

Induction of Phase I, II and III Drug Metabolism/Transport by Xenobiotics

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Drug metabolizing enzymes (DMEs) play central roles in the metabolism, elimination and detoxification of xenobiotics and drugs introduced into the human body. Most of the tissues and organs in our body are well equipped with diverse and various DMEs including phase I, phase II metabolizing enzymes and phase III transporters, which are present in abundance either at the basal unstimulated level, and/or are inducible at elevated level after exposure to xenobiotics. Recently, many important advances have been made in the mechanisms that regulate the expression of these drug metabolism genes. Various nuclear receptors including the aryl hydrocarbon receptor (AhR), orphan nuclear receptors, and nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) have been shown to be the key mediators of drug-induced changes in phase I, phase II metabolizing enzymes as well as phase III transporters involved in efflux mechanisms. For instance, the expression of CYP1 genes can be induced by AhR, which dimerizes with the AhR nuclear translocator (Arnt), in response to many polycyclic aromatic hydrocarbon (PAHs). Similarly, the steroid family of orphan nuclear receptors, the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), both heterodimerize with the retinoid X receptor (RXR), are shown to transcriptionally activate the promoters of CYP2B and CYP3A gene expression by xenobiotics such as phenobarbital-like compounds (CAR) and dexamethasone and rifampin-type of agents (PXR). The peroxisome proliferator activated receptor (PPAR), which is one of the first characterized members of the nuclear hormone receptor, also dimerizes with RXR and has been shown to be activated by lipid lowering agent fibrate-type of compounds leading to transcriptional activation of the promoters on CYP4A gene. CYP7A was recognized as the first target gene of the liver X receptor (LXR), in which the elimination of cholesterol depends on CYP7A. Farnesoid X receptor (FXR) was identified as a bile acid receptor, and its activation results in the inhibition of hepatic acid biosynthesis and increased transport of bile acids from intestinal lumen to the liver, and CYP7A is one of its target genes. The transcriptional activation by these receptors upon binding to the promoters located at the 5-flanking region of these CYP genes generally leads to the induction of their mRNA gene expression. The physiological and the pharmacological implications of common partner of RXR for CAR, PXR, PPAR, LXR and FXR receptors largely remain unknown and are under intense investigations. For the phase II DMEs, phase II gene inducers such as the phenolic compounds butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (tBHQ), green tea polyphenol (GTP), (-)-epigallocatechin-3-gallate (EGCG) and the isothiocyanates (PEITC, sulforaphane) generally appear to be electrophiles. They generally possess electrophilic-mediated stress response, resulting in the activation of bZIP transcription factors Nrf2 which dimerizes with Maf and binds to the antioxidant/electrophile response element (ARE/EpRE) promoter, which is located in many phase II DMEs as well as many cellular defensive enzymes such as heme oxygenase-1 (HO-1), with the subsequent induction of the expression of these genes. Phase III transporters, for example, P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and organic anion transporting polypeptide 2 (OATP2) are expressed in many tissues such as the liver, intestine, kidney, and brain, and play crucial roles in drug absorption, distribution, and excretion. The orphan nuclear receptors PXR and CAR have been shown to be involved in the regulation of these transporters. Along with phase I and phase II enzyme induction, pretreatment with several kinds of inducers has been shown to alter the

expression of phase III transporters, and alter the excretion of xenobiotics, which implies that phase III transporters may also be similarly regulated in a coordinated fashion, and provides an important mean to protect the body from xenobiotics insults. It appears that in general, exposure to phase I, phase II and phase III gene inducers may trigger cellular "stress" response leading to the increase in their gene expression, which ultimately enhance the elimination and clearance of these xenobiotics and/or other "cellular stresses" including harmful reactive intermediates such as reactive oxygen species (ROS), so that the body will remove the "stress" expeditiously. Consequently, this homeostatic response of the body plays a central role in the protection of the body against "environmental" insults such as those elicited by exposure to xenobiotics.

Key words: Phase I metabolizing enzymes, Phase II metabolizing enzymes, P-Glycoprotein, Multidrug resistance-associated protein, Organic anion transporting polypeptide 2, Aryl hydrocarbon receptor, Pregnane X receptor, Constitutive androstane receptor, Peroxisome proliferator activated receptor, Liver X receptor, Farnesoid X receptor, Retinoid X receptor, Nuclear factor-erythroid 2 p45-related factor 2

INTRODUCTION OF PHASE I, PHASE II DRUG METABOLIZING ENZYMES AND PHASE III DRUG TRANSPORTERS

Drug metabolizing enzymes (DMEs) play central roles in the metabolism, elimination and/or detoxification of xenobiotics or exogenous compounds introduced into the body (Meyer, 1996). In general, DMEs protect the body against the potential harmful exposure to xenobiotics from the environment as well as certain endobiotics. In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with diverse and various DMEs including phase I, phase II metabolizing enzymes as well as phase III transporters, which are present in abundance either at the basal uninduced level, and/or inducible at elevated level after xenobiotics exposure (Meyer, 1996; Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

Phase I DMEs consist primarily of the cytochrome P450 (CYP) superfamily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities (Gonzalez and Nebert, 1990; Guengerich, 2003; Meyer, 1996; Nebert *et al.*, 1991; Nelson *et al.*, 1996). More than thirty-six gene families have been described to date. Twelve families exist in all mammals, which comprise twenty-two subfamilies. In human, five CYP gene families, such as CYP1, CYP2, CYP3, CYP4 and CYP7 are believed to play crucial roles in hepatic as well as extra-hepatic metabolism and elimination of xenobiotics and drugs (Gonzalez and Nebert, 1990; Lewis, 2003; Nebert *et al.*, 1991; Nelson *et al.*, 1996; Pascussi *et al.*, 2003b; Simpson, 1997; Waxman, 1999).

The phase II metabolizing or conjugating enzymes, consisting of many superfamily of enzymes including sulfotransferases (SULT) (Banoglu, 2000; Weinshilboum

et al., 1997), and UDP-glucuronosyltransferases (UGT) (Innocenti *et al.*, 2002; King *et al.*, 2000; Mackenzie *et al.*, 1997; Tukey and Strassburg, 2000), DT-diaphorase or NAD(P)H:quinone oxidoreductase (NQO) or NAD(P)H:menadione reductase (NMO) (Jaiswal, 1994; Kong *et al.*, 2001a), epoxide hydrolases (EPH) (Guenther *et al.*, 1989; Hinson and Forkert, 1995), glutathione *S*-transferases (GST) (Moscow and Dixon, 1993; Schilter *et al.*, 1993; Tew and Ronai, 1999) and *N*-acetyltransferases (NAT) (Vatsis *et al.*, 1995). Each superfamily of phase II DMEs consists of families and subfamilies of genes encoding the various isoforms with different substrate specificity, tissue and developmental expression, as well as inducibility and inhibitory by xenobiotics (Hinson and Forkert, 1995; Schilter *et al.*, 1993). In general, conjugation with phase II DMEs generally increases hydrophilicity, and thereby enhance excretion in the bile and/or the urine and consequently a detoxification effect. Although under certain situations, conjugation with phase II enzymes could result in activated metabolites and increase toxicity (Chen *et al.*, 2000; Hinson and Forkert, 1995; Kong *et al.*, 2000; Rushmore and Kong, 2002; Schilter *et al.*, 1993). For example, reactive electrophiles are typically conjugated with glutathione (GSH) catalyzed by various GSTs, and have been implicated with the potential of forming reactive intermediates in particular when GSH levels in the cells are attenuated, consequently resulting in toxicological effects (Bolton and Chang, 2001; Bolton *et al.*, 2000). On the other hand, the SULT (Banoglu, 2000) and UGT (Sugatani *et al.*, 2001; Tukey and Strassburg, 2000) which catalyze sulfation and glucuronidation, may play important roles in the conjugation and ultimately excretion and elimination of many drugs and xenobiotics containing hydroxyl (OH) functional group either present in the parent structure and/or after biotransformation by the phase I enzymes such as the CYPs (Banoglu, 2000; King *et al.*, 2000; Schilter *et al.*, 1993; Simpson, 1997).

