REGULATION OF CYTOCHROME P450 3A4 GENE EXPRESSION THROUGH MODULATING PREGNANE X RECEPTOR TRANSCRIPTIONAL ACTIVITY BY NF-κB, ARYL HYDROCARBON RECEPTOR AND XENOBIOTICS

A Dissertation

by

XINSHENG GU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Toxicology

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ABSTRACT

Regulation of Cytochrome P450 3A4 Gene Expression Through Modulating Pregnane X Receptor Transcriptional Activity by NF-κB, Aryl Hydrocarbon Receptor and Xenobiotics. (August 2007)

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Cytochrome P450 3A4 (CYP3A4) is a key enzyme responsible for the metabolism of drugs and endogenous compounds in human liver and intestine. CYP3A4 gene expression is mainly regulated by Pregnane X receptor (PXR) which is a ligand-dependent nuclear receptor.

It is a long-standing observation that inflammatory responses and infections decrease drug metabolism capacity in human and experimental animals. In this study, I reported that NF-κB activation by LPS and TNF-α plays a pivotal role in the suppression of CYP3A4 through interactions of NF-κB with PXR/RXR complex. Inhibition of NF-κB by NF-κB specific suppressor SRIκBα reversed the suppressive effects of LPS and TNF-α. Furthermore, I showed that NF-κB p65 disrupted the association of PXR/RXRα complex with DNA sequences as determined by EMSA and chromatin immunoprecipitation assays. NF-κB p65 directly interacted with DNA binding domain of RXRα and DNA binding domain, hinge domain and ligand-binding domain of PXR and may prevent its binding to the consensus DNA sequences, thus inhibiting the transactivation by PXR/RXRα complex. This mechanism of suppression by NF-κB activation may be extended to other nuclear receptor-regulated systems where RXRα is a dimerization partner.

Many genes regulated by PXR and AhR are important for phase I, II and III drug metabolism. In this study I reported a crosstalk between PXR and AhR pathways. AhR physically and functionally interacted with PXR and enhanced the PXR transcriptional activity, and the interaction repressed the AhR transcriptional activity. AhR also

physically interacted with RXRα. The synergistic induction of Gsta1 in the liver of mice by PCN and TCDD might assume a different mechanism. The results suggested the metabolism kinetics of mixture drugs was different from and more complicated than that of single compound.

Using a HepG2 cell-based PXR-driven CYP3A4-Luciferase assay, I reported that E/F domain of PXR was responsible for ligand-dependant activation. A/B domain was necessary for co-activating the ligand-dependent activation and D domain was suppressive. High doses of Valerian Root extraction were PXR-dependent CYP3A4 inducers. Green tea polyphenols, aflatoxin B1, $CuSO_4$ and $MnCl_2$ enhanced the PXR transcription activity activated by rifampicin. The results suggested PXR-mediated drug metabolism kinetics altered on xenobiotic exposure.

DEDICATION

To my wife, Liming Huang

My daughter, Tianyun Gu

My parents, Lianming Gu and Qinfen Wang

My sisters Jianhua Gu, Meihua Gu, Xiuhua Gu and Jinhua Gu

My father- and mother-in-law, Rixing Huang and Shuzhi Qi

My brothers in law, Manxing Du, Guilin Jiang, Youquan Gu and Xian Xu

For their love, support, patience, and friendship.

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1.1.1 Xenobiotics

A xenobiotic is a chemical compound found in an organism but not normally produced or expected to be present in it. It can also include substances which are present in much higher concentrations than are usual. Xenobiotics are ubiquitous including both manufactured and natural chemicals such as as drugs, industrial chemicals, pesticides, pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites, and toxins produced by molds, plants, and animals. Human may be exposed to xenobiotics every day through inhalation, ingestion and dermal absorption.

For various practical purposes, xenobiotics are usually classified into several categories. Xenobiotics can be classified into organic and inorganic chemicals according to their molecular structure (Casarett et al., 2001). The number of inorganic chemicals is relatively limited (Table 1). However, the number of xenobiotic organic chemicals is much larger.

