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Genomic Profiling in Nuclear Receptor-Mediated Toxicity

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ABSTRACT

Nuclear receptors (NRs) are attractive drug targets due to their role in regulation of a wide range of physiologic responses. In addition to providing therapeutic value, many pharmaceutical agents along with environmental chemicals are ligands for NRs and can cause adverse health effects that are directly related to activation of NRs. Identifying the molecular events that produce a toxic response may be confounded by the fact that there is a significant overlap in the biological processes that NRs regulate. Microarrays and other methods for gene expression profiling have served as useful, sensitive tools for discerning the mechanisms by which therapeutics and environmental chemicals invoke toxic effects. The capability to probe thousands of genes simultaneously has made genomics a prime technology for identifying drug targets, biomarkers of exposure/toxicity and key players in the mechanisms of disease. The complex intertwining networks regulated by NRs are hard to probe comprehensively without global approaches and genomics has become a key technology that facilitates our understanding of NR-dependent and -independent events. The future of drug discovery, design and optimization, and risk assessment of chemical toxicants that activate NRs will inevitably involve genomic profiling. This review will focus on genomics studies related to PPAR, CAR, PXR, RXR, LXR, FXR, and AHR.

Keywords. Toxicogenomics; nuclear receptor; transcription profiling; PPAR; CAR; PXR; AHR.

NUCLEAR RECEPTORS AND THEIR ROLE IN MEDIATING PHYSIOLOGIC, THERAPEUTIC, AND TOXIC RESPONSES

Nuclear receptors are a group of ligand-activated transcription factors, which are responsible for regulating expression of genes associated largely with metabolism, developmental function, and cell differentiation. Nuclear receptor-targeted pharmaceuticals are estimated to be 10–15% of the $400 billion global pharmaceutical market. A number of therapeutic compounds including antibiotics, anticonvulsants, hypolipidemics, and cancer therapies target nuclear receptors. They are prime candidates as drug targets for several reasons. Because the ligands are small and lipophilic, they resemble endogenous inducer compounds. Many nuclear receptors have tissue-specific expression that is crucial for the specificity of drug action. Also, nuclear receptors play key roles in many physiological processes where phase I and II metabolism genes are involved. These include synthesis and metabolism of steroids, vitamin D, cholesterol, lipids, and bile acids (Waxman and Azaroff, 1992; Mangelsdorf and Evans, 1995; Honkakoski and Negishi, 2000).

Nuclear receptors are also inadvertent targets of numerous anthropogenic environmental toxicants (e.g., phthalates, dioxins). While the mechanisms by which these chemical agents mediate toxicity are still largely unknown, nuclear receptors have been shown to play key roles in their pathophysiological effects. Thus, determining the human health risk, particularly of low-dose chronic exposures to nuclear receptor ligands is of increasing priority to environmental regulatory agencies.

The nuclear receptor superfamily of proteins is subdivided into 6 subfamilies, based on their amino acid sequence similarities (Mangelsdorf et al., 1995; Aranda and Pascual, 2001). The general structure of all nuclear receptors is very similar, and encapsulates three functional domains: (1) a ligand-binding/dimerization domain, (2) a DNA-binding/weak dimerization domain, and (3) transactivation domains. The ligand-binding and dimerization domain at the carboxy terminus of the receptor is where endogenous or xenobiotic ligands bind resulting in activation of the receptor.

Many receptors require heterodimerzation with retinoid x receptor (RXR) (Mangelsdorf and Evans, 1995). The DNA-binding domain of the receptor is responsible for recognition of a receptor-specific response element consisting of a specific sequence of nucleotides in the promoter region of the target gene. The transactivation domains consist of a ligand-independent transcription activation function (AF)-1 and a ligand–dependent transcription AF-2. AF-1 is located

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Abbreviations: aryl hydrocarbon receptor, AHR; constitutive androstane receptor, CAR; cytochrome P450s, CYP450s; farnesoid x receptor, FXR; liver x receptor, LXR; peroxisome proliferators, PPs; peroxisome proliferator activated receptors, PPARs; phenobarbital, PB; pregnane x receptor, PXR; retinoid x receptor, RXR; 2′,3′,7,8′-tetrachlordibenzo-*p*-dioxin, TCDD.

at the amino acid terminus of the receptor and is a target for kinase mediated phosphorylation cascades, which can affect transcriptional activity in the absence of ligand-binding. AF-2 is located at the carboxy terminus that also harbors the ligand-binding site. AF-2 can interact with other transcription factors to form a complex of co-activators, which regulate histone acetyltransferase activity, or co-repressors, which regulate histone deacetylase activity (Glass and Rosenfeld, 2000; Steinmetz et al., 2001).

In common among most nuclear receptors is that they transcriptionally regulate a number of proteins involved in xenobiotic metabolism, particularly phase I cytochrome P450 (CYP450) enzymes (Figure 1) (Waxman, 1999; Honkakoski and Negishi, 2000; Johnson et al., 2002). Because of the redundancy in regulation of CYP450s and many other genes, identifying individual nuclear receptors that are responsible for a specific therapeutic or toxic effect can be challenging. Not only do nuclear receptors share transcriptional targets, but many serve as transcriptional inducers of one another. Also, ligands are often not selective for one particular nuclear receptor target, but rather are partial or full agonist for a number of receptors.

In light of these complexities, new methods have been developed for studying global chemical-induced changes in macromolecules (genes, proteins, metabolites) that collectively define an organ's response. Genomic profiling involves use of microarray technology (but has recently been broadened to include multiplex RT-PCR assays) to measure transcript levels in tissue or cell culture. Because of the high-throughput capabilities and data-rich output, gene expression profiling is an efficient and useful alternative to traditional methods in toxicology.

Microarrays as a Tool for Mechanistic and Predictive Toxicology

Microarray technology has proven to be a powerful tool for simultaneous collection of large amounts of data on expression of tens of thousands of genes. Traditional methods for measuring transcript levels (i.e., quantitative RT-PCR, northern blot analysis, RNase protection assay), while well-established and robust, simply cannot provide the same high-volume assaying capabilities that microarrays offer. Furthermore, with the recent completion of the mouse genome project in 2002 (Gregory et al., 2002) and the completion of the human genome shortly thereafter (Collins et al., 2003), understanding how gene-environment interactions affect health outcomes and using model systems to characterize these relationships is now more attainable than ever before.

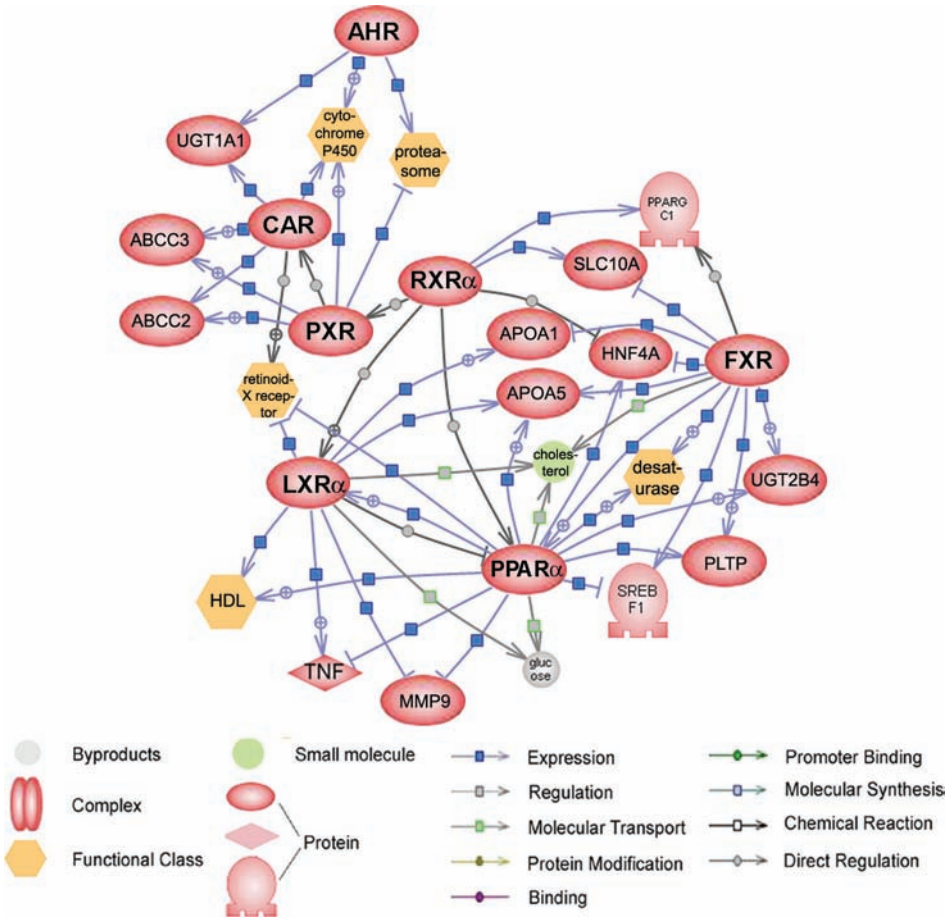

FIGURE 1.—Cross-talk and co-regulation among nuclear receptors. This and other schemes in this review were prepared using PathwayStudio 4.0 software (Ariadne Genomics, Rockville, MD) (Nikitin et al., 2003). Using Medscan natural language processing, information from all abstracts on PubMed and other public data sources was extracted to assemble molecular networks. Designation of nodes and edges is indicated at the bottom of the figure.

For chemical compounds in which the mechanism of toxicity is well-characterized and key players are known, the more focused view that traditional assays offer is suitable; however, for most chemical toxicants, the molecular events leading to toxicity or involved in disease progression are not well-defined. In these cases, the genome-wide view of gene expression that microarrays offer is more ideal. Because toxicogenomics is a relatively novel technology, there are a number of limitations that must be resolved before array data is widely accepted.

Microarray studies have been touted as being highly sensitive for detecting toxic responses at much earlier time points and/or at lower doses than histopathology, clinical chemistry or other traditional toxicological assays can detect (Heinloth et al., 2004; Morgan et al., 2005). However, based on the nature of the assay, measurements of extreme levels of gene expression—low or high—are thought to be unreliable. Also, the reproducibility of microarray experiments has raised concerns. "Batch effects" based on the day, user, and laboratory environment have been observed in array datasets (Baker et al., 2004; Bammler et al., 2005). To address these concerns, confirmation of microarray-derived gene expression profiles is typically performed using quantitative real-time polymerase chain reaction (RT-PCR) or Northern blot analysis.

Another limitation to widespread use of genomics technology is the high cost. The price and availability of microarrays in early years of the technology were prohibitive. With the establishment of genomic core facilities at universities and research institutes and growing competition in the private sector, the cost of array experiments is now more reasonable. Given the high return of data on investment, it is likely that this technology will continue to be used widely.