Phase III transporters, including P-glycoprotein (P-gp)

(Brinkmann and Eichelbaum, 2001), multidrug resistance-associated protein (MRP) (Kerb *et al.*, 2001), and organic anion transporting polypeptide 2 (OATP2) (Tirona and Kim, 2002) are expressed in many tissues such as the liver, intestine, kidney, and brain, where they provide a formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion (Brinkmann and Eichelbaum, 2001; Kim, 2003; Mizuno *et al.*, 2003; Staudinger *et al.*, 2003). P-gp was first reported to be associated with multidrug resistance (MDR) in cancer chemotherapy. P-gp and MRP utilize the energy from the hydrolysis of ATP to substrate transport across the cell membrane, and are called ATP binding cassette (ABC) transporters (Mizuno *et al.*, 2003). ABC transporters belong to one of the largest superfamilies of proteins, and either import or export a broad range of substrates that include amino acids, ions, sugars, lipids, xenobiotics, and many therapeutic drugs (Dean *et al.*, 2001; Kerb *et al.*, 2001). There are only exporters in the eukaryotes. In human, 46 ABC transporters have been identified (Dean *et al.*, 2001; Mizuno *et al.*, 2003). All ABC transporters are composed of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). The NBD is also called an ABC, is the hallmark feature of this transporter family. The role of TMD is to recognize and mediate the passage of substrates across the cell membranes (Dean *et al.*, 2001; Kerb *et al.*, 2001). Along with P-gp (or MDR1; ABCB1, the MDR subfamily includes MDR3 (ABCB4), BSEP (or SP-gp; ABCB11) (Brinkmann and Eichelbaum, 2001). The MRP subfamily consists of MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11), and MRP9 (ABCC12) (Brinkmann and Eichelbaum, 2001; Dean *et al.*, 2001; Kerb *et al.*, 2001; Mizuno *et al.*, 2003). MRP1 and MRP3 are typically located on the basolateral membrane of polarized cells, whereas MRP2 is generally localized to the apical membrane (canalicular in liver), which implies that in liver, MRP2-mediated transport leads to increased excretion into bile, but MRP1- and MRP3-mediated transport into blood leads to increased excretion into the urine. Organic anion transporting polypeptide 2 (OATP2; SLC21A5) is a member of the organic anion transporting polypeptide family that mediates sodium- and ATP-independent transport of a variety of structurally unrelated endogenous and exogenous compounds, including conjugated and unconjugated bilirubin, conjugated steroids, neutral compounds, some type II organic cations, thyroid hormones T3 and T4, and bile salts (Reichel *et al.*, 1999; Shitara *et al.*, 2002). OATP2 is localized in the hepatic sinusoidal membrane, with selective expression in the midzonal to perivenous hepatocytes. P-gp, MRP and OATP2 are all expressed on the brush-border membrane of the intestinal

enterocytes, and excrete their substrates as well as xenobiotics/drugs into the lumen, resulting in a potential limitation of net absorption of drugs (Dean *et al.*, 2001; Kerb *et al.*, 2001; Kim, 2003; Mizuno *et al.*, 2003).

Therefore, the regulation of gene expression of various phase I, phase II DMEs and phase III transporters has potential impact on the metabolism, elimination, pharmacokinetics/dynamics, toxicokinetics/dynamics, drug-drug interactions of many therapeutic agents, as well as their ability in the protection of the human body against exposure of environmental xenobiotics (Guengerich, 2003; Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

RECEPTORS INVOLVED IN THE REGULATION OF PHASE I, PHASE II METABOLIZING ENZYMES AND PHASE III TRANSPORTERS

The human body has evolved versatile inducible metabolizing enzymes and efflux transporters to facilitate the metabolism and elimination of potentially harmful drugs, and/or xenobiotics that are introduced from the environment. The enzymatic system includes phase I enzymes, such as CYP superfamily (Guengerich, 2003; Lewis, 2003), as well as phase II enzymes, such as GST and UGT (Mackenzie *et al.*, 1997; Tew and Ronai, 1999). The efflux transporter system includes phase III ABC proteins, such as P-gp, MRP2, and OATP2 which remove the parent drugs, metabolites, and xenobiotics from cells (Dean *et al.*, 2001; Lewis, 2003; Meyer, 1996; Mizuno *et al.*, 2003; Wang and LeCluyse, 2003). In order to understand the regulation of gene expression of phase I, phase II metabolizing enzymes and phase III efflux transporters, one would need to address the signaling mechanism involving the aryl hydrocarbon receptor (AhR) (Hahn, 2002; Rowlands and Gustafsson, 1997), the orphan nuclear receptors (Moore *et al.*, 2000; Wang and LeCluyse, 2003), and other relevant transcription factors and/or signal transduction cascades (Kumar and Thompson, 1999; Wang and LeCluyse, 2003) at the molecular level.

The AhR and orphan nuclear receptors comprise a gene superfamily encoding the transcription factors that sense endogenous, such as small lipophilic hormones, and exogenous, such as drugs, xenobiotics and transfer into cellular responses by regulating the expression of their target genes (Levine and Perdew, 2001; Wang and LeCluyse, 2003). Regulation of gene expression at the transcriptional level by AhR and orphan nuclear receptors plays a crucial role in the metabolism and clearance of drugs and xenobiotics that are introduced into the body for the purpose of protection the body from the environmental insults (Li *et al.*, 1998; Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

Aryl hydrocarbon receptor

AhR is a member of the basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) gene superfamily of transcription factor, and has been studied for more than 30 years (Hahn, 2002; Rowlands and Gustafsson, 1997). AhR is known to recognize a range of chemical structures, including non-aromatic and non-halogenated compounds (Elferink, 2003; Hahn, 2002; Rowlands and Gustafsson, 1997). The AhR is highly polymorphic, especially when compared with orphan nuclear receptors, such as PXR and CAR (Honkakoski *et al.*, 2003; Willson and Kliever, 2002). The bHLH motif exists in many transcription factors such as Myc and Max that functions as sequence-specific transcriptional regulation. This motif plays a role in both DNA binding (basic region) and protein dimerization (HLH) (Hahn, 2002; Huang *et al.*, 2004; Kikuchi *et al.*, 2003).

The Ah receptor nuclear translocator (Arnt), is not required for nuclear translocation per se, but is required to generate an AhR-Arnt complex with a greater affinity for nuclear extracts upon cell disruption (Heid *et al.*, 2000; Kikuchi *et al.*, 2003). The unliganded AhR is found almost exclusively in the cytoplasm of the cell, and treatment with ligand causes a time-dependent movement of the AhR into the nucleus. The Arnt protein, on the other hand, is found to be exclusively nuclear with or without ligand. Thus, ligand may serve to initiate translocation of the AhR to the nucleus where dimerization of these two partners can occur. In Arnt-deficient cells, the AhR can still translocate to the nucleus in the cell, a process therefore independent of Arnt (Hahn, 2002; Kikuchi *et al.*, 2003; Levine and Perdew, 2001).

AhR can bind to DNA as a heteromeric complex. It was demonstrated that both the bHLH and PAS domains are required for DNA-binding, and thus presumably for dimerization with Arnt. The basic region of Arnt is not required for dimerization, but both helix regions, and either the *N*-terminal or *C*-terminal half of the PAS domain, are essential. The AhR and Arnt proteins have a single transactive domain (TAD) in their C-terminals, comprising amino acids 521-640 in the AhR and amino acids 582-774 in Arnt (Huang *et al.*, 2004; Kikuchi *et al.*, 2003).

The unliganded AhR exists in the cytosol complexed with a dimer of Hsp90, which maintains the AhR in a ligand-binding conformation and prevents nuclear translocation and/or dimerization with Arnt (Heid *et al.*, 2000). The hydrophobic AhR ligands enter the cell by diffusion and are bound by the Hsp90-associated AhR. Ligand binding causes a conformational change resulting in a receptor species with an increased affinity for DNA and a much slower rate of ligand dissociation. This event is associated with nuclear translocation and an exchange of Hsp90 for Arnt (Hahn, 2002; Heid *et al.*, 2000). It has been shown

that in a purified system the AhR-Hsp90 complex is not dissociated by the addition of ligand. Ultimately, the recognition of DRE enhancer sequences by the AhR-Arnt complex results in the transactivation of target genes (Hahn, 2002; Heid *et al.*, 2000; Levine and Perdew, 2001).

Orphan nuclear receptors

Orphan receptor is a subclass of nuclear receptors that binds to steroid-based ligands, such as cortisol, estradiol, progesterone, aldosterone, testosterone and vitamin D. All the known orphan receptors share two modulatory domains, one is the highly conserved DNA-binding domain (DBD), the other is the ligand binding domain (LBD) (Kumar and Thompson, 1999; Wang and LeCluyse, 2003). The DBD is characterized by two C4-type zinc fingers, links the receptor to the specific promoter regions of its target genes, termed hormone response element (HRE) or xenobiotic response element (XRE). The DBD can recognize the response elements that contains one or two consensus core half-sites related to the hexamer ACAACA (steroid receptors) or AGGTCA (estrogen receptors and so on) (Kumar and Thompson, 1999; Wang and LeCluyse, 2003). Different orphan nuclear receptors bind to their response element either as homodimers, as heterodimers with the RXR, or as monomers. The LBD is located in the carboxy-terminal portion of the receptor, and not only serves as a docking site for ligands, but also contains dimerization motifs: transcriptional activation domains, such as the activation function 2 (AF-2) helix, and the sequence mediating the nuclear localization of the receptor. Ligand binding induces significant conformational changes in the folding the LBD, and leads to the recruitment of coactivator proteins and co-integrators, and transactivation of the target genes (Kumar and Thompson, 1999; Wang and LeCluyse, 2003).