Xenobiotics can be classified into herbicide, food additives, household chemicals, industrial chemicals, medicine, misused chemicals, poisonous plants and animal toxins etc. according to their applications and sources.

Pesticides include insecticides, botanical insecticides, herbicides, fungicides, fumigants, rodenticides. The insecticides can be organochlorine compounds, anticholinesterase agents, pyrethroid esters, avermectins, nitromethylenes, chloronicotinyl, phenylpyrazoles according to their chemical constitutes (Casarett et al., 2001).

Household chemicals include paints, paint cleaners, removers, strippers, solvents (such as chlorinated hydrocarbons, aromatic hydrocarbons, alcohols, glycols, glycol

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This dissertation follows the style and format of Gene.

ethers, fuels and fuel additives, carbon disulfide), and thinners, antifreeze and deicers, glue, cleansing agents, detergents, bleach, corrosive acids and alkalis, rug cleaners and rug deodorants, all-purpose cleaners and polishes, glass cleaners, disinfectants, dangerous mixtures, mothballs, cosmetics, smoke from fires, tobacco smoke, indoor air, combustion gases, formaldehyde. Food related xenobiotics include chemicals involved in producing crops such as fertilizers growth regulators, food processing such as food additives, toxic substances unintentionally resulting from food production, vitamins and minerals(Schiefer et al., 1997).

Table 1 Toxic inorganic xenobitics

Biological properties	Metals
major toxic metals with multiple effects	Lead (Pb), Mercury (Hg), Nickel (Ni), Arsenic (As), Arsine, Beryllium (Be), Cadmium (Cd), Chromium (Cr)
essential metals with potential for toxicity from elevated exposures	Cobalt (Co), Trivalent Chromium, Cr (III), Copper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn), Molybdenum (Mo), Selenium (Se), Zinc (Zn)
metals related to medical therapy	Aluminum (Al), Bismuth (Bi), Gallium (Ga), Gold (Au), Lithium (Li), Platinum (Pt) and Related Metals,
minor toxic metals	Antimony (Sb), Barium (Ba), Germanium (Ge), Indium (In), Silver (Ag), Tellurium (Te), Thallium (Tl), Tin (Sn), Titanium (Ti), Uranium (U), Vanadium (V)

Industrial chemicals include environmental pollutants and waste chemicals such as PBBs, PCBs, dioxins and furans, estrogens in the environment, pulp mill effluents, heavy metals, Radon and Radon decay products, Asbestos(Schiefer et al., 1997).

Medicine includes cough and cold preparations, analgesics, stimulants and sleep aids, antacids, antiseptics and astringents, miscellaneous compounds(Schiefer et al., 1997).

Misused chemicals range from household cleaners to all sorts of things, such as overuse of prescription drugs, coffee and alcohol, tobacco, street drugs, cannabis, cocaine, crystal meth, solvent abuse, heroin and other opiates, lysergic acid diethylamide, look-alike drugs and designer drugs, steroids, chemical weapon(Schiefer et al., 1997).

Naturally occurring toxins include bacterial endotoxin (such as liposaccharide), mycotoxin (such as aflatoxin), toxins from poisonous plants and toxins from animals, etc.. Poisonous plants can be found indoor, in the garden and on the farms, such as mushrooms. Animal toxins comes from Arthropods, Arachnida (Scorpions, Spiders, Latrodectus Species (Widow Spiders), Loxosceles Species (Brown or Violin Spiders), Steatoda Species (Cobweb Spiders), Cheiracanthium Species (Running Spiders), Phidippus Species (Jumping Spiders), Ticks), Chilopoda (Centipedes), Diplopoda (Millipedes), Insecta (Lepidoptera (Caterpillars, Moths, and Butterflies), Formicidae (Ants), Apidae (Bees), Heteroptera (True Bugs)), Reptiles (Lizards, Snakes)(Schiefer et al., 1997; Casarett et al., 2001).