Finally, improvements should continue to be made on statistical analysis and presentation of microarray data such that it is easy to interpret. Prior to the current advances in bioinformatics, the most common way of reporting results of microarray studies involved listing differentially expressed genes, with little information about the statistical significance or biological pathways with which the genes are associated. New mathematical and graphical approaches have been developed to improve data presentation and interpretation. Also, curated web-based tools and software applications have been developed to provide information on cellular location, physiological function, or disease association of a given gene. These approaches to analyzing array data, coined "pathway mapping" provide more biological relevance to the analyses.

Toxicogenomics studies thus far have largely supported what is already known for many chemical compounds, though opposing and inconclusive results have been presented. Until many of the above-mentioned shortcomings (i.e., cost, reproducibility, and data presentation/interpretation) and others are addressed, the great potential for toxicogenomics as a predictive and mechanistic tool in risk assessment may not be fully realized.

This review considers the current body of knowledge involving use of genomic profiling to understand the role of nuclear receptors, primarily as they relate to chemical-induced liver toxicity. Many of these studies have furthered our understanding of molecular mechanisms underlying xenobiotic-induced liver injury and ability to predict toxicity. Several nuclear receptors that are currently of great pharmacological or toxicological relevance, which include peroxisome proliferator activated receptors (PPARs), constitutive androstane receptor (CAR), pregnane X receptor (PXR), retinoid X receptor (RXR), liver X receptor (LXR), and farnesoid X receptor (FXR) are detailed in this review. The aryl hydrocarbon receptor (AHR), though not a nuclear receptor, will also be taken into consideration here. The estrogen receptor will not be discussed, since a comprehensive review on use of toxicogenomics to understand molecular mechanisms of toxicity by xeno-estrogens was recently published (Moggs, 2005).

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARS)

In general, PPARs are known for their role in fatty acid metabolism and glucose homeostasis (Dreyer et al., 1992; Schmidt et al., 1992). For this reason, they have been identified as useful drug targets for hyperlipidemia, diabetes and obesity. Of the 3 known isoforms of PPARs—α, β/δ and γ—PPARα and PPARγ have been studied most widely.

Pharmaceutical compounds that target PPARα exploit the induction of peroxisomal β-oxidation of fatty acids in the liver (Auboeuf et al., 1997). Main target genes of PPARα (Figure 2) include acyl-coA oxidase (*Aco*), carnitine palmitoyltransferase 1 (*Cpt1*), which are involved in β-oxidation and *Cyp4a1*, which is involved in ω-oxidation of fatty acids (Dreyer et al., 1992; Tugwood et al., 1992; Gulick et al., 1994; Aldridge et al., 1995; Picard and Auwerx, 2002). Treatment of patients who suffer from hyperlipidemia with PPARα-activator pharmaceuticals (e.g., clofibrate, gemfibrozil, ciprofibrate) results in a significant reduction of serum triglycerides and increase in HDL-cholesterol. In addition to therapeutics, a number of industrial compounds have been identified as ligands of PPARα, including phthalate esters, such as di-(2-ethylhexyl) phthalate (DEHP), trichloroethylene (TCE), perfluorooctanoic acid (PFOA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Reddy and Lalwani, 1983; Gonzalez et al., 1998). Long-chain saturated and polyunsaturated fatty acids are endogenous activators of PPARα (Vanden Heuvel et al., 2006).

PPARγ plays a major role in adipocyte differentiation and glucose and insulin homeostasis. It is mainly found in adipose tissue and cells of the immune system, although it is expressed to some extent in the intestines and liver (Kliewer et al., 1994; Braissant et al., 1996). Compounds that activate PPARγ, such as thiazoladinediones have proven to be a good treatment for conferring insulin-sensitivity to insulin-resistant diabetics (Alarcon et al., 2004; Vasudevan and Balasubramanyam, 2004). Endogenous ligands of PPARγ include fatty acid derivatives such as 15-deoxy-delta12,14-prostaglandin J2 (15d-PJ2) and eicosapentaenoic acid (Forman et al., 1995; Yu et al., 1995).

PPARβ/δ has been studied much less than the PPARα and PPARγ, and thus fewer ligands have been identified. PPARβ/δ is ubiquitously expressed, with relatively high levels in the brain, adipose tissue and skin (Amri et al., 1995; Braissant et al., 1996). It likely plays a minor role in fatty acid metabolism, as fatty acids are weak agonists of this receptor (Kliewer et al., 1994). It has been proposed that

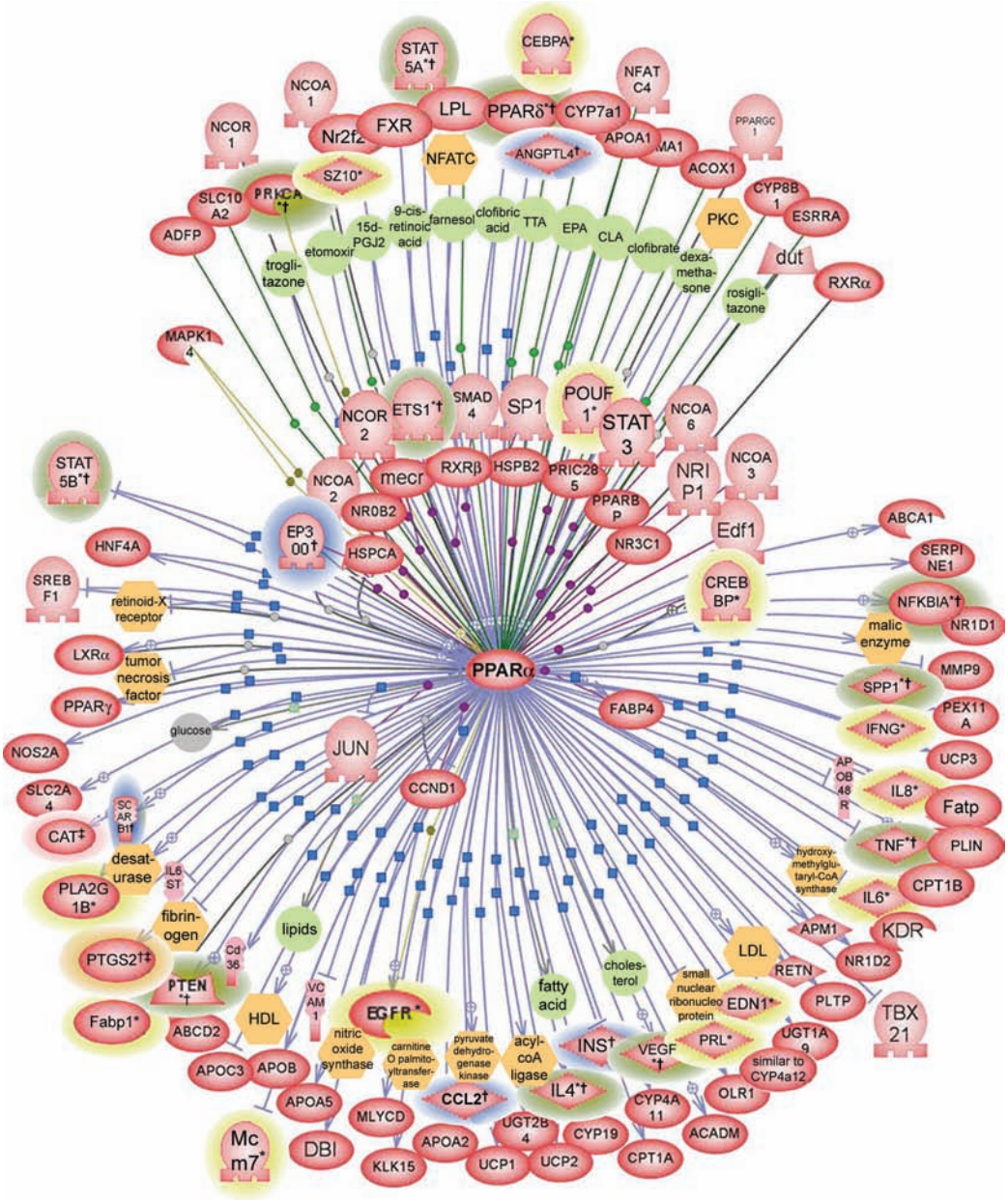

FIGURE 2.—Regulatory network modulated by PPARα consists primarily of mediators of fatty acid metabolism. On this and other figures, proteins involved in cell proliferation (denoted by "*" and highlighted yellow), apoptosis (denoted by "†" and highlighted blue) or oxidative stress (denoted by "‡" and highlighted red) were identified by searching for these terms in each proteins' GO Biological Processes using PathStudio software.

PPARβ/δ is involved in skin proliferation (Peters et al., 2000; Michalik et al., 2001). For this reason it has been considered as a potential drug target for wound healing and skin cancer (Tan et al., 2003; He et al., 2005). Studies have also suggested that PPARβ/δ is also involved in fatty acid and glucose metabolism (Luquet et al., 2005). *Ppar*β/δ knockout mice exhibit glucose intolerance and activation of PPARβ/δ improves insulin sensitivity in diabetic mice (Lee et al., 2006). These findings suggest that PPARβ/δ should be considered as a drug target for metabolic syndrome.

Gene expression profiling to investigate molecular mechanisms associated with toxic effects resulting from PPAR activation has been carried out to a large extent for PPARα and to a lesser extent for PPARγ and PPARβ/δ. Due to the dearth of genomics studies on PPARβ/δ and PPARγ, and the greater relevance of PPARα as a mediator of chemical-induced toxicity in liver, the remainder of the focus will be placed on the PPARα.

Gene Expression Profiling Supports a Nongenotoxic Mechanism of Action of PPARα-Agonists

PPARα agonists are collectively referred to as peroxisome proliferators (PPs) because of the hyperplastic effects on liver peroxisomes following exposure in rodents (Reddy and Krishnakantha, 1975). In addition to peroxisomal proliferation, the size and number of hepatocytes also increases, causing significant liver enlargement. Chronic administration of PPs in rodents leads to liver tumorigenesis by a nongenotoxic

mechanism (Kluwe et al., 1982; Marsman and Popp, 1994). Additionally, male reproductive and developmental toxicity and carcinogenic effects in testis, pancreas and kidney have also been associated with chronic administration of a number of PPARα-agonists (Kurokawa et al., 1988; Biegel et al., 2001; Peraza et al., 2005). While humans display the therapeutic effects of pharmaceutical PPARα targets, they are thought to be nonresponsive to the adverse effects. This may be due to species (rodent-to-human) differences in receptor expression, structure, function, and other factors (Mukherjee et al., 1994; Palmer et al., 1998; Lambe et al., 1999).