Pregnane X receptor (PXR)

PXR was first cloned from mouse liver, then its homologous counterparts in rat, rabbit and human were identified (Kliever *et al.*, 1998). Orthologous receptors from different species were given unrelated names at first due to the lack of a common nomenclature system. The human receptor of PXR has also been referred to as "steroid and xenobiotic receptor (SXR)" or "pregnane activated receptor (PAR)" (Xie *et al.*, 2000a, 2000b, 2001). A nomenclature system has been devised for the nuclear receptor superfamily recently. According to this system, PXR has been classified as NR112. The gene family is designated by an Arabic numeral, the superfamily is indicated by a capital letter, and individual gene members are identified by the second Arabic numeral (Dussault and Forman, 2002; Kliever *et al.*, 1998; Kumar and Thompson, 1999;

Wang and LeCluyse, 2003).

All PXR (human, mouse, rat and so on) are predominantly expressed in the liver and intestine, and to a lower level in the kidney and lung. The tissue-specific distribution pattern of PXR expression resembles that of CYP3A (Beigneux *et al.*, 2002; Coumoul *et al.*, 2002). There is more than 95% sequence homology in the DBD regions, but only 75-80% amino acid homology in the LBD of PXR between the different species (Dussault and Forman, 2002; Wang and LeCluyse, 2003).

Constitutive androstane receptor (CAR)

The orphan nuclear receptor CAR (NR113) was identified in 1994 (Baes *et al.*, 1994). It was originally defined as constitutively activated receptor, because it forms a heterodimer with RXR which binds to retinoic acid response elements and transactivates target genes in the absence of ligands (Honkakoski *et al.*, 1998b). CAR is mainly expressed in liver, and less abundance in the intestine (Wang and LeCluyse, 2003; Wei *et al.*, 2002). Two metabolites of androstane, androstanol and androstenol were found to be the endogenous CAR ligands. Both of them act as antagonists by dissociating CAR from its coactivator and inhibiting the transactivation of CAR instead of activating CAR (Fraser *et al.*, 2003; Goodwin *et al.*, 2002; Honkakoski *et al.*, 2003; Pascussi *et al.*, 2003b).

CAR is located in the cytoplasm of hepatocytes in the absence of ligands, and it is translocated into the nucleus after treatment with phenobarbital-like CYP2B inducers. Recent studies indicated that activation of CAR is a multistep process, the initial step is nuclear translocation, which can be independent of ligand binding, the final step is CaMK-dependent activation of this receptor (Bae *et al.*, 2004; Maglich *et al.*, 2002; Paquet *et al.*, 2000; Wang and LeCluyse, 2003).

Peroxisome proliferator activated receptors (PPAR)

Currently, three members of this nuclear receptor family have been identified as: PPAR α , PPAR β and PPAR γ (Gervois *et al.*, 2000; Gilde *et al.*, 2003). PPAR α is mainly expressed in the liver, heart, kidney, intestine and brown adipose tissue. PPAR β is widely expressed in most adult tissues, and the brain, kidney and intestine are the highest expressed tissues. PPAR γ is mainly expressed in the spleen, intestine and fat cells, and it is composed of two submembers, named PPAR γ 1 and PPAR γ 2. PPARs demonstrated distinct but overlapping physiological functions (Gilde *et al.*, 2003; Issemann and Green, 1990; Rushmore and Kong, 2002; Tugwood *et al.*, 1992; Wang and LeCluyse, 2003).

At the very beginning, PPAR α was found to be activated by compounds that cause proliferation of liver peroxisomes, hyperplasia and hepatic carcinogenesis in

rodents, however, subsequently, studies suggested that PPARs may play a crucial role in the regulation of lipoprotein and fatty acid metabolism (Gervois *et al.*, 2000; Gilde *et al.*, 2003; Schoonjans *et al.*, 1996; Yu *et al.*, 2003).

Liver X receptor (LXR)

Liver X receptors are transcription factors commonly known as cholesterol sensors. They are important regulators of transport and metabolism of sterols and fatty acids. There are two members of this family, LXR α and LXR β . LXR α and LXR β share a high degree of amino acid similarity (~80%) and are considered paralogues. Oxysterols including 24(*S*), 25-epoxycholesterol, 22(*R*)-hydroxycholesterol, and 24(*S*)-hydroxycholesterol, are natural ligands of LXRs. Some LXR-mediated genes include those associated with cholesterol and bile acid metabolism as well as those with fatty acid synthesis and regulation. LXR α is predominantly expressed in liver, lower level in kidney, spleen and intestine. On the contrary, LXR β is located in almost every tissue tested. LXRs are mainly located in the nucleus, and must heterodimerize with RXR for activation (Khan and Vanden Heuvel, 2003; Lehmann *et al.*, 1997; Menke *et al.*, 2002; Peet *et al.*, 1998; Venkateswaran *et al.*, 2000).

Farnesoid X receptor (FXR)

FXR was shown to be activated by supraphysiological concentration of farnesol in rats when it was originally identified. Similar to other orphan nuclear receptors, FXR is mainly expressed in liver and intestine, it heterodimerizes with RXR and binds to FXR response element (FXRE) in the promoter region of target genes. Recent reports showed that FXR was identified as a bile acid receptor, and was activated by physiological ligands resulted in the inhibition of hepatic bile acid biosynthesis and increased transport of bile acid from the intestine to the liver (del Castillo-Olivares and Gil, 2000; Makishima *et al.*, 1999; Wang *et al.*, 1999; Wang and LeCluyse, 2003).

Retinoid X Receptor (RXR)

There are three members of this family, RXR α , RXR β and RXR γ . RXR α is mainly expressed in the liver, muscle, kidney and lung, and to a lower level in the spleen, heart and adrenal gland, whereas RXR β is found in all tissues except the liver and intestine, and RXR γ is found in just a few tissues, such as skeletal muscle, heart and central nervous system (Mangelsdorf *et al.*, 1992; Mangelsdorf and Evans, 1995; Wang and LeCluyse, 2003; Zetterstrom *et al.*, 1996). The metabolite 9-*cis*-retinoic acid of vitamin A was identified as a high-affinity ligand of RXRs (Baes *et al.*, 1994). RXR can form heterodimers with other orphan nuclear receptors as a common partner, and the

formation of a heterodimer with RXR is a critical step for facilitating the specific binding and activation of all known orphan nuclear receptors (Mangelsdorf and Evans, 1995; Wang and LeCluyse, 2003; Zetterstrom *et al.*, 1996). There are two kinds of RXR heterodimers, nonpermissive and permissive. RXR is completely silent and can only be activated by the ligands of the partner orphan nuclear receptor in the nonpermissive heterodimers. RXR permissive heterodimers can be freely activated by ligands of both RXR and partner nuclear receptors, such as PXR/RXR, CAR/RXR, PPAR/RXR, LXR/RXR and FXR/RXR (Mangelsdorf *et al.*, 1992; Mangelsdorf and Evans, 1995; Wang and LeCluyse, 2003; Zetterstrom *et al.*, 1996). Because RXRs have a broad binding ability with most other orphan nuclear receptors, and affect the subsequent regulation of their target genes, so the RXRs are involved in the regulation of most drug metabolizing enzymes and transporter directly or indirectly. But the ultimate role of RXR heterodimer complexes appear to be multifaceted and yet uncertain (Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

REGULATION OF PHASE I DMEs

It appears that in general xenobiotics exposure can trigger certain "stress" response to the body, and consequently resulting in an increase in gene expression of

xenobiotic metabolizing enzymes or DMEs, so that the body will be able to remove the "stress insults" as fast as possible from the body (Fig. 1) (Kong *et al.*, 2001a, 2001b; Rushmore and Kong, 2002).