Xenobiotics also include physical agents, such as sunlight, radiation such as α particles Radium exposures (226, 228Ra), beta particles, positrons, and electron capture Radium exposure (224Ra), gamma-ray (Photon) emission Iodine (131I)(Schiefer et al., 1997).

Xenobiotics can be classified into organ- or biological function-targeted chemicals according to the location of action. Xenobiotics exert a variety of effects on biological systems. These effects may be beneficial, in the case of drugs, or deleterious, in the case of poisons, depending on the physicochemical properties of the xenobiotic. In many instances, chemical modification of a xenobiotic by biotransformation alters its biological effects. The organic chemicals especially lipophilic compounds are the subject of biotransformation. The metals themselves are unable to be biotransformed.

The biological effects are also dependent on the doses of the chemicals contacted. “Dose makes the poisons”, which is the key point of toxicology(Casarett et al., 2001).

1.1.2 Transference of xenobiotics in the body

Humans are constantly exposed toa broad range of xenobiotic chemicals in the environment surrounding them. The environment and the whole body are separated by the skin, respiratory tract and alimentary canal. The physiological systems and organs or tissues in the body are also separate. The communications between these separate

entities are implemented by the circulatory system. When xenobiotics enter our body by ingestion through gastrointestinal tract, by inhalation through respiratory tract, by contact with skin and by intravenous, intraperitoneal, subcutaneous and intramuscular administration, they were absorbed into the bloodstream and distributed throughout the body, including target organ or target tissue where they produce effects. Xenobiotics are removed from the systemic circulation by biotransformation, excretion, and storage at various sites in the body. Transference of the xenobiotics among the compartments and subcompartments characterize the disposition of xenobiotics in the body.

To transfer among the circulatory system and other physiological systems, xenobiotics usually pass through several layers of plasma membranes of a number of cells, such as the stratified epithelium of the skin, the thin cell layers of the lungs or the gastrointestinal tract, the capillary endothelium, and the cells of the target organ or tissue. The plasma membranes which xenobiotics pass through are similar. The cell membrane is an 8-nm thick structure of two layers of amphiphilic lipids with protein adhere to it. The bilayer consists of phospholipid with hydrophilic groups (phosphatidylcholine, phosphatidylethanolamine) heading on both the outer and inner surfaces of the membrane and hydrophobic fatty acids filling out the inner space. Proteins are inserted and cross in the bilayer. Some proteins are attached in the surface of the bilayer. These membrane proteins such as transporters facilitate active transference of xenobiotics across membrane. The membranes are semifluid at physiologic temperatures because of the structure and relative abundance of unsaturated fatty acids, facilitating more rapid active or passive transport.

The xenobiotics are transported through cell membranes by either active or passive transport. Passive transport includes simple diffusion and filtration, and does not require energy from the cell. Simple diffusion is the process of movement of substances from an area of high concentration to an area of lower concentration. Most xenobiotics cross membranes by simple diffusion. Small hydrophilic molecules with molecular weight less than 600 presumably permeate membranes through aqueous pores(Benz et al., 1980), The smaller a hydrophilic molecule is, the more readily it traverses membranes by

simple diffusion through aqueous pores. The hydrophobic molecules diffuse across the lipid domain of membranes. Filtration allows any solute small enough to pass through the pores when water flows in bulk across a porous membrane, whose size is different with different cells. An active transport system is facilitated by membrane-bound proteins, characterized by features of allowing chemicals to be moved against electrochemical or concentration gradients, being saturated at high substrate concentrations and selective for certain structural features of chemicals, and requiring expenditure of energy of the cell. Generally, a transporter forms a complex with substances on one side of the membrane, the complex transforms and carrys the substances to the other side of the membrane where they are released. The transporter then returns to the original surface to repeat the cycle. Xenobiotic transporters are responsible for the uptake of some chemicals into cells, and extremely important for the export of chemicals out of cells (Table 2) (Casarett et al., 2001; Klaassen, 2002; Klaassen and Slitt, 2005; Choudhuri and Klaassen, 2006).