An induction of cell proliferation, oxidative stress, and suppression of apoptosis are generally accepted as key steps in the mode of action of non-genotoxic carcinogens (Butterworth, 1990). Indeed, these responses were demonstrated in the acute, sub-acute and sub-chronic gene expression studies that were conducted to identify gene signatures associated with peroxisome proliferator-induced effects in liver.

Gene expression profiling along with pathway mapping as an unbiased way to identify gene signatures and temporal associations was used by several research groups to study acute and sub-acute effects of DEHP on mouse liver. Currie et al. (2005) reported that acute exposure to DEHP induces a 2-stage transcriptional response, one occurring at early time points (2 and 8 hours), and another occurring later (24 and 72 hours). This study suggests that many PPARα ligand-induced regulatory events occur prior to induction of cell proliferation and liver growth, which were detected 48 hours after dosing. Further analysis of early response genes demonstrated an endoplasmic reticulum-overload response, response to stress and negative regulation of protein kinase activity. The majority of genes with altered expression in response to DEHP were those encoding proteins within the peroxisome and microsome. A large set of DEHP-regulated biological processes included metabolism, particularly fatty acid, amino acid, steroid and bile acid metabolism were altered. Novel responses to DEHP-treatment included blood clotting, circadian clock and complement activation, which were commonly identified using other pathway mapping tools.

Gene expression data from subchronic studies feeding clofibrate and ciprofibrate also confirmed well-known effects of PPs. Kramer et al. evaluated dose-dependent changes in rat liver gene expression that resulted from 5 days of clofibrate (up to 80 mg/kg) treatment (Kramer et al., 2003). Analysis of transcriptional profiles obtained from cDNA arrays identified 163 genes whose expression was altered by clofibrate treatment. A majority of the genes were associated with metabolism. PPARα activation resulted in induction of fatty acid and energy metabolism and suppression of carbohydrate and amino acid metabolism. These data corroborate other genomics studies in which a role for PPARα in amino acid metabolism was demonstrated (Kersten et al., 2001).

Surprisingly, a strong induction in genes associated with cell proliferation was not observed by Kramer et al. (2003), even though immunohistochemical detection of proliferating cell nuclear antigen (PCNA) showed a dose-dependent induction of cell replication. The lack of a transcriptional signature for cell cycle regulation could be explained by the fact that peroxisome proliferator-induced cell proliferation is thought to rapidly increase, then decline after 4–7 days of treatment (Marsman et al., 1988). In addition, genes involved in apoptosis were found to be both up- and down-regulated and immunohistochemical analysis of caspase 3 (*Casp3*) revealed no changes in apoptosis. While this study reported a dose response in the transcriptional changes, cell proliferation and clinical chemistry, it is not possible to conclude whether PPARα activation, lipid metabolism-mediated effects, or secondary effects were responsible for these changes.

Microarray studies in mouse liver following 2 weeks of dietary treatment of potent peroxisome proliferator, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (WY-14,643) revealed similar results with altered expression of genes involved in lipid and glucose metabolism, transcription, apoptosis and cell cycle (Cherkaoui-Malki et al., 2001). A novel set of 27 peroxisome proliferator-regulated genes were identified and included cell death inducing DNA fragmentation factorα (*Cide*), retinoic acid early transcript γ (*Rae*γ), cell surface receptors *Cd39*,*Cd24*, pyruvate dehydrogenase-kinase-4 (*Pdk-4*), *Cyp2b9* and *Cyp2b10*. Furthermore, PPARα dependency was demonstrated for many of these genes, as no increase in expression was observed in *Ppar*α knockout, or *Ppar*α and *Aox* double knockout mice, but was observed in *Aox* knockout mice, which exhibit spontaneous tumors.

Transcriptional changes in genes involved in lipid and carbohydrate metabolism, cell proliferation, stress response, immune and inflammatory responses and transcription were identified in rat liver following subchronic (60 days) ciprofibrate treatment (Yadetie et al., 2003). Though only 8% of the 5,000 genes that were probed on the cDNA arrays demonstrated a significant change in expression, the pathways that were altered corroborate previously observed phenotypic changes associated with long-term peroxisome proliferators treatment (Lalwani et al., 1981; Marsman et al., 1988).

Genomic Analysis of PPARα-Mediated Liver Carcinogenesis

Lack of liver enlargement, unaltered cell proliferation and absence of tumors in *Ppar*α knockout mice provide unequivocal evidence that PPARα is important to the mechanism of carcinogenesis in rodents by peroxisome proliferators (Peters et al., 1997). Also, humanized mice expressing human *PPAR*α cDNA (*hPPAR*α) do not develop hepatocellular carcinoma in response to WY-14,643 at as high an incidence rate (5%) as *mPPAR*α mice (71%) suggesting that species differences in the receptor are likely responsible for differential susceptibility (Morimura et al., 2006).

Genomic studies have further demonstrated that the mechanism of carcinogenesis by peroxisome proliferators in rodents may not be relevant to humans who appear to be insensitive to peroxisome proliferator-mediated liver effects (Bentley et al., 1993; Gonzalez et al., 1998). Gene expression changes in clofibrate-induced hepatocellular carcinomas (HCC) were compared to 6 other mouse models of HCC and human HCCs in an effort to determine what molecular events, if any, are common among human and mouse cancers (Lee et al., 2004). Interestingly, gene expression profiles revealed that clofibrate induced HCCs and those spontaneously formed in *Aox*–null mice were the least similar to human HCC.

Identifying acute markers of hepatocarcinogenesis is important for improving mechanistic understanding and diagnostic capabilities. In a recent genomics study, early gene markers were identified in clofibrate-induced liver cancer. Diethylnitrosamine (DEN) was administered in a single dose as an initiating agent, followed by dietary feeding of clofibrate for up to 20 months (Michel et al., 2005). Microarray and RT-PCR analysis of liver tissue (including tumors) at early and later time points for up to 20 months found that expression of transferring growth factor beta-induced transcript, *Tsc22*, a previously identified potential marker of hepatocarcinogenesis, was consistently down-regulated over the 20 months. This effect was reversed when clofibrate diet was removed (Kramer et al., 2004). While the function of *Tsc22* is not completely known, previous studies with anti-cancer agents have shown an induction of *Tsc22* (Uchida et al., 2000; Hino et al., 2002).

Three other genes with similar expression patterns were identified as possible acute markers of hepatocarcinogenesis. These include fibroblast growth factor receptor subtype 4 (*Fgfr4*), dual specificity protein phosphatase 1 (*Dusp1*, also called *Mkp1*), and small conductance calcium activated potassium channel (*Kcnn2*), all of which have demonstrated modulation by glucocorticoids (Riva et al., 1998; Brem et al., 1999; Kotev-Emeth et al., 2002; Engelbrecht et al., 2003). These findings suggest that clofibrate not only directly regulates genes through PPARα, but may also have indirect effects through glucocorticoid receptor inhibition.

Gene Expression Profiling Reveals PPARα- and Species-Specific Targets in Liver

Toxicogenomic analysis has been used to discriminate between transcriptional responses to chemicals of different classes (de Longueville et al., 2003; Hayes et al., 2005). In a study that compared activators of PPARα and CAR receptors, genomic analysis was performed on mouse liver following subacute (24 hours), or subchronic (2 weeks) treatment with PPARα ligands, WY-14,643, clofibrate or gemfibrozil, or CAR-activator, phenobarbital (PB) (Hamadeh et al., 2002). All compounds caused an induction of genes involved in fatty acid metabolism, cell proliferation and acute phase proteins and a suppression of genes associated with gluconeogenesis. Despite the similarities in pathways that were altered, distinct differences in expression profiles of peroxisome proliferators versus PB were observed. PPARα-agonists produced a significantly greater increase in β-oxidation pathways, whereas PB caused a large response of genes encoding for detoxification and microsomal enzymes.

Principle component analysis (PCA) along with a pairwise correlation analysis of the treatment groups confirmed the greater similarity in gene expression profiles among compounds within the same class than between the two classes. Additionally, temporal changes in expression were observed as a result of peroxisome proliferators or phenobarbital treatment. Genes that demonstrate a delayed response to treatment (higher expression at 2 weeks) were identified as good candidate markers of toxicity and adaptation to exposure, while transiently altered genes (induced at 2 hours) likely constitute the initial response in liver following the first insult.

Different mammalian species have demonstrated varying degrees of sensitivity to PPs (Dirven et al., 1993; Graham et al., 1994; Mukherjee et al., 1994; Roberts et al., 2000). In a recent genomics study, cynomolgus monkeys were exposed to ciprofibrate for 4 or 15 days (Cariello et al., 2005). Similar to rodents, a dose-dependent increase in liver weight and number of peroxisomes was observed. Though rhesus monkey arrays are available and would have been the more appropriate choice, the authors chose to use human arrays assuming that a large number of transcripts would be still detected. Pathway mapping of differentially expressed genes found reported in the 15-day, high-dose group revealed that processes involving ribosomes, proteasomes, fatty acid metabolism, tryptophan metabolism and oxidative phosphorylation were significantly up-regulated.

Down-regulation in coagulation cascades was also observed which is consistent with the therapeutic effects of fibrates and confirms previous reports of reduced inflammatory response caused by peroxisome proliferator treatment (Yadetie et al., 2003). In contrast to rodents, regulatory genes such as *Nfκb*, *Jun* and *Cmyc*, which are thought to be involved in peroxisome proliferator-induced growth and anti-apoptotic effects, were down-regulated in cynomolygus monkey liver (Miller et al., 1996; Peters et al., 1998; Rusyn et al., 1998; Klaunig et al., 2003).

Comparisons of gene expression of β-oxidation between species demonstrated a greater induction in rodents than in primates. Also, decreased mRNA for growth response genes and induction of those involved in apoptosis suggests an anti-proliferative, pro-apoptotic response in primates. These mRNA expression data correlate well with the phenotypic response, as no proliferation was observed in primate livers measured by immunohistochemical detection of Ki-67 and by mitotic activity (Hoivik et al., 2004). This genomic study confirms the disparate response in rodents and primates to PPs, and further demonstrates the idea that primates are a less sensitive species.

In vitro genomic profiling has also confirmed many of the responses observed in vivo. Studies performed in rat (FaO) and human HepG2 liver-derived cell lines following 6 hours of treatment with WY-14,643 (50 μM) revealed few similarities in gene expression (Vanden Heuvel et al., 2003). Induction of lipid metabolism and suppression of signaling and growth factor response was observed in rat cell lines. A number of novel genes that have not previously been identified as being PP-responsive, including kinases and phosphotases were also regulated by WY-14,643 treatment in FaO cells. *Mkp1* was identified as a target of PPARα that affects the receptor's activity, thus participating as autoregulatory controller. *Mkp1* has been previously described as an early responsive gene to growth factor treatment (Sun et al., 1993) and more recently as a potential early marker of hepatocarcinogenesis (Michel et al., 2005). Human cell lines demonstrated little or no induction by WY-14,643, contrary to other studies performed using HepG2 cells (Tachibana et al., 2005). Use of doxycycline for receptor induction in other studies may explain these differences in gene response.