The steroid family of the orphan receptors, PXR and CAR can heterodimerize with the RXR, and have been shown to transcriptionally induced CYP3A (Anakk *et al.*, 2004; Coumoul *et al.*, 2002; Lehmann *et al.*, 1998) and CYP2B (Bae *et al.*, 2004; Beigneux *et al.*, 2002) gene expression by xenobiotics such as dexamethasone/rifampin type of compounds and phenobarbital-like compounds (Bae *et al.*, 2004; Coumoul *et al.*, 2002; Honkakoski *et al.*, 2003; Willson and Kliewer, 2002). PPAR is one of the very first members to be identified in this orphan nuclear receptor superfamily, and it can also heterodimerize with RXR, that was initially found to be activated by the lipid lowering agent fibrate-type of compounds and other chemicals. Previously it was found to increase the levels of peroxisomes in rodents, and later it was shown to increase the gene expression of CYP4A enzymes (Gervois *et al.*, 2000; Rushmore and Kong, 2002; Simpson, 1997; Zhou *et al.*, 2002). LXR (Menke *et al.*, 2002) and FXR (Wolters *et al.*, 2002) receptors are involved in the regulation of CYP7A in mediating the elimination of cholesterol and biosynthesis and excretion of hepatic bile acids. These diverse array of naturally occurring or synthetic compounds are primarily metabolized by CYP

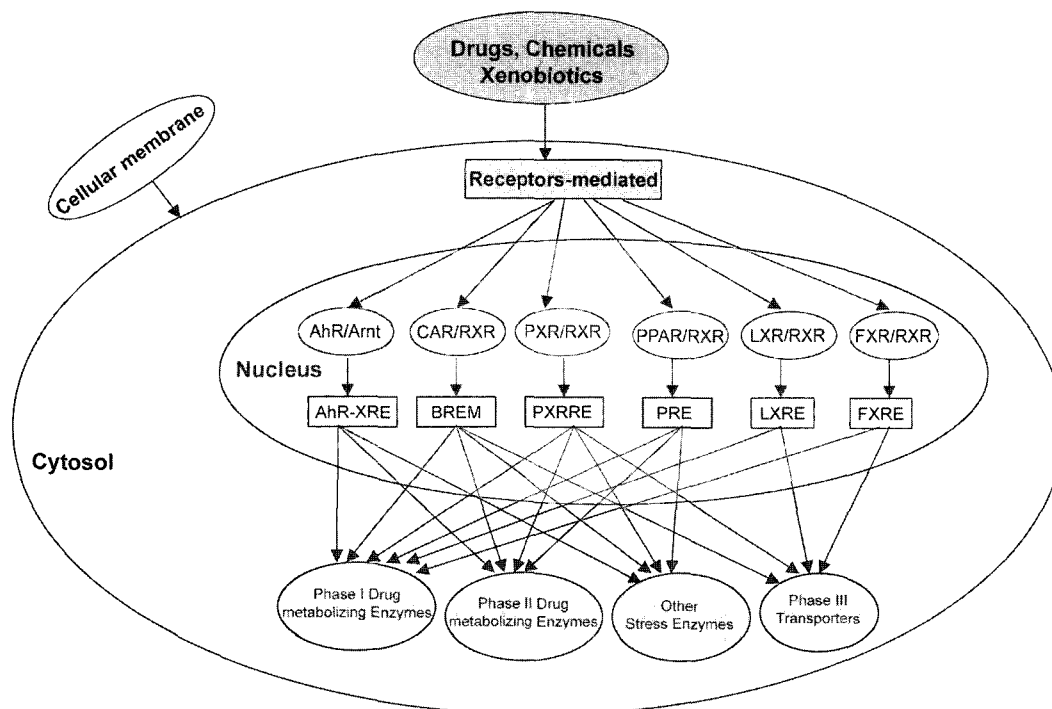


Fig. 1. A schematic representation of drugs/chemicals/xenobiotics-induced stress response leading to the activation of specific receptor-mediated gene expression of phase I drug metabolizing enzymes, the cytochrome p450s, phase II drug metabolizing enzymes, other stress enzymes, and phase III transporters, which result in the enhancement of detoxification of the xenobiotics and a potential homeostatic cell survival response.

enzymes in the body, and they range from endogenous compounds such as the steroids and cholesterol to drugs as well as potential carcinogens found in the environment (Lehmann *et al.*, 1997; Venkateswaran *et al.*, 2000; Wang *et al.*, 1999). The oxidized products are generally more polar, can be excreted directly and/or further conjugated by the phase II DMEs and ultimately eliminated from the body, and consequently detoxification (Hinson and Forkert, 1995; Meyer, 1996). However, in some situations, notably procarcinogens, they may be metabolized to more reactive species, and potentially promoting toxicity and carcinogenicity (Banoglu, 2000; Guengerich, 2003; Schilter *et al.*, 1993).

Regulation of CYP1 by AhR

When AhR is bound by polycyclic aromatic hydrocarbon (PAH), such as dioxin and 3-methylcholoranthrene (3-MC), AhR translocates from the cytoplasm to the nucleus, heterodimerizes with Arnt, and activates transcription through the XRE located in the promoters of CYP1 family genes (Li *et al.*, 1998; Nakajima *et al.*, 2003). The requirement of AhR in CYP1 expression induction was demonstrated in AhR-null mutant mice (Gonzalez and Fernandez-Salguero, 1998; Shimizu *et al.*, 2000). The expression of CYP1 genes induced by the AhR, in response to PAHs or halogenated aromatic hydrocarbon ligands such as benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or dioxin is well established (Gonzalez and Fernandez-Salguero, 1998; Levine and Perdew, 2001; Li *et al.*, 1998; Nakajima *et al.*, 2003; Shimizu *et al.*, 2000).

Regulation of CYP3A by PXR

Systematic deletion analysis has demonstrated that PXR response element (PXRRE) is located in the promoter region of CYP3A. The PXR response element is either a direct repeat of the half-site TGAACT spaced by three base pairs (DR3) or an everted or inverted repeat of the TGAACT half-site spaced by six base pairs (ER6 and IR6) (Dussault and Forman, 2002; Kliewer *et al.*, 1998; Wang *et al.*, 2003; Wang and LeCluyse, 2003; Xie *et al.*, 2000b). PXR can bind to and transactivate these response elements after activation by CYP3A inducers, and PXR is the predominant regulator of the xenobiotic-responsive expression of CYP3A genes (Anakk *et al.*, 2004; Coumoul *et al.*, 2002; Kliewer *et al.*, 1998; Xie *et al.*, 2001).

There are important species-specific PXR activation profiles to support the regulation of CYP3A by PXR. For example, rifampicin is a well-known and potent inducer of CYP3A in rabbit and human liver, but not in rat and mouse liver, and was found to be a potent activator of human and rabbit PXR, but not of rat or mouse PXR (Jones *et al.*, 2000). On the other hand, PCN is a potent

rat and mouse CYP3A inducer, but not of human or rabbit CYP3A, and was found also to be a potent rat PXR activator, having very little effect on human and rabbit PXR (Jones *et al.*, 2000; Staudinger *et al.*, 2001a, 2001b). Treatment of PXR-null mice with PCN failed to induce CYP3A expression providing definitive proof for PXR regulation of CYP3A expression (Staudinger *et al.*, 2001b; Xie *et al.*, 2000a). Replacement of the mPXR with its human orthologue resulted in the xenobiotic response in this humanized mouse, and the response to xenobiotic stimulation resembled that in human (Anakk *et al.*, 2004; Staudinger *et al.*, 2001b; Xie *et al.*, 2000a, 2001).

Regulation of CYP2B by CAR

CYP2B is potently induced by phenobarbital in most mammalian species. Study showed that CAR can bind to a 51-base-pair minimum sequence located in the 5'-flanking region of the CYP2B genes, and this sequence was required for phenobarbital induction, and was named as phenobarbital-response element module (PBREM) (Honkakoski and Negishi, 1997). PBREM is composed of two nuclear receptor binding sites (NR1 and NR2) as well as a nuclear factor 1 (NF1) binding site. Both NR1 and NR2 are DR4 motifs (Ramsden *et al.*, 1999; Sueyoshi *et al.*, 1999; Wang and LeCluyse, 2003). The highly conserved NR1 site is critical for conferring phenobarbital responsiveness, the function of NF1 site is still unclear (Honkakoski *et al.*, 1998b; Sueyoshi and Negishi, 2001). After transfection with some known nuclear receptors, such as RXR, CAR, or LXR, using PBREM reporter assay to study their ability to bind and transactivate the PBREM, only CAR was found to be able to stimulate PBREM reporter gene expression (Honkakoski *et al.*, 1998b). Subsequently, NR1-affinity chromatography was used to purify the protein that bound to PBREM, and that both binding assay and Western blot assay demonstrated that CAR was the protein that mediated the phenobarbital induction response (Honkakoski *et al.*, 1998b; Kawamoto *et al.*, 1999; Paquet *et al.*, 2000; Sueyoshi *et al.*, 1999; Sueyoshi and Negishi, 2001).