Table 2 Xenobiotic transport systems

Name (Abbreviation)	Function
Multi-drug–resistant protein or p-glycoprotein (mdr)	Decrease GI absorption , Blood-brain barrier, Biliary excretion, Placental barrier
Multi-resistant drug protein (mrp)	Urinary excretion, Biliary excretion
Organic-anion transporting polypeptide (oatp)	Hepatic uptake
Organic-anion transporter (oat)	Kidney uptake
Nucleotide transporter (nt)	GI absorption
Organic-cation transporter (oct)	Kidney uptake, Liver uptake, Placental barrier
Divalent-metal ion transporter (dmt)	GI absorption
Peptide transporter (pept)	GI absorption

In general, the Oatps, Oct1 and Oat2 mediate uptake of a large number of xenobiotics from blood into liver. Conversely, Mdrs, Mrps, and Bcrp mediate efflux of xenobiotics from liver into bile or blood. Microsomal enzyme inducers increase expression of various Oatps, Mrps, and Mdrs in liver(Klaassen and Slitt, 2005). ABCB1 (MDR1/P-

glycoprotein of subfamily ABCB), subfamily ABCC (MRPs), and ABCG2 (BCRP of subfamily ABCG), which belongs to the ATP-binding cassette (ABC) transporter family, constitute a large family of membrane proteins. These efflux transporters facilitate the movement of a variety of compounds out of cells through the membrane against a concentration gradient at the cost of ATP hydrolysis. Substrates of the ABC transporters include lipids, bile acids, xenobiotics, and peptides for antigen presentation. By facilitating the elimination of exogenous and endogenous compounds, these transporters reduce the body load of potentially harmful substances(Choudhuri and Klaassen, 2006).

Other transport processes include facilitated diffusion which is much like the active transport except that the substrate is not moved against an electrochemical or concentration gradient and the transport process does not require the input of energy. Nutrients are mainly transported by facilitated diffusion, whereas xenobiotics are rarely transported by facilitated diffusion. The removal of particulate matter from the alveoli by phagocytes and from blood by the reticuloendothelial system of the liver and spleen utilizes the mechanisms of phagocytosis and pinocytosis(Casarett et al., 2001).

The transference of the xenobiotics across membranes is affected by the lipophilicity of the xenobiotics, which is frequently expressed as octanol/water partition coefficient logP. The rate of transport of the nonionized form is proportional to its lipid solubility. The more lipophilic the xenobiotics, the more ready to pass through the cell membranes. Most xenobiotics consist of larger organic molecules with different degrees of lipophilicity.

The transference of the xenobiotics across membrane is also affected by the acidic or basic strenghth of the xenobiotics denoted by pKa and pKb, the pH at which a weak organic acid or base is 50 percent ionized. Many xenobiotics are weak organic acids or bases. They are ionized. The ionized form usually has low lipid solubility and thus does not permeate readily through the lipid domain of a membrane. In general, the nonionized form of weak organic acids and bases is to some extent lipid-soluble, resulting in diffusion across the lipid domain of a membrane. The molar ratio of ionized to non-

ionized molecules of a weak organic acid or base in solution depends on the ionization constant. There is a specific ionization constant with a specific weak acid or base.

Some transport of organic anions and cations (depending on their molecular weight) may occur through the aqueous pores, but this is a slow process (except for compounds of very low molecular weight), as the total surface area of aqueous pores is small compared with the total surface area of the lipid domain of a membrane. However, the rate of the transference should take into consideration the mass action law, surface area, and blood flow rate, which influence the balance of the xenobiotics on the both sides of the membrane.

1.1.3 Disposition of xenobiotics

When human are exposed to xenobitics, the rate of absorption is dependent on a number of factors including the route of exposure, solubility of the chemical and nutritional status of the individual. The absorbed xenobiotics distribute within the body, are biotransformed (metabolized) in the special sites, stored and/or excreted. The body biotransforms the xenobiotics mainly in the liver. The products of biotransformation may be excreted into urine, feces and/or air. Within the body, the xenobiotics may have biological effects on the body depending on their inherent properties, site specificity, the disposition and the dose. The disposition of the xenobiotics plays an important role in the the exertion of biological effects of the xenobitics. When the rate of absorption exceeds the rate of elimination, toxic xenobiotics may accumulate to a critical concentration at target site (s) and cause adverse effects on systems. In the case of therapeutic drugs, when the rate of absorption is lower than the rate of elimination, drugs may be eliminated before reach target site (s) or the effective concentration and has no therapeutic effecacy. Many chemicals have little biological effects but have to be activated by biotransformation into active metabolites, so the rate of production of active metabolites is critical. Thus, the rates of absorption, distribution, biotransformation, and excretion critically influence the biological effects of xenobiotics.