A genomics study in immortalized hepatocytes from *Pparα* wild type and knockout mice treated with WY-14,643, demonstrated a PPARα-dependent induction of ACO and genes regulating cell cycle, along with changes in other genes implicated in cancer, such as *JunB*, and retinoblastoma protein 1 (*Rb1*) (Tien et al., 2003). Also, the WY-14,643-induced

increase in growth rate was PPARα-dependent, but this response has not been observed in other pure cell cultures.

Previous studies conducted in vitro using primary cells have demonstrated that hepatocytes, in absence of Kupffer cells or TNFα, do not exhibit WY-14,643-induced cell proliferation (Parzefall et al., 2001). One major difference between primary and permanent cultures is the capacity for proliferation, with permanent cultures exhibiting higher cell turnover, which likely explains the difference between the observations in this study and historical data. This and many other factors make primary cultures a much better model of in vivo behavior compared to transformed cell lines.

Overall, gene expression profiles for PPARα-agonists effectively demonstrate dose-response, and clear temporal differences in transcriptional regulation of many biological pathways. Also, gene expression patterns of peroxisome proliferators could be distinguished from other classes of chemicals. Furthermore, comparative genomics show that peroxisome proliferator-induced liver tumors, unlike other murine models of hepatocarcinogenesis, are distinct from human liver tumors on the gene expression level. Despite the use of different techniques for functional categorization of genes, peroxisome proliferator-mediated induction of genes associated with fatty acid metabolism and cell proliferation was common among most studies. Also suppression of genes related to amino acid metabolism, carbohydrate metabolism and immune response were widely reported. Few studies provided statistical data alongside gene expression fold-changes, making it difficult to determine whether overrepresentation of genes in a specific pathway was statistically significant. Studies that employed pathway mapping tools offered the most comprehensive assessment of transcriptional responses to peroxisome proliferators and were able to identify novel signatures that are not typically associated with peroxisome proliferator-treatment.

CONSTITUTIVE ANDROSTANE RECEPTOR (CAR) AND PREGNANE X RECEPTOR (PXR)

CAR and PXR are expressed in liver, intestine, lung, and other tissues where they play an important role in xenobiotic sensing and act as master regulators of detoxifying phase I and II enzymes (Waxman, 1999). Because of these two traits, CAR and PXR have been characterized as "xeno-sensors" that protect the liver and other organs from potentially harmful compounds (Huang et al., 2003; Kretschmer and Baldwin, 2005). Physiological ligands of PXR (also known as steroid and xenobiotic receptor, or SXR) include corticosterone, progesterone, and precursors to pregnenolone (Kliewer et al., 1998; Lehmann et al., 1998; Jones et al., 2000). PXR also has a large number of exogenous ligands, many of which have been identified as endocrine-disrupting chemicals. The ligand-binding pocket of PXR is also larger than most other nuclear receptors, which may help explain its promiscuity (Watkins et al., 2001, 2003). PXR is located in the nucleus and has low basal activity (Moore et al., 2003).

In the nucleus, it dimerizes with RXR and binds to the a xenobiotic response element of PXR target genes (Blumberg and Evans, 1998). The primary role of PXR as an activator of xenobiotic metabolism became evident from PXR-mediated induction of *Cyp3a4* (Moore and Kliewer, 2000). CYP3A4 is the most abundant CYP450 in human liver (~30%) and is responsible for metabolism of about 50% of pharmaceuticals (Rendic and Di Carlo, 1997; Kliewer et al., 1999).

CAR is a less promiscuous receptor than PXR, with fewer known ligands (Moore and Kliewer, 2000; Moore et al., 2003). Phenobarbital (PB) is the prototypic CAR-activator, though it does not directly bind the receptor (Zelko et al., 2001). Unlike PXR, CAR is located in the cytoplasm and is constitutively expressed in absence of endogenous ligand. A number of other PB-like inducers of CAR activate a signal transduction pathway that causes CAR to translocate from the cytoplasm to the nucleus. There it heterodimerizes with RXR to effect transcription of target genes. In general the chemicals that activate CAR and PXR receptors vary widely in structure, although many share common features, such as their hydrophobic nature, low molecular weight and presence of either a ketone or hydroxyl group (Waxman and Azaroff, 1992; Schuster and Langer, 2005;).

It is well established that PXR and CAR regulate metabolism and elimination of many xenobiotics and endogenous compounds by inducing CYP450s, primarily CYP3A4 and CYP2B10, respectively (Waxman, 1999; Honkakoski and Negishi, 2000). As shown in Figure 3, genes encoding other CYP450s along with phase II enzymes, glutathione–S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs) and sulfotransferase (SULTs) are regulated by CAR and PXR. Transporters such as multi-drug resistance protein 1 (*Mdr1*) and organic anion transporter polypeptide (*Oatp2*) are induced by PXR-agonists, while multi-drug resistance-associated protein (*Mrp2*) gene expression increases as a result of CAR-activation (Geick et al., 2001; Hagenbuch et al., 2001; Synold et al., 2001; Kast et al., 2002). CAR- and PXR-mediated induction of these transporters, often collectively referred to as ATP-binding cassette (ABC) proteins, results in efflux of xenobiotics to the gut lumen or uptake into bile for ultimate elimination from the liver.

Regulation/Autoregulation of Nuclear Receptors by CAR and PXR

A number of genomics studies have been conducted to assess the role of CAR and PXR in normal liver physiology and in chemical-induced liver toxicity. Most have confirmed that CAR and PXR mediate induction of phase I and II enzymes and transporters (Xie et al., 2000; Maglich et al., 2002; Wei et al., 2002). In a study using RT-PCR, expression of about 40 genes involved in xenobiotic metabolism was measured to identify receptor-specific and tissue-specific gene signatures in mouse liver and intestines (Maglich et al., 2002). Expression profiles from *Pxr*-null and *Car*-null mice that were treated for 28 hours with either a PXR-agonist, pregnenolone-16a-carbonitrile (PCN, 100 mg/kg), or a CAR-agonist, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, 0.3 mg/kg), revealed that a number of enzymes and transporters induced in the small intestines were regulated by PXR, not CAR. Similarly, several genes induced in liver were regulated by CAR, but not PXR.

Autoregulation of PXR and cross-talk with CAR in mouse liver was demonstrated by PCN-induced expression of PXR and CAR mRNA in wild-type, but not PXR-null mice. Additional gene expression profiles were obtained from human hepatocytes of 2 different donors. Interestingly, 48 hours of hPXR-specific activator, rifampicin (10 μM) was able to

increase AHR-regulated *Cyp1a1* and *Cyp1a2* mRNA levels by as much as 24-fold over control, while phenobarbital induced both genes, but only 3-fold over control. PXR and CAR activators were also able to induce expression of AHR. The fact that PXR-activation by PCN did not induce *Ahr* or *CYP1a1* in mouse liver confirms that there are substantial species differences between human and mouse PXR receptor. PXR's autoregulatory response that was observed in mouse liver was much weaker in human hepatocytes and regulation of CAR by PXR was not observed.

Recent gene expression studies using CAR-activators further demonstrate the diverse role of CAR in normal liver function. Microrarray analysis of liver tissue from wild-type and *Car*-null mice treated with PB for 12 hours identified 144 significant genes, which grouped into distinct categories based on their dependence on CAR for altered expression (Ueda et al., 2002). Genes that were CAR-dependent for induction by PB were those associated with xenobiotic metabolism, including *Cyp2b10*, aldhehyde dehydrogenase (*Ald1*) and flavin containing monooxygenase 5 (*Fmo5*). Another group of genes, which required CAR for suppression, spanned a wide range of functions including signal transduction, fatty acid oxidation, energy metabolism, and cell surface communication. A third group of genes which were induced by PB in *Car*-null mice included *Cyp4a10* and *Cyp4a14*, which are PPARα-regulated genes (Johnson et al., 1996).

The enzymes encoded by these 2 genes are major microsomal peroxidases and may contribute to oxidative stress in the liver (Leclercq et al., 2000). Since *Cyp4a10* and *Cyp4a14* were suppressed in wild-type mice, CAR may act as a suppressor for oxidative stress, an idea consistent with previous findings (Sugatani et al., 2001). Finally, another set of genes were induced or repressed by PB, in a CAR-independent manner. Within this group of genes was aminolevulinate synthase 1 (*Alas-1*), a key gene in heme biosynthesis. Heme supply is an essential factor of CYP450 activity, and is thought to be coordinated with CYP450 induction (Iba et al., 1999). CAR-independent induction of *Alas-1* has been confirmed in other studies (Yamamoto et al., 2004). These results suggest that CAR-agonists elicit other pathways that regulate hepatic expression in addition to those mediated by CAR.

A network of genes regulated by PXR was identified in a study that used a humanized mouse model that expressed either a full-length or transcriptionally active variant of *hPXR* (Rosenfeld et al., 2003). These mice, termed VP-*hPXR* constitutively regulate PXR target genes, which eliminates the added variables that using ligand activators can create. From the 8700 sequence cDNA microarrays, 150 unique transcripts that were differentially expressed were identified. A number of distinct CYP450s, particularly those associated with ω-oxidation within the CYP4A family were suppressed in VP-*hPXR* mice. PPARα targets, *Aco* and *Cpt1* and apolipoproteins were also down-regulated, suggesting cross-regulation or antagonism of PPARα. A similar response has been observed with bile acid antagonism of PPARα (Schuetz et al., 2001; Sinal et al., 2001). Interestingly, *Car* expression was down-regulated, which conflicts with previous studies demonstrating induction of CAR by PXR agonists.

In an attempt to find overlapping genes, these expression data were compared to results from the abovementioned studies by Ueda et al. (2002) and Maglich et al. (2002). Only half of the chosen transcripts had synonymous expression among the 2 datasets, which could be due to the varying dosing regimens and different platforms used in the 3 studies. When these three data sets are considered collectively, PXR and CAR appeared to induce many common genes, including *Cyp2a4*, *Cyp2b10*, and *Cyp3a11*, *Aldh1a1* and *Ald1a7*, all of which are involved in Phase I metabolism, *Gsta1*, *Gstm1* and 3′-phosphoadenosine 5′-phosphosulfate synthase 2 (*Papss2*), which are involved in phase II conjugation, and *Mrp3* of phase III elimination. Suppression of PPARα pathways also seems to be a common effect of PXR and CAR activation. Signatures specific to PXR included induction of *carboxylesterase 2* (*Ces2*) and *3* (*Ces3*), *Ugt1a*, *Mdr1a*, *Mdr1b Oatp02*, and *Abcb9*. CAR-specific genes included *AhR, Cyp1a, esterase1, Fmo5*, betaine-homocysteine methyltransferase (*Bhmt), Sult1d1, and Mrp1, and Mrp2*. These genomics studies have been confirmatory of each other and have provided insight into co-regulated as well as PXR and CAR-specific regulatory pathways.

