivity to cocaine or acetaminophen [16,17]. Together, these results unequivocally show that CAR is a mediator of CYP2B induction.

Like PXR, the LBD of CAR is also divergent among species (72 % identity between human and mouse). Not surprisingly, CAR also exhibits a strong species specificity for activators. For example, a potent mouse CAR agonist TCPOBOP and the reverse agonist androstanol are inactive for human CAR, whereas the potent human CAR reverse agonist clotrimazol is inactive for the mouse receptor [18]. However, since most of published studies are with mouse CAR or mouse liver cells, the extent of the species variation and the role of the receptor in the process is not clear.

A potential major distinction of CAR from other receptors is its constitutive activity in the absence of ligand in nonhepatic cells. Indeed, only TCPOBOP and androstanes are known to bind mouse CAR directly and modulate interaction with coactivator molecules such as SRC-1 and GRIP1 [14,18–20]. Rather, an alternative activation pathway appears to be employed that is indirect and does not involve ligand binding [21]. In vivo or in cultured hepatic cells, CAR is maintained in an inactive state by being localized to the cytoplasm. The inducing compound, while not binding, triggers a cytoplasm to nuclear translation resulting in activation of target genes. Although, the mechanism by which

CAR activators stimulate nuclear translocation is not yet clear, it is likely that some ligands including PB may act indirectly by triggering a juxtamembrane cascade, perhaps by a kinase, that modulates the LBD or an associated protein [22]. Defining the molecular basis of this process is important in understanding the signaling cascade of CAR xenobiotic activators.

PXR AND CAR CROSS-TALK

As discussed above, PXR and CAR were originally characterized as independent regulators of the CYP3A and 2B genes, respectively, presumably through distinct classes of drugs. The xenobiotic response elements in these two classes of CYP genes are also distinct, furthering the concept of independent regulation. Surprisingly, recent observations suggest that there is significant cross regulation of CY2Bs and 3As by these two receptors [8], which appears to be achieved by adaptive recognition of the opposing response element.

As the identity of PXR and CAR targets increases, it becomes progressively clear that they induce largely overlapping, if not identical, sets of genes. In addition to CYP3A and CYP2B, PXR appears to regulate the entire array of genes involved in metabolism and clearance of xenobiotics. These include: (1) members of cytochrome P450 enzymes, CYP2C, CYP1A, CYP1B, CYP2A, and CYP4F, which play central roles in inactivating endogenous hormones or exogenous toxins; (2) other phase I reductases and hydrolases such as carboxylesterase, monoamine oxidase, catalase, flavin-containing monooxygenases (FMOs); (3) conjugating enzymes such as UDP-glucuronosyltransferase (UGT), cytosolic sulfotransferase (SULT), and GST, which solubilize hydrophobic compounds to prepare for clearance; (4) finally, the membrane-bound transporters such as MDR1 and MRP2, which act as efflux pumps to clear drugs and drug conjugates (reviewed in [4], also see refs. [7,23–25]). In addition, PXR may regulate inducible nitric oxide synthase (iNOS) involved in the inflammatory response [26]. Like PXR, CAR has recently been shown to regulate a similar array of xenobiotic genes including several CYP enzymes, aldehyde dehydrogenase, esterase, FMO, methyl transferase, GST [27], SULT [28], UGT [29], and MRP2 [30] as well as iNOS [26]. Analysis of the promoter regions of these xenobiotic genes and identification of receptor binding sites reveals that both PXR and CAR can adaptively bind to common response elements [26,30,31], indicating that the DNA-binding specificities of these xenobiotic receptors are somewhat promiscuous. Regulation of a network of metabolic genes by a xenobiotic receptor suggests the existence of coordinated molecular cascade of drug clearance (Fig. 1). Furthermore, the ability of the receptors to respond to an overlapping set of drugs suggests a metabolic fail-safe system that confers a second layer of metabolic immunity from potentially toxic foreign compounds.