1.1.3.1 Absorption

Absorption of xenobiotics refers to the process that the environmental xenobiotics cross body membranes and enter the bloodstream when human are exposed to the xenobiotics. The absorption happens mainly at three sites, the gastrointestinal (GI) tract, the lung and the skin.

The GI tract may be viewed as a tube traversing the body with its contents being considered exterior to the body. Many environmental xenobiotics enter the food chain and are absorbed together with food from the GI tract. The absorption of the xenobiotics in the GI tract can take place along the entire GI tract, even in the mouth and rectum. The number of xenobiotics actively absorbed by the GI tract is very low; most xenobiotics are transported across the gastrointestinal mucosa by passive diffusion which is dependent on surface area and site. Many xenobiotics are weak organic acids or bases, so their gastrointestinal absorption is dependent on the pH along the gastrointestinal tract. Particles appear to enter intestinal cells by pinocytosis, a process that is much more prominent in newborns than in adults (Williams and Beck, 1969). Many factors alter the GI absorption of xenobiotics. These factors include the physical properties of a compound, changes of the permeability of the intestinal wall, residency time in the GI tract, biotransformation in the enterocytes, dirrect excretion into the bile through the liver, and gastrointestinal flora which may biotransform the xenobiotics.

The xenobiotic gases, vapors of volatile or volatilizable liquids, and aerosols are absorbed by the lungs. The absorption of inhaled gases and vapor differs from that of aerosols. When inhaled, the gases and vapors pass through the nose which has the structure of turbinates to increase the surface area, water-soluble gases and highly reactive gases are retained in the nose. The other inhaled gases and vapors are usually absorbed in the lungs. When a gas is inhaled into the lungs, gas molecules diffuse from the alveolar space into the blood and then dissolve. A chemical diffuses rapidly in the lungs, partly because the distance for a chemical to diffuse is very short, and partly because chemicals absorbed by the lungs are removed rapidly by the blood (about three-fourths of a second). Except for some gases with a special affinity for certain body

components, the uptake of a gas by a tissue usually involves a simple physical process--dissolving. The end result is that gas molecules partition between the two phases: air and blood during the absorptive phase, and blood and other tissues during the distributive phase. For each gas, the solubility ratio (the blood-to-gas partition coefficient) is constant and unique, therefore the higher the inhaled concentration of a gas (i.e., the higher the partial pressure), the higher the gas concentration in blood. For a substance with a low solubility ratio, an increase in the respiratory rate or minute volume does not change the transfer of such a gas to blood. An increase in the rate of blood flow markedly increases the rate of uptake of that substance (perfusion-limited). For a gas with a high solubility ratio, the time required to equilibrate with blood is very much longer, the rate-limiting step of absorption is respiration. Increasing the blood flow rate does not substantially increase the rate of absorption (ventilation-limited). The blood carries the dissolved gas molecules to the rest of the body and gas molecules diffuse into the tissues. The blood unloading the part of the gas into the tissues returns to the lungs to take up more of the gas. The process continues until a gas reaches equilibrium between blood and each tissue according to the tissue-to-blood partition coefficients characteristic of each tissue. At this time, no net absorption of gas takes place. However, the change of exposure concentration, biotransformation and excretion occurring in the tissues make the absorption to continue.