CAR and PXR in Drug Induced Liver Injury

While phase I metabolism is a primary detoxification pathway for many xenobiotics, there are a number of hepatotoxicants that are biotransformed to toxic metabolites. In the case of model hepatoxicant acetaminophen (APAP), metabolism of the parent compound results in the formation of a highly reactive metabolites, quinine, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Thummel et al., 1993; Lee et al., 1996; Tonge et al., 1998). CAR and PXR have both been implicated in mediating toxicity of APAP. *Car*-null mice given acute (2 hours) or subacute (24 hours) doses of APAP (500 mg/kg) were resistant to APAP-induced liver injury (Zhang et al., 2002) and *Cyp1a2* and *Cyp3a11* mRNA expression was abrogated in the absence of CAR. *Pxr*-null mice given a slightly lower dose (350 mg/kg) showed mild necrosis, but all other responses observed in wild-type mice were abrogated in the absence of PXR (Guo et al., 2004). Although PXR does not appear to modulate APAP-induced liver injury as directly as CAR, these results clearly show that PXR-activation contributes to toxicity of this compound. It is presumed that CAR-mediated induction of *Cyp1a2* and *Cyp3a4* promotes the conversion of APAP to the toxic intermediates.

This mechanism of toxicity explains why pretreatment with CYP450 inducers such as alcohol or phenobarbital exacerbates APAP-induced liver injury in both humans and rodents (Burk et al., 1990; Kostrubsky et al., 1997; Pirotte, 1984; Sinclair et al., 1998). Similar studies using other hepatotoxicants such as carbon tetrachloride (CCl_4) have also demonstrated a role for CAR in mediating toxicity (Yamazaki et al., 2005). To date, there is no published report of a genomic study that would specifically address the role of CAR in APAP- or CCl_4-induced liver injury.

A group of environmental chemicals whose toxicity is thought to be a result of PXR and CAR-activation includes azole fungicides, also known as conazoles. These compounds were developed as pharmaceuticals for treating fungal infections and are currently used as pesticides (Sheehan et al., 1999). Previous reproductive toxicity tests in rats using a number of conazoles reported testicular atrophy, prostate atrophy, reproductive failure and cancers of the thyroid and pituitary gland and liver. Conazoles have demonstrated

CYP450 modulation, which led to the idea that their toxicity may be nuclear receptor-mediated (Hurley, 1998; Nelson et al., 2004).

Two recent toxicogenomics studies were conducted to assess the effects of 4 triazoles (a subset of conazoles) on expression of CYP450s and other genes in rat liver and testis (Tully et al., 2006), and in mouse liver (Goetz et al., 2006). Rats treated with fluconazole (up to 50 mg/kg), propiconazole (up to 150 mg/kg), myclobutanil (up to 150 mg/kg) or triadimefon (up to 115 mg/kg) for 14 days had significantly enlarged livers, a response which was not observed at the lowest of the 3 doses. No changes in testis weight were observed. Global gene profiles showed that expression of 376 of 1137 genes was altered in liver and 357 of 2249 in testis by at least one treatment group. In both liver and testis, the majority of differentially expressed genes were selectively induced by only 1 triazole. Strong induction of only 2 genes, *Ces2* and UDP-glucuronyltransferase (*Udpgtr*) was observed across all treatments.

Induction by multiple triazoles of 4 of 6 genes encoding CYP3A enzymes was detected in liver using microarrays and RT-PCR. High concordance in liver expression of genes encoding CYP450 enzymes was observed across all triazoles. Also, clustering of 26 differentially expressed genes in liver that are regulated by CAR or PXR revealed a very homogenous profile across all triazoles, suggesting a common mechanism of action among the fungicides that may involve CAR or PXR activation. Neither CYP450s nor other CAR/PXR-regulated genes were uniformly expressed across all treatments in testis, providing some evidence that the mechanism of toxicity in this organ may not be nuclear receptor-mediated, at least directly.

In mouse liver, following the same dosing regimen as used in rats, expression of genes encoding CYP450s and other CAR or PXR-regulated genes varied by triazole treatment (Goetz et al., 2006). Comparison of mouse and rat data from these 2 studies revealed a common induction of *Ces2,* solute carrier organic anion transporter 1a4 *(Slco1a4),* and genes encoding CYP3A family enzymes by at least 3 of the 4 triazoles, but few other similarities were observed. From these genomics studies it may be concluded that triazoles induce compound specific pathways in rat liver, but also uniformly induce pathways involving CYP450s and CAR/PXR-associated enzymes and transporters that are likely important to the mechanism of action of triazoles. The role of CAR and PXR in mouse liver injury by these agents is less conclusive, given the variable induction of genes related to xenobiotic metabolism and requires further studies.

Elucidating the Mechanism of CAR-Mediated Carcinogenesis and Identifying Markers for Predictive Toxicology

CAR-activators comprise an important class of hepatocarcinogens, which, similar to peroxisome proliferators, act by a nongenotoxic mechanism (Butterworth, 1990). Subacute administration of these compounds results in liver enlargement, hepatic hyperplasia, and induction of metabolizing enzymes, with prolonged treatment ultimately leading to liver tumor formation (Diwan et al., 1992; Whysner et al., 1996). In an effort to identify early markers of chemical-induced carcinogenicity, CAR-activator, PB along with other nongenotoxic carcinogens (clofibrate, bemitradine, doxylamine, or methapyrilene), genotoxic carcinogens (tamoxifen or 1-acetylaminofluorene) and noncarcinogenic compounds (4-acetylaminofluoorene or isoniazid) were administered to rats for 5 days (Kramer et al., 2004) and gene expression was correlated with hepatic carcinogenicity. Of 105 hybridizations, 2 genes that correlated well with carcinogenic potential were NADPH cytochrome P450 oxidoreductase (*Cyp-r*), which was induced by carcinogens and *Tsc22*, which was suppressed. *Cyp-r* catalyzes electron transfer from NADPH to heme oxygenase and likely reflects the role oxidative stress in rodent hepatic carcinogenesis. As previously mentioned, *Tsc22* may be involved as an apoptotic protein (Uchida et al., 2000; Hino et al., 2002).

Studies of CAR-mediated hepatocarcinogenesis, using TCPOBOP or PB in rats and mice demonstrated a strong correlation between *Cyp2b1* induction and tumor promotion (Diwan et al., 1992). Additionally, PB was an effective inducer and tumor promoter in mice and rats, while TCPOBOP was effective only in mice. *Car*-null mice have unequivocally shown that hypertrophic, hyperplastic, and carcinogenic effects of these compounds are CAR-mediated (Yamamoto et al., 2004). Because of the species difference in receptor ligands and target genes, humanized CAR mice have recently been developed to better understand the human relevance of hepatic effects of PB-like compounds that are observed in mice. The *hCAR* mice, when treated with PB (0.05%) for 1 week exhibit increased proliferation, liver enlargement and induction of *Cyp2b10* and mouse double minute 2 (*Mdm2*), a gene that is involved in inhibiting *p*53-mediated cell growth arrest (Huang et al., 2005).

These new studies suggest that humans may be a sensitive and susceptible species to hepatocarcinogenesis by PB and PB-like compounds. Although there are reports which support these animal studies and link long-term PB treatment with liver cancer (Vazquez and Marigil, 1989), PB and PB-like compounds are not typically associated with increased cancer incidence in humans.

While it is clear that CAR is involved in the mechanism of action of PB-induced rodent carcinogenesis, the molecular events leading to cancer are still not known. Cell proliferation is an important event in carcinogenesis because it is a critical mechanism for driving clonal expansion of mutated or differentiated cells (Columbano et al., 1981; Butterworth, 1990). Microarray studies have helped to identify genes that are involved in the proliferative response associated with CAR-activation. Genes with a strong immediate-early induction in response to proliferation-inducing treatments, partial hepatectomy or TCPOBOP have been identified using cDNA arrays. An induction in *Gadd45β* occurred only 3 hours after treatment. *Gadd45* is a growth arrest and DNA-inducible gene with anti-apoptotic activity that is often mediated by NF-κB (De Smaele et al., 2001; Vairapandi et al., 2002). To determine if *Gadd45β* induction in response to CAR-agonists was TNFα-mediated as suggested by previous studies (Akerman et al., 1992; Yamada et al., 1997), a follow-up study was conducted in *Car*-null and *Tnfr*-null mice treated with TCPOBOP. These results confirmed that the immediate induction of *Gadd45β* and other early response genes by TCPOBOP required CAR and was independent of TNFα (Columbano et al., 2005).

Oxidative stress and induction of metabolizing enzymes are also thought to play a key role in the mode of action of nongenotoxic carcinogens (Klaunig and Kamendulis, 2004). Genomic profiling using amplified fragment length polymorphism (ALFP) in rat liver following sub-chronic (13 weeks) revealed 168 sequence "contigs," overlapping clones that represent a continuous region of DNA, with altered expression of 2-fold or greater (Elrick et al., 2005). Pathway mapping confirmed an induction of genes encoding for CYP2B family enzymes, UGTs and a number of other xenobiotic metabolizing enzymes. Expression of a select group of these genes was confirmed by RT-PCR. Transcription profiles from ALFP, when compared with 5 day PB-treated rat liver expression profiling by a standard microarray procedure (Kramer et al., 2004), revealed concordant expression of genes involved in xenobiotic metabolism and stress response, strengthening the argument that oxidative stress as a result of enzyme induction plays a major role in hepatocarcinogenesis by nongenotoxic compounds. Gene expression patterns associated with cell cycle, apoptosis and cellular metabolism genes, contrasted between the 2 studies. These differences may be explained by the shift in cell turnover rate that occurs between subacute and chronic treatment with nongenotoxic carcinogens (Marsman et al., 1988).

These genomics studies have significantly added to the body of knowledge on CAR and PXR by confirming the role of PXR and CAR in xenobiotic metabolism, identifying other nuclear receptor co-regulators, providing important insight into the mechanism of action of CAR- or PXR-activators and identifying novel and early-responding genes that may be involved in CAR-mediated liver toxicity or carcinogenesis.