Regulation of CYP4A by PPAR

PPAR is activated by a ligand-induced conformational structure change, and binds to specific upstream region of its target genes referred to as peroxisome proliferator response elements (PPREs) (Lambe and Tugwood, 1996; Tugwood *et al.*, 1996). CYP4A could be induced by a number of peroxisome proliferators, such as clofibrate, via the activation of PPAR α . CYP4A plays a central role in the hydroxylation of fatty acid derivatives and cholesterol metabolism (Rushmore and Kong, 2002; Simpson, 1997; Yu *et al.*, 2003; Zhou *et al.*, 2002).

Regulation of CYP7A by LXR and FXR

LXR can recognize a direct repeat of two similar hexanucleotide half-sites separated by 4 base pairs (DR4) in the upstream regions of their target genes, referred to as an LXRE (Lehmann *et al.*, 1997; Wang and LeCluyse, 2003). The endogenous oxysterols, such as the metabolites of cholesterol, are selective LXR ligands. The cholesterol is metabolized to hydrophilic bile acids by CYP7A (cholesterol 7 α -hydroxylase), and a cholesterol-rich diet in rat can upregulate CYP7A (Jelinek *et al.*, 1990; Jelinek and Russell, 1990; Menke *et al.*, 2002; Peet *et al.*, 1998; Wang and LeCluyse, 2003). CYP7A was recognized as the first target gene of LXR (Lehmann *et al.*, 1997). The DR4-LXRE is located in the proximal promoter region of CYP7A, and it can be specifically bound and activated by LXR (Menke *et al.*, 2002; Peet *et al.*, 1998). LXR α knockout mice are phenotypically normal when fed with low cholesterol diet, but cholesterol accumulation, chronic hepatomegaly development and liver function impairment occurred as compared to their wild-type counterparts when the knock out mice were fed with a high cholesterol diet (2%), because the LXR α knockout mice could not regulate the CYP7A gene expression and the bile acid biosynthesis (Peet *et al.*, 1998). The LXRE of CYP7A is a much stronger response element for transcription activation by LXR α than by LXR β . Although LXR β expression is normal in LXR α knock out mice, its presence could not prevent the cholesterol accumulation and liver function disorder when these mice were fed with high cholesterol diet (Lehmann *et al.*, 1997; Venkateswaran *et al.*, 2000; Wang and LeCluyse, 2003).

The binding and activation of FXR by bile acids accumulation was followed by the transcriptional activation of ileal bile acid-binding protein (IBABP), that resulted in the increase of bile acid reabsorption (Makishima *et al.*, 1999; Wang and LeCluyse, 2003). FXR can negatively regulate CYP7A expression by binding to its bile acid ligands, but until now there is no evidence showing that FXR can bind directly to the CYP7A promoter region. FXR is the main regulator in facilitating bile acid reabsorption (IBABP activation) and it is an inhibitor of CYP7A (cholesterol hydroxylase) (del Castillo-Olivares and Gil, 2000; Denson *et al.*, 2001; Wang and LeCluyse, 2003).

Cross-talk among the orphan nuclear receptors

The individual response elements of the different phase I CYP genes can be activated by more than one single nuclear receptor, and it is commonly referred to as "cross-talk". Recent study found that PXR can bind to the PBREM located in the 5'-flanking region of the CYP2B (Pascussi *et al.*, 2003b; Sueyoshi and Negishi, 2001; Wang and LeCluyse, 2003). Dexamethasone is a ligand for mouse PXR but not an activator for mouse CAR, study

revealed that it could potentially induce CYP2B10 expression in mouse hepatocytes (Wang and LeCluyse, 2003; Wei *et al.*, 2002). PXR activators such as rifampin, phenobarbital, phenytoin and clotrimazole (an PXR activator but CAR deactivator) can efficiently induce CYP2B6 expression in human hepatocytes (Honkakoski *et al.*, 1998a, 1998b; Xie *et al.*, 2000b). All PXR activators can transactivate CYP2B6 hPBREM reporter gene expression after cotransfection of hPXR with the CYP2B6 hPBREM or NR1 reporter vectors in human hepatocytes. A distal xenobiotic responsive enhancer module (XREM) was found to be located in the promoter of the CYP2B6 gene recently, and both PXR and CAR can bind to and activate this novel XREM (Wang *et al.*, 2003). Transfection of both PBREM and XREM was found to maximally activate CYP2B6 reporter gene (Wang *et al.*, 2003; Wang and LeCluyse, 2003). All these results strongly support the notion that PXR plays an important role in the regulation of CYP2B gene. Both CYP2C8 and CYP2C9 expression can be induced by PXR activators such as rifampin, SR-12813 and paclitaxel in human hepatocytes, suggesting that a PXR response element may be present in the promoter region of these genes (Pascussi *et al.*, 2003b; Wang *et al.*, 2003). To date, several DR4 and DR5 elements have been found in the upstream 5'-flanking region of CYP2C9 start site. The role of PXR in the transcriptional regulation of CYP2C gene expression is still unclear, and needs further investigation of the upstream region of the CYP2C gene promoters (Ferguson *et al.*, 2002; Pascussi *et al.*, 2003b; Wang and LeCluyse, 2003). CYP7A was also reported to be regulated by PXR (Staudinger *et al.*, 2001a, 2001b; Wang and LeCluyse, 2003; Waxman, 1999).

Although CAR and PXR were originally identified as the regulators of CYP2B and CYP3A, respectively, there are a lot of cross-talk between the induction of these target genes by these two compounds. This is due in part to the fact that both CAR and PXR can recognize other response elements such as DR3, DR4 or ER6, and resulting in the induction of CYP3A and CYP2B by either common or selective ligands. Both CAR and PXR can regulate the CYP3A and CYP2B gene expression by their specific ligands in CV-1 cells as well as in hepatocytes (Wang *et al.*, 2003; Wang and LeCluyse, 2003; Xie *et al.*, 2000b, 2001).

UGTs play an important role in phase II metabolism, and they are mainly expressed in the liver. Phenobarbital has been used for the treatment of Crigler-Najjar syndrome for quite some time, and it was reported to induce UGT1A1 (Innocenti *et al.*, 2002; Sugatani *et al.*, 2001). UGT1A1 is the specific isozyme responsible for bilirubin conjugation and detoxification. A 290-base-pair distal enhancer containing three putative nuclear receptor motifs was found

and identified to be necessary for UGT1A1 induction by phenobarbital, and it was considered to be correlated with the regulation by CAR (Pascucci *et al.*, 2003a; Ritter *et al.*, 1999; Sugatani *et al.*, 2001; Wang and LeCluyse, 2003).

Overall, the transcriptional regulation of phase I drug metabolizing enzymes is a multifaceted and complicated process. A single orphan nuclear receptor may mediate the induction of multiple target genes, and conversely, a single gene may be coregulated by multiple orphan nuclear receptors and ligands.

REGULATION OF PHASE II DMEs

The role of phase II conjugation in the metabolism of drugs and xenobiotics in the human body has been studied for a long time however, the mechanism of phase II genes regulation remains unclear until recently. Many structurally unrelated chemicals including the PAHs, barbiturates and many naturally occurring cancer chemopreventive agents including phenolic antioxidants, isothiocyanates and flavonoids were all found to induce phase II genes (Chen *et al.*, 2000; Hu *et al.*, 2004; Keum *et al.*, 2003; Owuor and Kong, 2002; Schilter *et al.*, 1993; Shen *et al.*, 2004). Further studies of the promoters of

some of the phase II genes revealed the existence of several *cis*-acting regulatory elements, such as the anti-oxidant response element (ARE)/electrophile response element (EpRE), xenobiotic-responsive element (XRE)/aromatic hydrocarbon responsive element (AhRE), activator protein-1 (AP-1), and nuclear factor-kappa B (NF- κ B) binding sites in their 5'-flanking regulatory region (Hu *et al.*, 2004; Itoh *et al.*, 1997; Keum *et al.*, 2003; Kong *et al.*, 2001a; Rushmore and Kong, 2002; Shen *et al.*, 2004). Most recent findings suggest and support the key role of the ARE/EpRE in the regulation of expression of some phase II genes such as NQO, GST, and UGT by phenolic antioxidants and other naturally occurring cancer chemopreventive agents (Chen *et al.*, 2000; Hu *et al.*, 2004; Keum *et al.*, 2003; Kong *et al.*, 2001a, 2001b; Owuor and Kong, 2002; Rushmore and Kong, 2002; Shen *et al.*, 2004). Recently, several ARE/EpRE-binding proteins have been proposed and identified, including the members of basic leucine zipper transcription factor (bZIP) family, Nrf1, Nrf2, and small Maf proteins. A nuclear protein ARE-BP1, has also been described to bind constitutively to the ARE-inducible sequence, the GC box, and to be activated by tBHQ possibly through a post-translational mechanism (Itoh *et al.*, 1997; Owuor and Kong, 2002), the exact