The particle size and water solubility of chemicals present in aerosols influence the rate of absorption. Particles 5 μm or larger usually are deposited in the nasopharyngeal region. Particles of 2 to 5 μm are deposited mainly in the tracheobronchiolar regions of the lungs, from which they are cleared by retrograde movement of the mucus layer in the ciliated portions of the respiratory tract. These particles eventually may be swallowed and absorbed from the GI tract. Particles 1 μm and smaller penetrate to the alveolar sacs of the lungs. They may be absorbed into blood or cleared through the lymphatics after being scavenged by alveolar macrophages.

The skin is relatively impermeable and therefore is a relatively good barrier for separating organisms from their environment. However, some chemicals can be

absorbed by the skin in sufficient quantities to produce systemic effects. To be absorbed through the skin, a xenobiotic must pass through the epidermis or the appendages (sweat and sebaceous glands and hair follicles) whose cross-sectional area is probably between 0.1 and 1.0 percent of the total skin surface. Chemicals are absorbed mainly through the epidermis, which constitutes the major surface area of the skin. Chemicals that are absorbed through the skin have to pass through several cell layers (a total of seven) before entering the small blood and lymph capillaries in the dermis. The rate-determining barrier in the dermal absorption of chemicals is the uppermost layer of the epidermis, the stratum corneum (horny layer), consisting of densely packed keratinized cells that have lost their nuclei and thus are biologically inactive. It is clear that all xenobiotics move across the stratum corneum by passive diffusion. Generally, polar substances appear to diffuse through the outer surface of protein filaments of the hydrated stratum corneum, whereas nonpolar molecules dissolve in and diffuse through the lipid matrix between the protein filaments. The rate of diffusion of nonpolar toxicants is proportional to their lipid solubility and is inversely related to their molecular weight. However, the rate of dermal penetration of highly lipophilic or hydrophilic chemicals is very limited(Weber et al., 1991). Human stratum corneum displays significant differences in structure and chemistry from one region of the body to another, and these differences affect the permeability of the skin to chemicals. The permeability of the skin depends on both the diffusivity and the thickness of the stratum corneum. Agents such as acids, alkalis, water and solvents increase skin permeability. The second phase of percutaneous absorption consists of diffusion of the toxicant through the lower layers of the epidermis (stratum granulosum, spinosum, and germinativum) and the dermis, which contain a porous, nonselective, aqueous diffusion medium. Xenobiotics pass through this area by diffusion and enter the systemic circulation through the numerous venous and lymphatic capillaries in the dermis. The rate of diffusion depends on blood flow, interstitial fluid movement, and perhaps other factors, including interactions with dermal constituents.

Naturally, xenobiotics enter the bloodstream after absorption through the skin, lungs, or GI tract. For the purpose of studies, xenobiotics are administrated to laboratory animals by intraperitoneal, subcutaneous, intramuscular, and intravenous routes. The intravenous route introduces the toxicant directly into the bloodstream, eliminating the process of absorption. Intraperitoneal injection of xenobiotics into laboratory animals is also a common procedure. It results in rapid absorption of xenobiotics because of the rich blood supply and the relatively large surface area of the peritoneal cavity. In addition, this route of administration circumvents the delay and variability of gastric emptying. Intraperitoneally administered compounds are absorbed primarily through the portal circulation and therefore must pass through the liver before reaching other organs. Subcutaneously and intramuscularly administered toxicants are usually absorbed at slower rates but enter directly into the general circulation. The rate of absorption by these two routes can be altered by changing the blood flow to the injection site. The route of administration may or may not change the biological effects of the xenobiotics.

1.1.3.2 Distribution

After entering the blood by absorption or intravenous administration, a xenobiotic is available for distribution (translocation) throughout the body. Distribution usually occurs rapidly. The rate of distribution to organs or tissues is determined primarily by blood flow and the rate of diffusion out of the capillary bed into the cells of a particular organ or tissue. In general, the initial phase of distribution is dominated by blood flow, whereas the eventual distribution is determined largely by affinity of a xenobiotic for various tissues and/or proteins within the tissue. The penetration of xenobiotics into cells occurs by passive diffusion or special transport processes. Some xenobiotics such as very polar molecules and ions do not readily cross cell membranes and therefore have restricted distribution. Small water-soluble molecules and ions diffuse through aqueous channels or pores in the cell membrane. Lipid-soluble molecules readily permeate the membrane itself. Very polar molecules and ions of even moderate size (molecular weight of 50 or more) cannot enter cells easily except by special transport mechanisms because they are surrounded by a hydration shell, making their actual size much larger.