RETINOID X RECEPTOR (RXR)

RXR is a common binding partner for a number of receptors, including PPAR, CAR, PXR, LXR, and FXR and is also capable of forming homodimers (Mangelsdorf and Evans, 1995). For these receptors, transcription of target genes requires the formation of an RXR-NR complex, which binds to the response element in the promoter region of the target gene. PPAR and FXR, among others have been identified as permissive binding partners with RXR. In this case, a heterodimer can be activated independently by an agonist for the primary receptor (i.e., PPAR, CAR, FXR, etc), by an RXR-agonist or by both to cause synergistic effects. RXR heterodimers that contain nonpermissive partners can only be activated by the partner receptor's agonist but not by RXR agonists.

Three distinct RXR isoforms (-α, -β and -γ) have been characterized in vertebrates. All are expressed ubiquitously, though β and γ have no apparent role in liver, as observed in *RXR*β- and *RXR*γ-null mice (Kastner et al., 1996). The alpha isoform is the most highly expressed variant in liver and plays a central role in regulating bile acid, cholesterol, fatty acid, steroid and xenobiotic metabolism, and homeostasis (Mangelsdorf and Evans, 1995). Many efforts to identify endogenous ligands and the physiological function of RXR revealed a central role of these receptors as the body's lipid sensors. Ligands of RXRα include both endogenous (e.g., vitamin A, docosahexaenoic acid), and xenobiotic (e.g., bexarotene) compounds (Shulman and Mangelsdorf, 2005). Because RXRα is a requisite heterodimer for a number of other nuclear receptors, it also regulates a number of genes involved in cholesterol, lipid and glucose homeostasis (Figure 4). RXRα has been considered as a potential drug target for metabolic syndrome, which is characterized by hypertension, insulin resistance, obesity and hyperlipidemia (Kliewer et al., 1992; Shulman and Mangelsdorf, 2005). Only a few genomics studies to date have been conducted, mainly to characterize the role of RXRα in modulating glutathione synthesis.

RXR as Mediator of Glutathione Homeostasis

Glutathione (GSH) is an important endogenous antioxidant that is responsible for scavenging electrophiles produced as a result of phase I metabolism. A role for RXRα in regulating GSH homeostasis was demonstrated using a mouse model for *Rxr*α deficiency in hepatocytes (Wu et al., 2004). This hepatocyte-specific knockout whole animal model was developed as a result of embryonic lethality caused by total gene knockout (Kastner et al., 1994; Sucov et al., 1994; Kastner et al., 1996). To further assess *Rxr*α–regulated pathways, cDNA microarray analysis was performed. Over 280 of 15,000 expressed sequence tags had significantly altered expression in *Rxr*α-deficient hepatocytes as compared to wild-type cells (Wan et al., 2000a). A small subset of genes associated with GSH synthesis was significantly down-regulated in *Rxr*α-deficient mice and hepatic GSH was greatly reduced. *Cyp1a2* and *Cyp3a11* mRNA was also significantly lower compared to wild-types. *Rxr*α-deficient hepatocytes were more sensitive to oxidative stress by *t*-butylhydroperoxide compared to wild-types.

Interestingly, the *Rxr*α-deficient mice were protected from acetaminophen (APAP)-induced hepatotoxicity after 24 hours of treatment with 500 mg/kg (i.p.). It was suggested that the reduced expression of *Cyp1a2*, the enzyme that metabolizes APAP at high doses to toxic metabolite NAPQI (Snawder et al., 1994), is protective in *Rxr*α-deficient mice despite the concomitant GSH depletion in liver.

In a similar study confirming the role of RXRα in regulating hepatic GSH levels, mRNA levels encoding for glutathione-S-transferase (GST) family enzymes were measured by Northern blot analysis (Dai et al., 2005). Additionally, APAP metabolites in liver of hepatocyte-specific *Rxr*α-deficient mice were measured. Despite the decrease in hepatic GSH compared to normal levels in wild-types, RXRα deficiency caused an enhancement in APAP-GSH conjugation. Mutant mice possessed 7-fold higher APAP-GSH concentrations in liver as compared to wild-type mice, but concentrations of sulfonation and glucuronidation metabolites were no different. Northern blot analysis of mRNA levels for GSTs demonstrated a significant difference in basal expression of 13 of 15 genes measured between *Rxr*α-deficient mice and wild-type mice. GST is responsible for catalyzing the conjugation of APAP metabolite, NAPQI with GSH. It is possible that the increase in APAP-GSH conjugation may be attributed to the up-regulation of specific GST μ2, μ4, α1/2, and μ1/2.

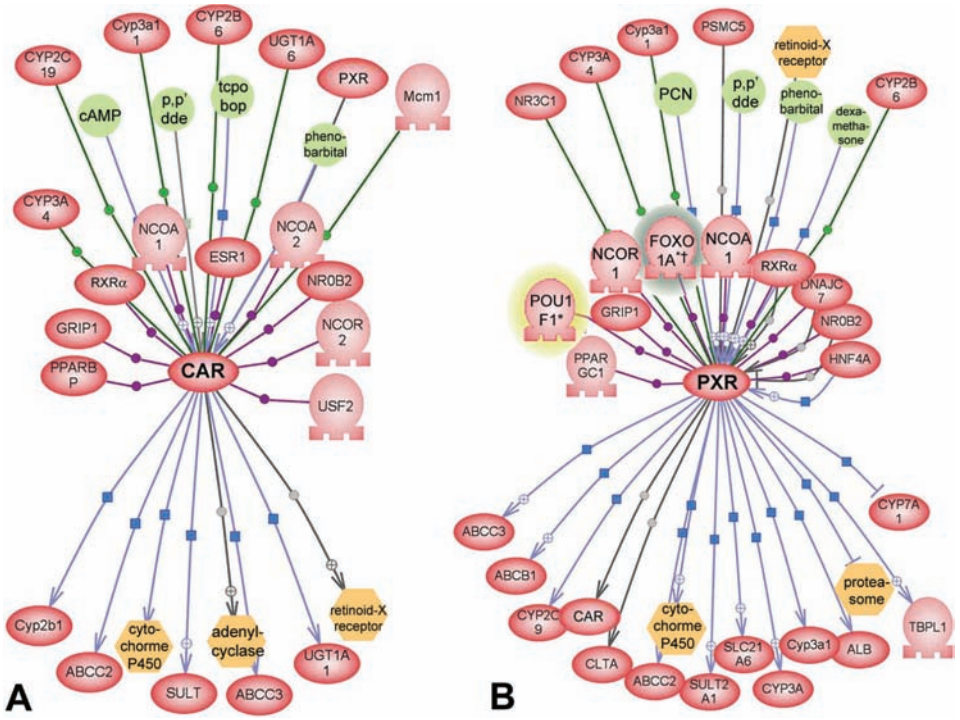

FIGURE 3.—Regulatory networks controlled by CAR (A) and PXR (B) reveal induction or direct regulation of a number of phase I and II enzymes and phase III transporters.

Role of RXRα in PPARα-, CAR-, and PXR-Mediated Toxicity

RXR*α* forms a heterodimer with a number of other nuclear receptors that mediate subchronic and chronic liver toxicity. PPAR*α* agonist, WY-14,643 and CAR agonists, phenobarbital and TCPOBOP, are nongenotoxic liver carcinogens that cause morphological changes in liver shortly after administration (Platt and Cockrill, 1967; Marsman et al., 1988). Studies using knock-out mouse models confirm the causal role of CAR receptor activation in liver carcinogenesis by PB-like compounds (Yamamoto et al., 2004). Because receptor activation for both PPAR*α* and CAR requires heterodimerization with RXR*α*, it was hypothesized that RXR*α* deficiency may result in protection against WY-14,643 or PB-induced liver toxicity. While no subchronic or chronic high-throughput gene expression studies have been conducted to characterize the role of RXR*α* in nuclear-mediated liver toxicity, a number of studies have assessed basal and toxicant-induced expression of specific CYP450s along with morphological changes of liver.

From studies that measured expression of PPAR*α* target genes and other genes related to fatty acid metabolism in hepatocyte *Rxrα*-deficient mice fed WY-14,643 (0.1% w/w), it was determined that PPAR*α* -agonists are not dependent on RXR*α* and perhaps RXR*β* or RXR*γ* can serve as substitutes, though much less plentiful in the liver than RXR*α* (Wan et al., 2000b). With respect to CAR- and PXR-specific gene targets, hepatocyte *Rxrα*-deficient mice exhibit significantly lower basal levels of *Cyp2b10* and *Cyp3a4*. Furthermore, CAR and PXR-agonists did not cause liver enlargement in these mutant mice, but were still capable of enhancing expression of *Cyp2b10* and *Cyp3a4*, respectively, demonstrating that *Rxrα*-deficiency is likely not protective of long-term effects of these compounds in liver.

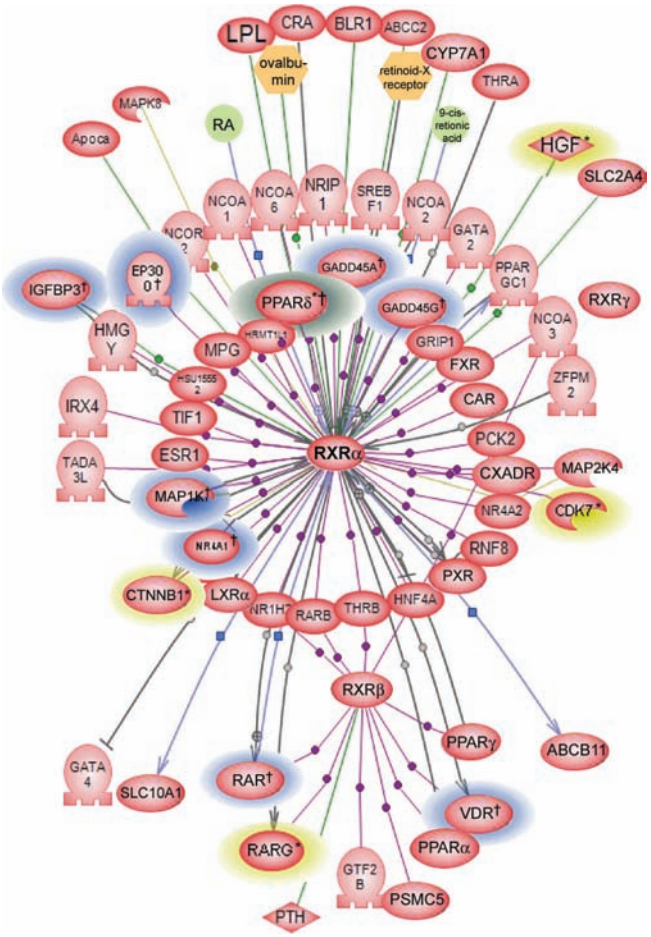

FIGURE 4.—Regulatory network modulated by RXR*α* displays the many other nuclear receptors with which it binds.