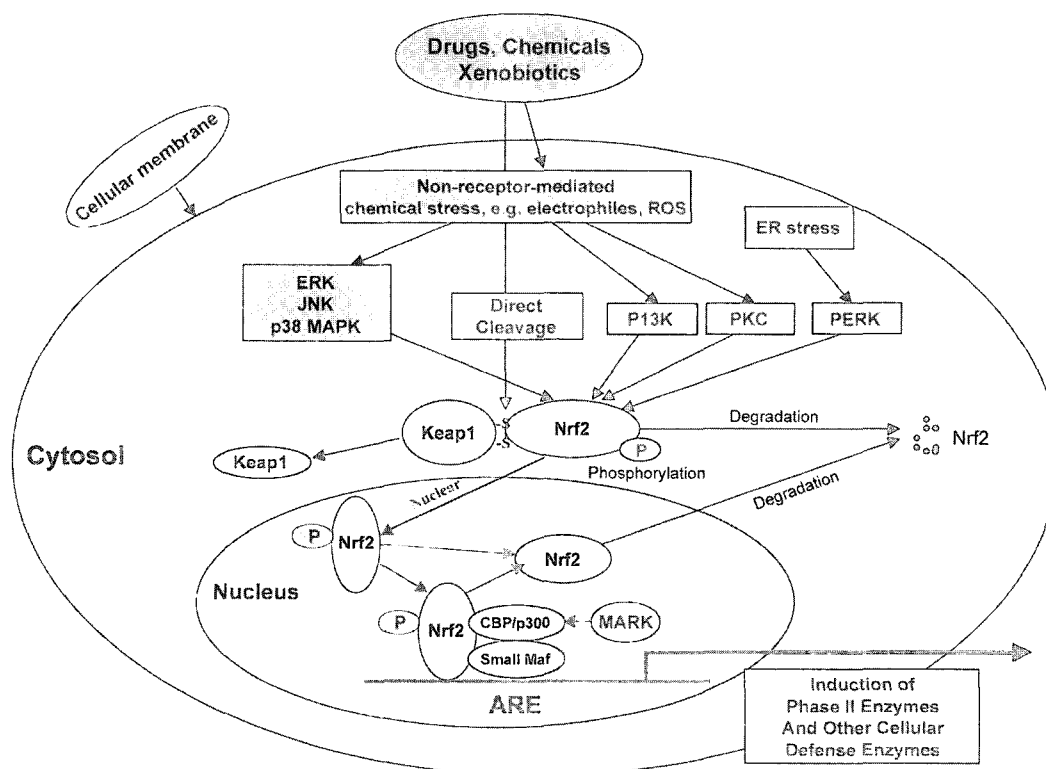


Fig. 2. A schematic representation of drugs/chemicals/xenobiotics induces stress response leading to the potential sulfhydryl modification of Keap1-Nrf2 and/or activation of the signaling pathways such as the non-receptor-mediated MAPK (ERK, JNK, and p38), PKC, PI3K and PERK. The activation of these signaling pathways leads to the activation of transcription factors such as Nrf2/Maf and increase in ARE-mediated gene expression including the phase II DMEs (GST, NQO, UGT) as well as other cellular defensive enzymes (GCL, HO-1), which ultimately results in the increase of detoxification of the xenobiotics and/or generated ROS, leading to a potential homeostatic cell survival response.

identity of this protein is still unclear, presumably could be related to Nrf2/Maf complex. The central role of Nrf2 in the transcriptional activation of ARE-reporter genes has been confirmed recently in other ARE-mediated genes including human γ -glutamylcysteine ligase (GCL), and mouse heme oxygenase-1 (HO-1) (Chen *et al.*, 2000; Kong *et al.*, 2000, 2001a, 2001b; Owuor and Kong, 2002; Shen *et al.*, 2004). The induction of NQO and GST by the phenolic antioxidant BHA was largely eliminated in the intestine and liver of Nrf2^{-/-} mice, and the gene expression of several detoxification enzymes including NQO was markedly reduced in the lung of Nrf2^{-/-} mice (Itoh *et al.*, 1997). This lack of phase II DME induction in Nrf2^{-/-} mice strongly suggests that Nrf2 is the most likely transcriptional factor involved in the transcription activation of ARE-mediated phase II genes and cellular defense genes induction (Chan and Kan, 1999; Itoh *et al.*, 1997; Kwak *et al.*, 2001).

Questions remain over the past few years as to how Nrf2 is transcriptionally activated by such diverse chemical compounds. Several models have been proposed and put forward as depicted in Fig. 2 and the biological reality probably involve the convergent of some or all of these multiple signaling pathways depending on the chemical structures, cell or tissues types, the gene of interest and in conjunction with other signaling events that are yet to be uncovered.

Previously, our group has shown that the mitogen-activated protein kinase (MAPKs) are involved in the regulation of the ARE in a Nrf2-dependent manner using transient transfection studies as well as kinase specific chemical inhibitors (Yu *et al.*, 2000a). We found that the extracellular signal-regulated kinase 2 and 5 (ERK2, ERK5), and *c*-Jun *N*-terminal kinase 1 (JNK1) upregulated the ARE (Keum *et al.*, 2003; Shen *et al.*, 2004; Yu *et al.*, 1999), while the p38 MAPK appears to suppress it (Yu *et al.*, 2000b).

The phosphatidylinositol 3-kinase (PI3K) has been postulated to be a positive regulator of ARE in IMR-32 neuroblastoma by the use of PI3K chemical inhibitor, wortmannin (Lee *et al.*, 2001). Kang *et al.* provided further evidence that PI3K may be involved in Nrf2 nuclear translocation in response to tBHQ-induced oxidative stress in conjunction with cytoplasmic actin rearrangement (Kang *et al.*, 2002). Furthermore, Huang *et al.* have reported that protein kinase C (PKC) can directly phosphorylated Nrf2 (Huang *et al.*, 2000) and Ser-40 appears to be a site of potential phosphorylation (Huang *et al.*, 2002). Furthermore, Cullinan *et al.* have indicated that Nrf2 is directly phosphorylated by PERK, a transmembrane transcription factor, following the accumulation of unfolded proteins in the endoplasmic reticulum (ER) (Cullinan and Diehl, 2004; Cullinan *et al.*, 2003). Taken together, these results suggest that multiple kinase pathways are involved in the tran-

scriptional activation of ARE.

The most compelling regulatory mechanism of activation of Nrf2 other than phosphorylation, have been reported recently. Dinkova-Kostova *et al.* have shown that phase II inducers, most of which are strong electrophiles, can result in direct cleavage of Nrf2-Keap1 complex by modifying Keap1 at cysteine residues through Michael reaction (Dinkova-Kostova, 2001, 2002a, 2002b). To support this hypothesis, Wakabayashi *et al.* recently shown that two of the 15 cysteine residues (Cys273 and Cys288) in Keap1 may play an important role in releasing Nrf2 in response to electrophiles and oxidative stress via the formation of an intermolecular disulfide bridge, at least in the test tube (Wakabayashi *et al.*, 2004). Questions remain whether this will occur in cells or *in vivo* tissues. In addition, the presence of an ARE-like sequence in the promoter region of Nrf2 have also been shown, which may be responsible for sustaining the duration of ARE activation by providing the binding site of Nrf2 itself, as a feedback control mechanism (Kwak *et al.*, 2002). Interestingly, strong phase II inducers such as cadmium, tert-butylhydroquinone (tBHQ), and beta-naphthoflavone (β -NF) did not affect Nrf2 mRNA level in certain cell types, but attenuated ubiquitination and proteosomal degradation of Nrf2, implying that the activity of Nrf2 may not be determined transcriptionally but perhaps post-translationally, at least in these cells types (Alam *et al.*, 2003; Nguyen *et al.*, 2003; Stewart *et al.*, 2003). Future studies in *in vivo* animal or in human into the activation of these multiple signaling pathways by xenobiotics will yield better insights into mechanisms of activation of Nrf2 leading to the induction of phase II drug metabolizing enzymes as well as cellular defensive enzymes and their biological consequences in the protection against environmental insults.