Xenobiotics distribute throughout the body with the flow of the body water. Total body water may be divided into extracellular water and intracellular water. Extracellular water consists of plasma water and interstitial water. The concentration of a xenobiotic in blood depends largely on what compartment of the water it distributes. The concentration of a chemical would be high in the plasma if the chemical were distributed into plasma water only, and much lower if it were distributed into a larger pool, such as extracellular water or total body water.

The distribution of chemicals in the body is governed largely by the solubility of the chemical and the rate of blood flow into the body. Xenobiotics which are relatively insoluble, such as chlorinated pesticides may partition into fats. Most chemicals pass through the liver, which has a high rate of blood flow, where the chemical structure is altered by phase I and phase II metabolism. Binding to proteins and other biological molecules generally increases the retention of xenobiotics.

A chemical in storage is assumed to be in equilibrium with the free fraction of the xenobiotic in plasma. As a chemical is biotransformed or excreted from the body, more is released from the storage site. As a result, the biological half-life of compounds stored in lipid or bone can be very long. The initial phase of distribution is determined primarily by blood flow to the various parts of the body. Therefore, a well-perfused organ such as the liver may attain high initial concentrations of a xenobiotic. However, the affinity of less well perfused organs or tissues may be higher for a particular xenobiotic, causing redistribution over time.

Several plasma proteins such as albumin bind xenobiotics as well as some physiologic constituents of the body. The liver and kidney have a high capacity for binding a multitude of chemicals. Highly lipophilic toxicants are distributed and concentrated in body fat. Toxicants appear to accumulate in fat by dissolution in neutral fats. Compounds such as fluoride, lead, and strontium may be incorporated and stored in bone matrix. Skeletal uptake of xenobiotics is essentially a surface chemistry phenomenon, with exchange taking place between the bone surface and the fluid in contact with it. Deposition and storage of toxicants in bone may or may not be detrimental. Xenobiotics

may be redistributed after the initial phase of distribution which is primarily determined by blood flow. If the affinity of a chemical to the less perfused is high, the chemical will redistribute.

Blood-brain barrier and the placenta barrier are special structures limiting the xenobiotics from entering brain and fetus, respectively.

1.1.3.3 Metabolism (biotransformation)

Most xenobiotics are lipid soluble and thus have affinity to absorb to tissues and organs. In some cases, xenobiotics can be reabsorbed prior to excretion and thus may be retained in the body for an extended time. Biotransformation is a process that changes the lipophilic xenobiotics to relatively more hydrophilic metabolites, facilitating elimination. In the absence of biotransformation, lipophilic xenobiotics would accumulate in the body and potentially reach toxic concentrations. Thus, xenobiotic biotransformation is an essential mechanism for maintaining homeostasis of organisms. However, biotansformation does not have a minimal affect on the elimination of volatile compounds.

In most cases, biotransformation terminates the specific effects of a xenibiotic by chemical modifications to facilitate elimination. In many instances, chemical modification of a xenobiotic by biotransformation alters its biological effects, not only its pharmacokinetic behavior. In the case of pharmacology, some drugs must undergo biotransformation to exert their pharmacodynamic effect. In the case of toxicology, many xenobiotics must undergo biotransformation to exert their characteristic toxic or tumorigenic effect. That is, it is the metabolite of the xenobiotics, and not the xenobiotics themselves that exerts the specific biological effects.

The chemical conversion of xenobiotics into polar molecules is catalyzed by a broad range of biotransforming enzymes (drug-metabolizing enzymes). Phase I and phase II enzymes which play central roles in the biotransformation, metabolism and/or detoxification of xenobiotics in the liver and other tissues. Enzymes catalyzing biotransformation reactions often determine the intensity and duration of biological

action of xenobiotics(Jakoby, 1980; Anders et al., 1981; Jakoby et al., 1982; Katåo et al., 1989). The reaction of biotransformation and drug metabolism is described in 1.1.4.