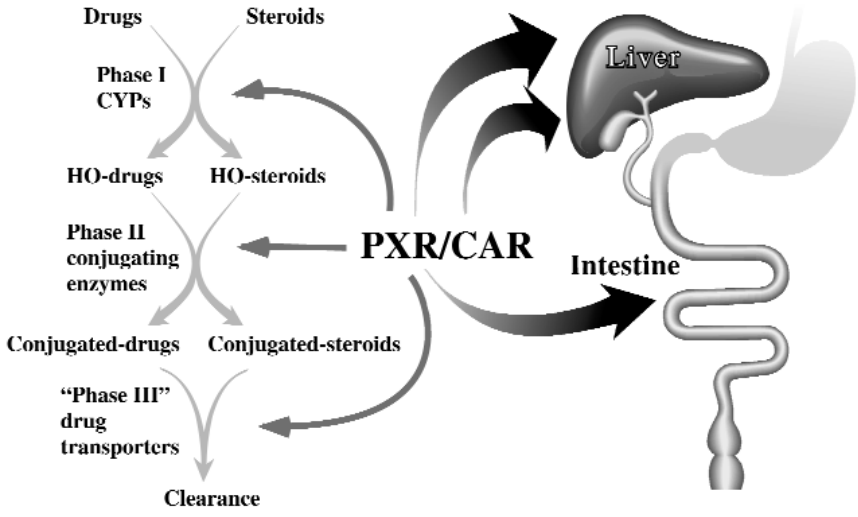

Fig. 1 Schematic representation of the mammalian xenobiotic response. PXR and CAR function as master regulators of xenobiotic response by activating both phase I and II xenobiotic enzymes, as well as the drug transporters. PXR is expressed throughout the enterohepatic axis, whereas CAR is predominantly expressed in the liver. Abbreviations: HO, hydroxylated.

MULTIPLICITY OF LIGANDS FOR XENOBIOTIC RECEPTORS

As a xenobiotic receptor, it is not surprising that PXR binds a diversity of structurally unrelated chemicals. In fact, X-ray crystal structures of the PXR LBD have revealed that its ligand-binding pocket is relatively large (1150 $Å^3$) compared to most other nuclear receptor LBDs, and can accommodate even a single hydrophobic ligand in multiple configurations [32]. The absence of a highly constrained pocket allows for molecular flexibility and plasticity of ligand recognition, in a fashion that is somewhat reminiscent of the low substrate specificity of xenobiotic enzymes. For example, the human receptor is activated by antibiotic macrolide rifampicin, certain cholesterol lowering drugs such as SR12813 and the statins, the antidepressant herb St. John's Wort, the antineoplastic drug paclitaxel, and the antimycotic clotrimazole (see Fig. 2, reviewed in ref. [4]). With relevance to endocrine disruption, human PXR is activated by numerous endogenous steroids including corticosterone and estradiol as well as other estrogenic chemicals including diethylstilbestrol, the phytoestrogen coumestrol [5], bisphenol A, a substance widely used in the food industry and in dentistry [33], and organochlorine pesticides such as chlordane, dieldrin, and endosulfan [34]. Other environmental contaminants including endocrine-disrupting chemicals such as nonylphenol and phthalic acid, the nonplanar polychlorinated biphenyls (PCBs), and the organochloride pepticides such as *trans*-nonachlor and chlordane have been shown to activate mouse PXR [35]. In addition, an antagonistic ligand that can block PXR-mediated induction of CYP3A, ecteinascidin-734, has also been identified [7]. Thus, in principle, it should be possible to design specific drugs that could selectively inhibit or promote the xenobiotic response.

The list of compounds that elicit CAR activity is small compared to the multiplicity of ligands that activate PXR. As mentioned above, mouse CAR is indirectly activated by PB and directly activated by TCPOBOP. Other mouse CAR activators include the antipsychotic chlorpromazine, plant products picrotoxin and camphor, and pesticides including PCBs, dieldrin, DDT, and methoxycholar and its metabolites (reviewed in ref. [13]). In addition, mouse CAR is repressed strongly by progesterone and androstanes while it is activated by estrogen [36]. Human CAR does not respond to these steroids and the difference has been attributed to residue 350 in the LBD (threonine in mouse and methionine in human) [37]. As mentioned above, human CAR is activated by PB, but not by TCPOBOP. To date, there is no known agonistic ligand that directly binds to the human receptor.