REGULATION OF PHASE III TRANSPORTERS

The major determinants of the *in vivo* systemic bioavailability of many drugs are due in part to the physico-chemical properties (solubility, ionization, lipophilicity), intestinal absorption/permeability and the intestinal/hepatic first-pass effect. The P-glycoprotein (P-gp) or multidrug resistant (MDR) protein is usually coexpressed and co-induced with CYP3A in the liver and intestine. It plays an important role in reducing drug absorption and enhancing drug elimination back to the gut lumen, and it appears that it may be regulated by PXR (Johnson *et al.*, 2003; Perloff *et al.*, 2004). P-gp is expressed at the apical surface of the intestinal enterocytes, where it mediates the efflux of xenobiotics into the intestinal lumen before these compounds can access the portal and subsequent systemic circulation. PXR ligands such as rifampicin, SR-12813, 5 β -pregnane-3-20-dione, clotrimazole, mifepristone and

nifedipine have been demonstrated to induce *MDR1* in hepatocyte and colon cancer cell lines (LS180 and LS174T) (Kullak-Ublick and Becker, 2003; Song *et al.*, 2004; Wang and LeCluyse, 2003). Constitutively activated hPXR expressed in LS180 cells was able to induce both P-gp and CYP3A expression without specific ligand binding. It appears that a similar PXR-dependent mechanism may be also involved in *MDR1* induction as compared to CYP3A induction. Endogenous *MDR1* gene is highly inducible by rifampin in human colon carcinoma cell line LS174T, using DNA binding and transfections assays. A DR4 nuclear receptor response element in the upstream enhancer at about 28 kilobase pairs was identified as a distinct PXR binding site that was essential for *MDR1* induction by rifampin (Geick *et al.*, 2001).

Most recently, many studies have demonstrated that PXR activation results in the induction of several other transporters including OATP2 (Staudinger *et al.*, 2001a, 2001b), MRP2 or canalicular multispecific organic anion transporter (cMOAT) (Kast *et al.*, 2002), and MRP3 (Kullak-Ublick *et al.*, 2004; Teng *et al.*, 2003). MRP2 is mainly expressed in liver, intestine, and kidney tubules, Similar to Pgp, MRP2 is localized to the apical membranes of these tissues (Chan *et al.*, 2004). MRP2 is originally designated as the cMOAT, is responsible for the biliary excretion of organic anions including leukotriene C4 (LTC4), divalent bile salts, and phase II glutathione, glucuronide, and sulfate conjugates. Absence of this transporter in hepatocytes is believed to be the reason for the defect in biliary excretion of organic anions in patients with Dubin-Johnson syndrome (Konig *et al.*, 1999a, 1999b). It appears that the substrates of MRP2 and Pgp to some degree are overlapping. The co-localization of MRP2 and Pgp at the apical membrane may be important in drug disposition, and may present a formidable barrier to the absorption of many drugs (Chan *et al.*, 2004). Co-expression of MRP2 with relevant phase II metabolizing enzymes such as GST and UGT, which are found to be expressed notably at sites where CYP3A4, Pgp and MRP are found including the liver, intestine and kidney, it is possible that MRP2 and GST, UGT may play a synergistic role in mediating drug elimination (Chan *et al.*, 2004; Coles *et al.*, 2002; Turgeon *et al.*, 2001). FXR, PXR and CAR appear to be responsible for induction of *Mrp2*/*MRP2* mRNA in rat, mouse and human hepatocytes (Kast *et al.*, 2002). Kast *et al.* found that *MRP2* mRNA levels were induced following treatment of human or rat hepatocytes with FXR ligands and PXR or CAR agonists. The dexamethasone- and pregnenolone 16 α -carbonitrile-dependent induction of *MRP2* expression was not evident in hepatocytes derived from PXR null mice. In contrast, induction of *MRP2* by phenobarbital, an activator of CAR, was comparable in wild-type and PXR null mice. An unusual

26-bp sequence was identified 440 bp upstream of the *MRP2* transcription initiation site that contains an everted repeat of the AGTTCA hexad separated by 8 nucleotides (ER-8). PXR, CAR, and FXR bound with high affinity to this element as heterodimers with the RXR. Furthermore, the isolated ER-8 element was capable of conferring PXR, CAR, and FXR responsiveness with the heterologous thymidine kinase (TK) promoter. Mutation of the ER-8 element abolished the nuclear receptor response. These studies demonstrate that *MRP2* may be regulated by three distinct nuclear receptor signaling pathways that converge on a common response element in the 5'-flanking region of this gene (Kast *et al.*, 2002).

MRP3 is a basolateral efflux transporter that transports bile acids as well as several clinically important anionic drugs such as etoposide, methotrexate, and glucuronide conjugates. The expression of *MRP3* in rat and human liver is low under normal conditions but is induced during cholestasis and in the absence of *MRP2* or bile salt export pump. Up-regulation of this transporter appears to compensate for the diminished ability to excrete organic anions into bile. For example, *MRP3* expression is increased in patients with Dubin-Johnson syndrome to compensate for a deficiency in biliary excretion of organic anions (Konig *et al.*, 1999a, 1999b). Elevated expression of *MRP3* is also observed in the naturally occurring *MRP2*-deficient *eisai* hyperbilirubinemic rats (Hirohashi *et al.*, 1998). *MRP3* is also believed to play an important role in the enterohepatic circulation of bile salts (Chandra and Brouwer, 2004). PXR is also activated by bile acids, which likely to prevent their accumulation to toxic levels (Staudinger *et al.*, 2001b; Xie *et al.*, 2001). When the human hepatoma cell lines HuH7 and HepG2 were treated with PXR activators including clotrimazole, rifampicin, 17 β -hydroxy-11 β -[4-dimethylaminophenyl]-17 α -[1-propynyl]estra-4,9-dien-3-one (RU486), metyrapone, nifedipine, lithocholic acid, and PCN, the levels of *MRP3* mRNA were induced 1.6- to 8-fold in a dose-dependent manner. Corresponding decreases in the multidrug resistance-associated protein-dependent cellular retention of 5-carboxyfluorescein were also seen in the treated HuH7 cells. In vivo studies demonstrated increased PXR mRNA and induction of *MRP3* mRNA in the livers of wild-type mice treated with the PXR activator RU486. On the other hand, *MRP3* induction was not seen in the RU486-treated PXR-null mice. These results suggest that PXR activation may play an important role in the regulation of *MRP3* expression (Teng *et al.*, 2003). Deletion analysis of the *Mrp3* promoter identified a basal transcription element at -123/-106, two negative response regions at -2723/-1128 and -530/-443, respectively, as well as two positive response regions at -1063/-943 and -302/-157. Site-directed mutagenesis analysis and gel mobility shift assays provided

evidence for Sp1 and Sp3 binding within the -123/-105 regions. These studies indicated that Sp1 and Sp3 may be involved in the regulation of the rat *Mrp3* gene (Tzeng and Huang, 2002). Both MRP3 mRNA level and the promoter activity of MRP3 were increased about 3-fold in human colon cells by the bile acid chenodeoxycholic acid (CDCA), and that the putative bile salt-responsive elements were found to be located in the region -229/-138, which include two alpha-1 fetoprotein transcription factor (FTF)-like elements. Construct of a specific mutation in the consensus sequence of FTF elements showed no increase in the basal transcriptional activity following CDCA treatment. In electrophoretic mobility shift assay with nuclear extracts, specific binding of FTF to FTF-like elements was observed when treated with CDCA. The expression of FTF mRNA levels was also markedly elevated after treatment with CDCA, and overexpression of FTF specifically activated the MRP3 promoter activity about 4-fold over the basal promoter activity. These results suggest that FTF may play an important role in the regulation of MRP3 expression (Inokuchi *et al.*, 2001).

Selective activation of PXR or CAR induced OATP2 and MRP3 expression in wild-type mice but not in PXR knock out (PXR-KO) mice (Staudinger *et al.*, 2003). Treatment of wild-type mice with the PXR-selective activator PCN resulted in robust increases in *Oatp2*, *Mrp3*, and *CYP3A* gene expression levels. Treatment of wild-type mice with the CAR activator phenobarbital induced only slight increases in *Oatp2*, *Mrp3*, and *CYP3A* gene expression levels. In contrast to treatment with phenobarbital, treatment of wild-type mice with the CAR-selective activator 1,4-bis[2-(3,5-dichlorophenyl)oxy]benzene (TCPOBOP) potently induced increases in *Oatp2*, *Mrp3*, and *CYP3A* gene expression levels. There were no changes in *Oatp2*, *Mrp3*, and *CYP3A* gene expression when PXR-KO mice were treated with PCN, however, phenobarbital treatment of PXR-KO mice produced relatively obvious increases in *Oatp2*, *Mrp3*, and *CYP3A* gene expression when compared with the phenobarbital-treated wild-type mice (Staudinger *et al.*, 2003), suggesting the importance of CAR and PXR in the co-regulation of these transporters. In the same study, MRP2 expression was significantly induced by phenobarbital and this was similarly reported in rat liver that MRP2 (cMOAT) was found to be induced by phenobarbital using microarray gene chip study (Rushmore and Kong, 2002).