Liver X Receptor (LXR) and Farnesoid X Receptor (FXR)

LXR and FXR serve as master regulators of cholesterol and bile acid homeostasis, respectively. Endogenous ligands for LXR primarily include oxysterols (e.g., 24(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol) (Janowski et al., 1996; Lehmann et al., 1997). This receptor is widely expressed in the liver and intestines (Lu et al., 2001). LXR forms a heterodimer with RXR, which can be activated by both LXR- and RXR-specific ligands. It is thought that the heterodimer is prebound to DNA but is complexed with co-repressors. Binding of RXR or LXR agonists releases co-repressors and recruits co-activators for initiation of transcription (Chen and Evans, 1995; Glass and Rosenfeld, 2000). FXR is activated by primary and secondary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) (Makishima et al., 1999; Parks et al., 1999). It, too, is widely expressed in the liver and intestines, which is consistent with its physiological function and activates transcription of its target genes in a fashion much like LXR.

LXR and FXR are rather connected in their functions. As a modulator of oxysterol levels, LXR is responsible for regulating cholesterol synthesis and metabolism. Accumulation of cholesterol can lead to a number of deleterious hepatic, cardiovascular, and neurological effects (Carleton et al., 1991; Dietschy and Turley, 2004; Miller and Chacko, 2004; Pauli-Magnus et al., 2005). The endogenous mechanism for eliminating potentially toxic sterols in the liver is by converting them to bile acids. Amphiphilic bile acids solubilize cholesterol and eliminate it in bile (Chiang, 2003). Bile acids are also important for absorption of fat-soluble vitamins, A, D, E, and K. Because accumulation of bile salts in liver is also toxic and can lead to interhepatic cholestasis, tight regulation of their synthesis and circulation ensures that a nontoxic intracellular level is maintained (Pauli-Magnus et al., 2005). FXR accomplishes this by inducing feedback repression and feed-forward regulatory loops to suppress bile acid synthesis (Eloranta and Kullak-Ublick, 2005). It is not then surprising that targets of LXR and FXR, shown in Figure 5 are largely those associated with bile acid synthesis, efflux transport, lipoprotein metabolism, and fatty acid metabolism. A role for LXR in immune response has also been reported (Castrillo et al., 2003; Joseph et al., 2004).

Diverse Roles of LXR and FXR in Lipid, Bile Acid and Glucose Metabolism

One of the most important gene targets of both LXR and FXR is *Cyp7a1*, which catalyzes the rate-limiting step in the pathway of cholesterol conversion into bile acids. LXR has an inductive effect on the enzyme, while FXR activation is suppressive. Many other transcription factors, mostly nuclear receptors, transcriptionally regulate *Cyp7a1* (Lehmann et al., 1997; Parks et al., 1999; Marrapodi and Chiang, 2000; Chen et al., 2001) FXR and LXR also regulate expression of transporters but for differing purposes. LXR modulates reverse transport of cholesterol by up-regulation of ABC transporters. ABCA1 induces apolipoprotein-mediated efflux of cholesterol and lipid-loaded macrophages back to the liver to prevent accumulation on arterial walls as foam cells.

Oxysterols induce ABCA1 to a greater extent in the intestines than in the liver (Repa et al., 2000; Singaraja et al., 2001). FXR regulates transport of bile salts between the liver and intestines during enterohepatic circulation. Hepatic efflux involves the bile salt export pump (BSEP, also known as ABCB11). In the intestinal lumen, activation of FXR suppresses sodium-dependent bile salt transporters, effectively decreasing bile acid absorption. Finally, bile salts are reabsorbed from portal circulation into the hepatocyte. Sodium-dependent taurocholate co-transporting peptides (NTCO) and sodium-independent organic anion transporting peptides (OATPs) are responsible for facilitating hepatic uptake. Through tight regulation of these transporters, FXR-activation can prevent bile-acid induced liver toxicity (Kalaany and Mangelsdorf, 2006).

There are few gene expression profiling studies in liver to date that involve LXR or FXR. One study, by Anderson et al. (2004), used oligonucleotide arrays to identify a gene network regulated by RXR, LXR and PPARα in mouse liver. The LXR-agonist N-(2,2,2-trifluoroethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl) phenyl] sulfonamide (T0901317) induced genes involved in fatty acid β- and ω-oxidation and transport and triglyceride synthesis. Expression of other genes associated with fatty acid metabolism, including sterol regulatory element binding transcription factor 1 (*Srebf1*) and phospholipids transfer protein (*Pltp*) was enhanced by LXR activation, and to a lesser degree by PPARα activation. It is thought that LXR and PPARα regulate fatty acid metabolism by two different pathways, and that functional antagonism of the receptors occurs to prevent these pathways from being activated simultaneously (Ide et al., 2003; Yoshikawa et al., 2003). Bile acid synthesis genes, *Cyp7a1* and *Aldh3a2* were also induced by T0901317, although *Cyp7b1* was suppressed. The small subset of genes that were altered across all treatment groups had a very similar expression pattern, which provides further evidence of overlapping gene regulation.

Both LXR and FXR have also been implicated in glucose metabolism. Previous studies show that LXR or FXR activation in liver results in suppression of gluconeogenesis. In fact, one study demonstrated that activation of LXR improves insulin sensitivity of diabetic insulin-resistant rats (Cao et al., 2003). Genomics studies were conducted to further investigate this response and to identify novel LXR-regulated genes in liver, white adipose or brown adipose (Stulnig et al., 2002) that may be involved in glucose synthesis. Mice treated with T0901317 for 7 days exhibited a significant increase in genes associated with sterol biosynthesis/metabolism, lipid metabolism, heme synthesis, and detoxification. An induction, as high as 8-fold, in genes associated with peroxisomes support previous findings that LXR and PPARα share a significant number of transcriptional targets (Anderson et al., 2004). LXR-dependent suppression of phosphoenolypyruvate carboxykinase (*Pepck*) suggests a novel role for the receptor in gluconeogenesis. Despite the down-regulated expression of glycolytic enzyme in adipose tissues, based on the response in liver, LXR may be an interesting drug target for patients who are insulin resistant.

Aryl Hydrocarbon Receptor (AHR)

AHR is a basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) transcription factor that mediates toxicity

of a number of environmental toxicants. AHR does not belong to the nuclear receptor superfamily, but because of its similarities in function, it is often taken into consideration along with nuclear receptors. In its inactive form, AHR resides in the cytoplasm in association with a complex of heatshock proteins, hsp90, co-chaperone p23 and immunophilin-like protein, XAP2. Upon ligand binding, the complex translocates to the nucleus where it heterodimerizes with Ah receptor nuclear translocater (ARNT) (Hankinson, 1995). In mammals, the receptor is expressed in the liver, lung, and mammary glands. Several endogenous tryptophan-related intermediates have been suggested to be either AHR agonists or pro-agonists, including tryptamine (TA), indole acetic acid, indole-3-pyruvate, and 2-(1′H-indol-3′-carbonyl)-thiazole-4-carbozylic acid methyl ester, which is likely a product of tryptophan metabolism (Heath-Pagliuso et al., 1998; Song et al., 2002; Bittinger et al., 2003).

A number of persistant environmental contaminants including polychlorinated biphenyls (PCB), aromatic hydrocarbons (PAH) and polyhalogenated dioxins are also known agonists of AHR. The chemical compound 2′,3′,7,8′-tetrachlordibenzo-*p*-dioxin (TCDD) is a prototypic activator of AHR and is classified as a human carcinogen (Manz et al., 1991). Despite an aberrant liver phenotype of reduced blood flow to certain regions of the liver, resulting in necrotic lesions (Bunger et al., 2003; Harstad et al., 2006), *Ahr*-null mice have been used to prove that AHR mediates proliferative effects of TCDD (Tijet et al., 2006). Additionally, a mouse model in which AHR is constitutively expressed demonstrates a high incidence of TCDD-induced tumors (Moennikes et al., 2004). Activation of AHR results in induction of primary gene targets (Figure 6), which encode phase I enzymes, CYP1A1, CYP1A2, and phase II GSTs and UGTs (Schrenk, 1998; Mimura and Fujii-Kuriyama, 2003).

AHR Activation Alters Xenobiotic Metabolism, Protein Synthesis and Cell Adhesion

Two studies used genomic profiling to identify novel genes associated with TCDD exposure in human HepG2 cells. In the first study, cells exposed to 10 nM of TCDD for 8 hours suppressed many genes associated with calcium-dependent regulation, protein trafficking, membrane integrity, and DNA stability (Puga et al., 2000). An equal number of genes were up-or down-regulated in pathways involved with cell proliferation, apoptosis, cell adhesion cancer, and metastasis. In another study, HepG2 cells were treated for 18 hours with 10 nM TCDD (Frueh et al., 2001). While over half of the differentially expressed genes were expressed sequence tags of unknown function, a number of genes, similarly found by Puga et al., were those associated with cell adhesion and pathways involved in cell proliferation.

Serial analysis of gene expression was used to assess gene expression changes in mouse liver following 7-day feeding of TCDD (20 μg/kg bw) (Kurachi et al., 2002). TCDD-mediated alteration in metallothionein expression, and genes involved in xenobiotic metabolism and protein synthesis was reported. Aside from these responses, gene expression profiles from this study differed from the abovementioned in vitro studies, which is not all surprising given that responses observed in culture often do accurately reflect gene response following in vivo exposures.

AHR-Mediated Hepatoxicity

The role of AHR in dioxin-induced effects was evaluated in *Ahr*-null mice 19 hours following a toxic dose (1000 μg/kg) of TCDD. Of the significantly altered genes, 392 were associated with genotype alone and 456 were TCDD-induced AHR-dependent responses. As expected, TCDD caused an induction of *Cyp1a1* in wild-type but not *Ahr*-null mice. Altered expression of this and other genes was confirmed by RT-PCR. Functional categorization of AHR-dependent genes affected by TCDD revealed an induction of fatty acid metabolism, protein synthesis, and electron transport. Analysis of chromosomal location for responsive genes merely demonstrated that genes were widely dispersed among different chromosomes.

Dose-dependent and time-dependent differences in gene expression have provided significant insight into the mechanism of TCDD-induced steatosis and hepatotoxicity. In 2 separate studies, a single dose of TCDD resulted in significantly altered gene expression in liver as early as 4–6 hours after treatment, and was sustained at least 7 days after treatment (Boverhof et al., 2005; Fletcher et al., 2005). Boverhof et al. used several doses ranging from 0–300 ug/kg and time points from 2–168 hours. A dose-dependent increase in *Cyp1a1* and *Cyp1a2* was observed in TCDD-treated mouse liver. Similarly a dose-dependent suppression in gluconeogenic pathways was observed, which has been previously reported (Viluksela et al., 1995).