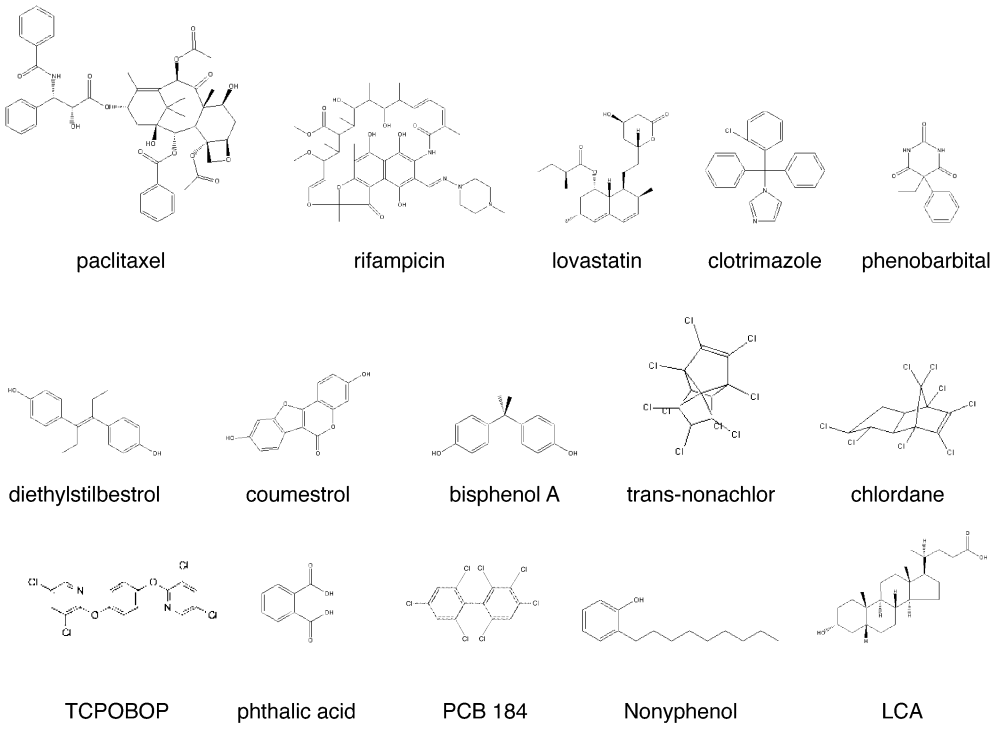

Fig. 2 Structures of representative compounds that activate PXR. Note that phenobarbital and TCPOBOP also activate CAR, whereas clotrimazole represses its constitutive activity.

The number of PXR and CAR ligands will continue to grow and the identification of endogenous as well as environmental chemicals that modulate the xenobiotic receptors will advance our understanding of the mechanisms by which environmental compounds affect our endocrine balance and may offer novel strategies for anticipating or preventing chemical toxicity.

BILE ACID REGULATION BY PXR/FXR/VDR

In addition to environmental toxins, our body is continuously exposed to a variety of toxic endogenous chemicals. For example, the secondary bile acid, lithocholic acid (LCA), is generated from non-toxic bile acids by intestinal bacteria and its elevation is implicated in pathogenesis of cholestatic liver disease and colon cancer. Because LCA is generated in an external cavity by a foreign organism, it is electively a xenobiotic compound.

The observation that LCA can induce CYP3A expression led to the suggestion that PXR may modulate this induction to reduce hepatotoxicity [9,10]. Three lines of evidence support this notion. First, LCA and its direct metabolite 3-keto LCA directly bind to and activate PXR. Second, in vivo activation of PXR by administration of an agonistic ligand (PCN) or by expressing a constitutively active form of PXR in the liver of transgenic mice results in marked resistance to LCA toxicity in rodents. Third, the potent CYP3A inducer and agonist for human PXR, rifampicin, has been reported to be effective in treating pruritus associated with chronic cholestasis. Detoxification of LCA by PXR appears to be mediated by the combined induction of CYP3A and the cytosolic sulfotransferase ST2A, both of which convert LCA to nontoxic metabolites [9,10,24]. Thus, the drug clearance pathway regulated by PXR can be utilized to detoxify endogenously produced toxins.

In addition to PXR, at least two other closely related nuclear receptors appear to contribute to bile acid regulation. Like PXR, the vitamin D receptor (VDR), was recently shown to bind secondary bile

acids such as LCA and can induce CYP3A in the intestine [38]. This may help to explain why vitamin D3 is protective in certain animal models of LCA-induced colon cancer. Studies from a number of laboratories have shown that primary bile acids are under homeostatic control by the farnesoid X receptor, FXR, that activates a distinct set of target genes controlling bile acid synthesis and transport (reviewed in ref. [39]). Thus, the nuclear receptors, PXR, VDR and FXR, appear to collaborate to protect our body from accumulation of toxic bile acids via the control of their synthesis and degradation. It is currently unknown whether CAR plays any role in bile acid clearance.

GENETIC POLYMORPHISM IN XENOBIOTIC RECEPTORS AND RECEPTOR BINDING-SITES

Genetic polymorphisms or single nucleotide polymorphisms (SNPs) that alter expression level or activity of gene products are likely to have a large influence on the susceptibility of individuals to environmental toxicants such as carcinogens or EAS. Such polymorphisms could also predispose individuals to unexpected adverse drug reactions that create major clinical risks. In relation to the xenobiotic response, changes in metabolic enzymes or drug transporters can have a large impact on inactivation and elimination of toxins. In fact, polymorphisms have widely been detected in genes encoding drug clearance components including cytochrome P450 enzymes, phase II conjugating enzymes and drug transporters (reviewed in refs. [40,41]). Emergence of PXR as a master regulator of these pathways has led to the speculation as to whether polymorphisms in PXR or PXR-binding sites in target genes could be, in part, responsible for variations in drug response. Recent studies indicate that this may be the case.