OATP2 is localized to the hepatic sinusoidal membrane, with selective expression in the midzonal to perivenous hepatocytes (Reichel *et al.*, 1999). Expression of OATP2 has also been detected in the brain and retina (Gao *et al.*, 2002). Treatment of rats with PXR activator PCN, significantly enhances the rat *oatp2* gene expression (Guo *et al.*, 2002a, 2002b). Four potential PXR response elements

(PXREs) were identified in the 5'-flanking region of the *oatp2* gene. One element (DR3-1) is located approximately ~5000 bp with three more (DR3-2, DR3-3, and DR3-4) clustered at about ~8000 bp. Results from electrophoretic mobility shift assays showed that the PXR-RXR heterodimer binds to the DR3-2 with the highest affinity, to the DR3-4 and DR3-1 with a lower affinity, and weakly or not at all to the DR3-3. Furthermore, a series of partial deletions of the 5'-flanking region illustrated that both the proximal and distal clusters of PXREs are required for maximal induction of rat OATP2 by PCN (Guo *et al.*, 2002a, 2002b).

COORDINATED REGULATION OF PHASE I, PHASE II DMES AND PHASE III TRANSPORTERS

Many phase I and phase II enzyme inducers share common mechanisms of transcriptional activation and share a similar battery of genes that are coordinately regulated. Many phase II metabolites were found to be transported out of the cells by P-gp, MRPs, and OATP2. Along with the phase I and phase II enzyme induction, pretreatments with several types of inducers have been shown to alter the excretion of xenobiotics, which implies that phase III transport processes may also be similarly regulated. Whether these phase I and phase II enzyme inducers coordinately regulate the so-called phase III transporter genes requires further studies, and such information would add to our knowledge of the disposition and elimination of xenobiotics.

3-methylcholanthrene (3MC) can induce the expression of *CYP1A1*, *CYP1A2* and *CYP1B1* by activating the AhR regulating transcription of the CYP1 genes. *CYP1A1* is undetectable in the liver of control rats but was found to be highly induced in the liver of 3MC treated rats. Induction of *CYP1A2* and *CYP1B1* were also observed in the livers from rats treated with 3MC (Rushmore and Kong, 2002). Induction of several phase II enzymes, *UGT1A6*, *GSTA1*, *GSTA2*, and *GSTM1* was also observed in the liver recovered from rats treated with 3MC. 3MC is known to induce expression of the UDP-glucuronosyl transferase gene *UGT1A6* (Bock *et al.*, 1998) and glutathione-S-transferase gene *GSTA1* (Rushmore *et al.*, 1990) by an AhR-dependent mechanism (Rushmore and Kong, 2002). The *GSTA2* and *GSTM1* genes are both induced by the *CYP1A1* epoxide and hydroxylated metabolite(s) of 3MC. Both genes contain an ARE domain in their promoter regions. This *cis*-acting element has been shown to be responsive to the diol metabolites of 3MC that can redox cycle and produce a pro-oxidative environment (Rushmore and Kong, 2002), presumably analogous to the electrophilic actions of phenolic anti-

oxidants BHA or tBHQ as described before. The glutathione-S-transferases *GSTA2* and *GSTM1* were previously observed to be 3MC-inducible in cultured rat hepatocytes (Maheo *et al.*, 1997). High levels of *mdr* mRNAs were observed by Northern blotting in two independent rat liver epithelial (RLE) cell lines after treatment with 3MC. 3MC-mediated *mdr* mRNA induction was demonstrated to be dose-dependent, it occurred through enhanced expression of the *mdr 1* gene, and paralleled the induction of the P-gp protein expression (Fardel *et al.*, 1996).

Phenobarbital is a transcriptional inducer of the rat *CYP2B1*, *CYP2B2* and *CYP3A1* genes. CAR is a nuclear receptor that interacts with RXR to form CAR-RXR heterodimers, which bind to the PBRE in response to phenobarbital treatment. *CYP2C7* is also reported to be induced by phenobarbital, but primarily in female rather than in male rats (Honkakoski and Negishi, 1997; Rushmore and Kong, 2002; Waxman, 1999). Induction of several phase II enzymes was observed after treatment with phenobarbital. A significant increase in the specific mRNA for microsomal epoxide hydrolase (EHm), *UGT2B1*, *GSTA1*, *GSTA2*, *GSTA3*, and *GSTM1*, was also observed in the livers recovered from rats treated with phenobarbital. Phenobarbital is known to induce the expression of CYP2B gene by the CAR-dependent mechanism. No PBRE sequence has been identified in the promoters for any of the phase II enzymes to date. In addition to the phase I and phase II enzyme induction, MRP2 expression was significantly induced by Phenobarbital (Staudinger *et al.*, 2003). MRP3 was also reported to be induced by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), a CAR and CYP2B inducer. However, the data suggested that CAR may not play a key role in phenobarbital-induced MRP3 (Xiong *et al.*, 2002).

Eighteen different microsomal enzyme inducers including TCDD, indole-3-carbinol, phenobarbital, diallyl sulfide, spironolactone, dexamethasone, diethylhexylphthalate, ethoxyquin, oltipraz, and acetylsalicylic acid were selected based upon six major proposed mechanisms of drug-metabolizing enzyme induction (AhR ligands, CAR activators, PXR ligands, PPAR ligands, EpRE activators, and CYP2E1 inducers), and they did not markedly increase the expression of MRP1 or MRP2 (Cherrington *et al.*, 2002). However, MRP3 expression was significantly increased by each of the CAR activators (phenobarbital, 390%; 2,2',4,4',5-pentachlorobiphenyl (PCB99), 580%; and diallyl sulfide, 540% over control), and an EpRE activator oltipraz (670% over control) in the livers of the rats. MRP3 was not similarly induced in kidney and large intestine, demonstrating that the coordinate inducibility of MRP3 may be specific to the liver. Additional evidence suggests that MRP3, which is under the transcriptional regulation of CAR, may have liver-specific induction of

MRP3 by CAR activators, because CAR is expressed almost exclusively in liver. The authors conclude that rat hepatic MRP3 is induced by CAR activators, thus enhancing the vectoral excretion of some phase II metabolites from the liver to the blood (Cherrington *et al.*, 2002). CAR has been described as a cellular sensor that is capable of responding to chemical toxicity and mediating CYP2B family induction (Honkakoski *et al.*, 1998b; Kawamoto *et al.*, 1999; Sueyoshi *et al.*, 1999). Activation of this cellular sensor leads to an increase expression of MRP3, which may lead to an enhanced ability of the liver to eliminate organic anions into the sinusoidal blood, thereby reducing hepatic toxicity.

OATP2 levels were decreased 56 to 72% by the AhR ligands (TCDD, indole-3-carbinol, and b-naphthoflavone), increased 84 to 132% by the CAR ligands (phenobarbital, diallyl sulfide, and PCB 99), increased 230 to 360% by PXR ligands (PCN, spironolactone, and dexamethasone), and no changes on OATP2 levels by PPAR ligands and ARE/EpRE activators were observed (Guo *et al.*, 2002a, 2002b). There was no correlation between *Oatp2* mRNA levels with the altered OATP2 protein levels, for example, among the PXR ligands, only PCN increased *oatp2* mRNA levels, but spironolactone and dexamethasone did not. Furthermore, only PCN, but not spironolactone and dexamethasone, increased the transcription of the *oatp2* gene as shown by the increase amount of mRNA. These authors concluded that OATP2 may be coordinated regulated by the PXR-CYP3A inducers, and that the regulation of OATP2 by these inducers occurs at both the transcriptional and post-translational levels (Guo *et al.*, 2002a, 2002b).

In summary, the importance of the coordinated regulation of the phase III transporter with the phase I and phase II drug metabolizing enzymes needs further investigation. The addition of phase III transporters, such as P-gp, MRPs and OATPs, regulation of gene expression by receptors such as PXR and CAR, will lend better understanding to the biological functions of PXR and CAR as a chemical/xenobiotics sensor, however, most importantly, as a mean of managing the protection of the drug or xenobiotics exposure from the environment. Future studies will shed light on the roles of other receptors, transcription factors and signaling cascades in the coordinated regulation of phase I, II and III drug metabolism/transport by endogenous compounds as well as by exogenous agents including environmental and nutritional, in the triggering of diseases and or toxicities induced by these chemicals.

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