A temporal response to TCDD revealed a small set of immediate-early response genes that were induced at 2 hours, an early response set of genes showing highest expression between 4–12 hours and a set of genes with delayed response. The large majority of genes was induced at 4 hours and sustained at least 168 hours after treatment, indicating that changes in gene expression occurred prior to histopathological alterations that were noticeable at 18 hours. Pathway mapping suggested an early, sustained induction of genes encoding reactive metabolizing enzymes and pathways leading to synthesis of protective antioxidants. Reactive oxygen species (ROS) are likely a product of TCDD-induced CYP1A enzymes (Bagchi et al., 2002).

Expression of genes involved in fatty acid uptake and metabolism was also elevated, along with serum levels of free fatty acids in serum and triglycerides. Induction of immune response occurred at later time points (72 and 168 hours) and was likely an AHR-independent response to fat accumulation and ROS. Also genes involved in development and differentiation were up-regulated. Rats treated with TCDD (40 μg/kg) for 6–168 hours showed a similar suppression in gluconeogenesis and induction of fatty acid metabolism genes *Aco* and *Cpt1*, though the majority of other genes associated with lipid metabolism were down-regulated (Fletcher et al., 2005).

In contrast, cell differentiation and immune responses were largely suppressed. It is likely that rats do not exhibit the same degree of TCDD-injury as mice since ALT levels progressively decreased over 7 days of treatment. Furthermore, hypertrophy was the only pathological change in rat liver 7 days after treatment, where as mice fed a slightly lower

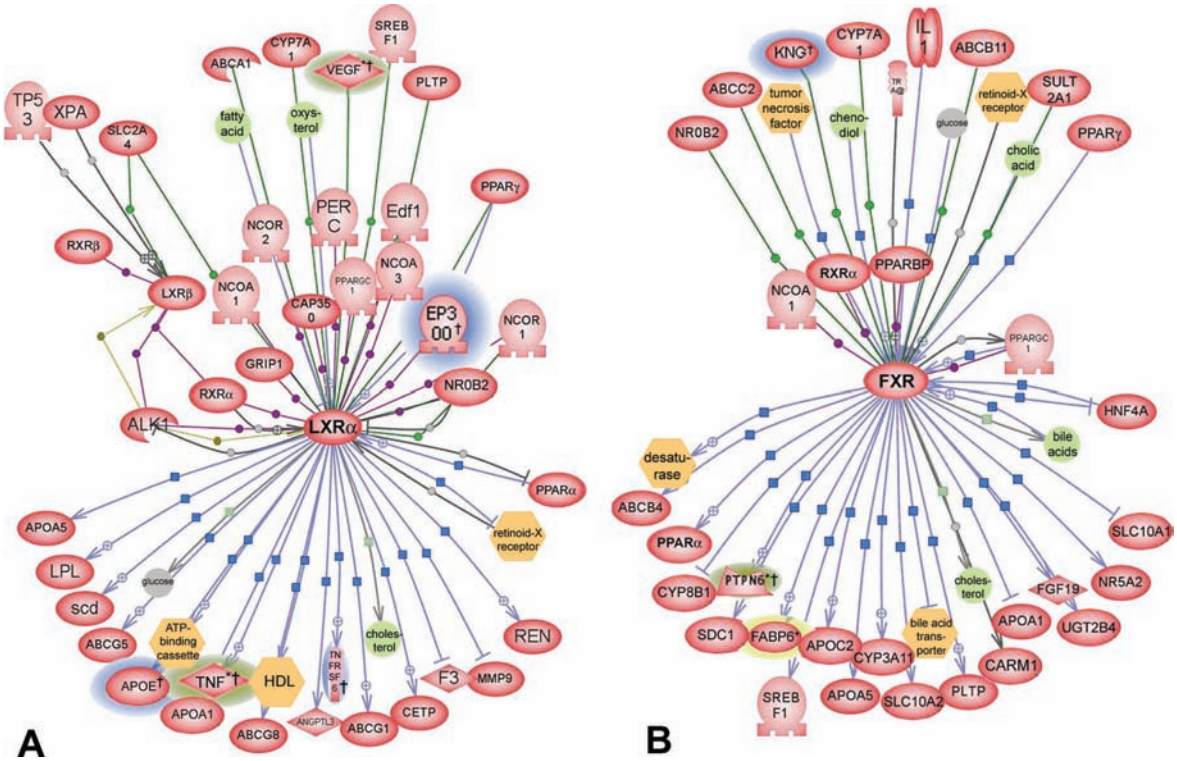

FIGURE 5.—Genes modulated by LXRα (A) and FXR (B) are associated with cholesterol, bile acid, and fatty acid metabolism.

dose (30 μg/kg) exhibited single cell apoptosis, immune cell and fat accumulation and mild injury as indicated by elevated ALT.

In an attempt to identify genes that respond to specific environmental AHR-agonists, rats were treated for 13 weeks with TCDD 3,3′4,4′,5,5′-pentachlorobiphenyl (PCB123), 2,3,4,7,8 pentachlorodibenzofuran (PeCDF) or 2,2′,4,4′,5,5′hexachlorobiphenyl (PCB153), the latter of which has no affinity to AHR (Vezina et al., 2004). Of the AHR-agonists, expression patterns produced in liver by PeCDF and PCB126 were more similar to one another than to TCDD. AHR-agonists caused a consistent up-regulation of known AHR target genes *Cyp1a1*, *Cyp1b1* and *Ugt1*. Novel genes whose expression was altered as a result of AHR-activation include carcinoembryonic-cell adhesion molecule (*Ccam4*) and adenylated cylcase-associated protein 2 (*Cap2*), neither of which is transcriptionally regulated by AHR directly, as they do not possess a dioxin response element (DRE) in their promoter.

The potent PAH 3-methylcholanthrene (3MC) is also an activator of AHR and has been used as model PAH to investigate chemical carcinogenesis. To identify genes with early, sustained response to 3MC, gene expression was measured in rat liver after 1, 15, or 28 days of treatment (Kondraganti et al., 2005). Expression profiles revealed a small set of early response genes with marked induction. In addition to induction of AHR target genes, α-1 acid glycoprotein (*Agp*) and orosomucid 1 (*Orm1*) were early responding genes to 3MC whose expression remained elevated for 15 days. The presence of these two novel genes suggest that the initial response to the 3MC is an inflammatory immune response (Stanley et al., 1988; Ceciliani et al., 2002; Hochepied et al., 2003).

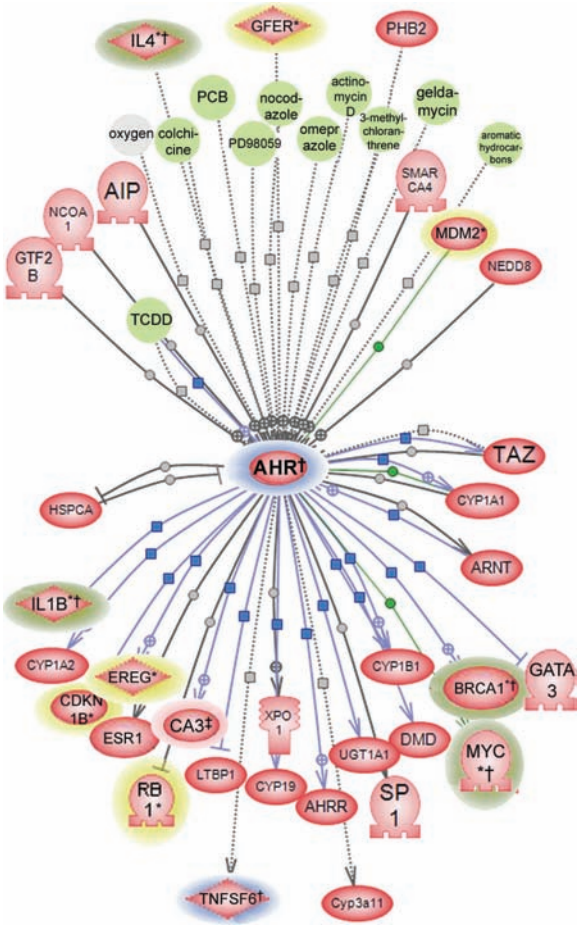

FIGURE 6.—AHR network includes phase I and II enzymes along with genes involved in a wide range of other cellular functions.

Another study compared altered gene expression in rat liver caused by the thienopyridine inhibitor, A-277249, designed as a potential therapeutic to reduce adhesion molecules in inflammatory diseases (Waring et al., 2002). Histopathology in rats treated with A-27749 showed hypertrophy and hyperplasia. Global gene expression from cDNA arrays was compared with a panel of 15 hepatotoxicants. The expression pattern for A-277249 at a high dose most resembled that of AHR activators 3MC and Aroclor 1254, with all three compounds exhibiting a strong induction of *Cyp1a.* These similarities confirm a role for AHR in modulation of cell adhesion and suggest that A-277249 or its metabolites activate AHR.

Nonmammalian systems that are sensitive to dioxins and other environmental AHR-agonists have also been used to study liver injury. Recently, hepatic gene expression profiling in medaka fish revealed an initial stress response following TCDD exposure (Volz et al., 2006). Using a custom gene array, expression profiles were obtained at 1, 5, 9, or 13 days posttreatment, with the strongest transcriptional response occurring at day 1. A number of genes associated with acute phase response, inflammation, oxidant defense, cell adhesion, metabolism, ion transport, and cell cycle regulation were induced prior to morphological changes in liver. Other genes associated with liver gene repair, responded at 5 days and persisted to day 13.

Use of toxicogenomics in studying AHR-mediated toxicity has revealed an induction of pathways similar to those activated by other nuclear receptors, which include early immune response, xenobiotic and fatty acid metabolism, cell proliferation, and oxidative stress. Genomic profiling demonstrated the capability to identify AHR-dependent pathways, reveal known and novel genes involved in AHR-mediated toxicity and demonstrate similar gene profiles across species.

In summary, genomic profiling of nuclear receptor-mediated responses to chemical toxicants has been useful in (1) confirming the known role of nuclear receptors in maintaining homeostatic conditions and meditating toxicity, (2) distinguishing chemicals with similar or diverse mechanisms of toxicity, and (3) identifying potential gene markers of toxicity. Furthermore, these studies have demonstrated that genomics can effectively be used in vitro and across nonrodent animal models to confirm mechanisms of toxicity. Given the high degree of agreement among studies and lack of statistical information regarding the significance of altered expression, it is unclear as to whether many of pathways reported are in fact key to the mechanism of action of nuclear receptor-activators, or reported based on the bias of the authors. More widespread implementation of pathway mapping techniques into genomics will provide unbiased statistically sound results, making cross-study comparisons more reliable.

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