In one study, PXR coding exons and flanking regions from over 200 patients were sequenced and a total of 28 SNP sites were identified including six (three in the N-terminal region and three in the LBD) that alter amino acid coding. Interestingly, one allele with an amino acid alteration in the LBD shows 3-fold higher activity compared to the normal allele when rifampicin was used as an agonist, but appears to be slightly less active when corticosterone was used. This observation is consistent with the idea that polymorphisms in the LBD could directly contribute to individual differences in the xenobiotic response profile [42]. In an independent study, the promoter, coding exon and flanking region sequences were analyzed in over 100 patients and 38 SNP sites including three non-synonymous changes in the coding region were identified. Interestingly, several of the noncoding SNPs located within the promoter or introns correlated with either enhanced or reduced inducibility of PXR target genes, suggesting that such SNPs could indeed alter drug response in human populations [43].

The molecular mechanisms of polymorphic expression of adult CYP3A7 represent an example of polymorphisms in xenobiotic receptor binding sites. While initially characterized as a fetal-specific isoform, CYP3A7 expression was also detected in adult livers but in a highly polymorphic manner. A survey of more than 300 patients has identified the CYP3A7*1C allele as a consistent marker of increased CYP3A7 expression both in the liver and the intestine [44,45]. By recombining a part of the CYP3A7 promoter with the corresponding region of CYP3A4, the CYP3A7*1C allele acquires the proximal ER6 response element of CYP3A4. Both PXR and CAR efficiently bind and activate the CYP3A4-ER6 element providing a molecular basis for increased response associated with the CYP3A7*1C allele [45].

REGULATION OF XENOBIOTIC RECEPTOR EXPRESSION

Several compounds are known to induce PXR or CAR mRNA indicating another level of control. For example, the glucocorticoid receptor (GR) can induce expression of PXR, CAR, and their heterodimeric partner RXR in cultured cells [46,47]. In addition, in the rodent liver PXR expression is auto-induced by PCN and induced by PPARα specific drugs such as perfluorodecanoic acid and clofibrate [48]. In theory, induction of the xenobiotic receptors could potentiate induction of downstream target genes. However, as both PXR and CAR are abundantly expressed in the liver, the effect of further in-

duction is not clear. Perhaps, this regulation is more significant for those individuals who have alleles of xenobiotic receptors with a low constitutive level or weak activity. The levels of hepatic PXR and CAR mRNA have also been reported to be down-regulated in response to inflammatory signals [49,50]. Further studies are expected to reveal the relevance of xenobiotic receptor regulation, and its impact on drug metabolism.

PERSPECTIVE: THE POTENTIAL INFLUENCE OF XENOBIOTIC RECEPTOR ON THE ACTION OF EASs

From the available studies, it is likely that many industrial and natural EASs bind and activate either PXR, CAR, or both. How might xenobiotic receptors and the pathways regulated by these receptors influence the action of EASs? PXR and CAR most likely play a protective role against most EASs by promoting their detoxification and/or clearance. However, in rare, but relevant cases, PXR and CAR may inadvertently promote the deleterious effects exerted by some EASs. First, detoxification reactions, such as CYP3A induction, are also known to activate certain substrates to carcinogenic and/or cytotoxic products. For example, the first step in aflatoxin metabolism creates a reactive adduct for DNA, enabling its activity as a hepato-carcinogen. Thus, activation of PXR or CAR may enhance the toxicity of some environmental chemicals. Second, because steroids and thyroid hormones are metabolized and typically inactivated by enzymes such as CYPs, UGTs, and SULTs, constitutive activation of PXR or CAR by environmental chemicals could alter endocrine systems. In fact, the chronic activation of PXR in transgenic mouse livers results in increased corticosterone in serum and urine and by extension most likely stimulates the production of gonadal steroids [31]. Furthermore, prototypical chemical activators for PXR or CAR induce increased metabolism and decreased level of thyroid hormones, consequential increase in thyroid stimulating hormone and thyroid hypertrophy in rats [51]. Further studies are required to clarify the effect of chronic activation or inactivation of the xenobiotic receptors on global endocrine physiology and disease progression.

In summary, we believe that the xenobiotic regulation of drug clearance by nuclear receptors will be an emerging and exciting field of research in the coming years. The results of these studies will greatly advance our understanding of the complexity of xenobiotic regulation and their implication in human physiology, pathology, pharmaceutical development as well as enable a broad assessment of environmental risks.

NOTE ADDED IN PROOF

A specific human CAR agonist has recently been identified [52].

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