1.1.3.4 Excretion

Xenobiotics may be eliminated from the body by several pathways. Most water soluble xenobiotics are excreted though urinary elimination. Metabolites excreted in urine are more hydrophilic than the absorbed form. The kidney is a very efficient organ for the elimination of xenobiotics from the body. The major functions of the kidney are to retain large biological molecules, maintain chemical balance in the blood, and facilitate the eliminateion of water soluble chemicals. Xenobiotic metabolites are separated from biological molecules in urine through glomerular filtration, tubular excretion by passive diffusion, and active tubular secretion.

Many of the less soluble xenobiotics that are either not metabolized or only partially metabolized are eliminated in the feces. The biliary route of elimination is perhaps the most important contributing source to the fecal excretion of xenobiotics and is even more important for the excretion of their metabolites. The liver cells can extract compounds from portal blood and prevent their distribution to other parts of the body. Furthermore, the liver is the main site of biotransformation of toxicants and the metabolites thus formed may be excreted directly into bile. Xenobiotics and/or their metabolites entering the intestine with bile may be excreted with feces. Once a compound is excreted into bile and enters the intestine, it can be reabsorbed or eliminated with feces. Furthermore many chemicals in feces are transferred directly from blood into the intestine by passive diffusion. In some instances, rapid exfoliation of intestinal cells also may contribute to the fecal excretion of some compounds. Intestinal excretion is a relatively slow process. Therefore, it is a major pathway of elimination only for compounds that have low rates of biotransformation and/or low renal or biliary clearance. The rate of intestinal excretion of some lipid-soluble compounds can be substantially enhanced by increasing the lipophilicity of the GI contents. Active secretion of organic acids and bases also has been demonstrated in the large intestine. A considerable proportion of fecally excreted xenobiotic is associated with the excreted

bacteria which may biotransform the xenobiotics. Chemicals originating from the nonabsorbed portion of an oral dose, the bile, or the intestinal wall are taken up by these microorganisms according to the principles of membrane permeability(Casarett et al., 2001).

Substances that exist predominantly in the gas phase at body temperature are eliminated mainly in exhaled air. These substances seem to be eliminated by simple diffusion. Elimination of gases is roughly inversely proportional to the rate of their absorption. No specialized transport systems have been described for the excretion of xenobiotics by the lungs. Therefore, gases with low solubility in blood, are rapidly excreted, those which have much higher solubility in blood, are eliminated very slowly by the lungs. Undoubtedly, this prolonged retention is due to deposition in and slow mobilization from adipose tissue of these very lipid-soluble agents. The rate of elimination of a gas with low solubility in blood is perfusion-limited, whereas that of a gas with high solubility in blood is ventilation-limited.

Liquid aerosols and particles can be removed from the alveoli. The removal of particulate matter from the alveoli (usually less than 1 μm in diameter) appears to occur by three major mechanisms. First, particles may be removed from the alveoli by a physical process to the mucociliary escalator of the tracheobronchial region. From there, they are transported to the mouth and may be swallowed. Second, particles from the alveoli may be removed by phagocytosis of the mononuclear phagocytes (the macrophages). They apparently migrate to the distal end of the mucociliary escalator and are cleared and eventually swallowed. Third, removal may occur via the lymphatics. The endothelial cells lining lymphatic capillaries are permeable for very large molecules (molecular weight 10^6) and for particles, although the rate of penetration is low for particles with a molecular weight above 10, 000. Nevertheless, the lymphatic system plays a prominent role in collecting high-molecular-weight proteins leaked from cells or blood capillaries and particulate matter from the interstitium and the alveolar spaces. Particulate matter may remain in lymphatic tissue for long periods, and this explains the name “dust store of the lungs.” For the reasons discussed above, the overall removal of

particles from the alveoli is relatively inefficient. The rate of clearance by the lungs can be predicted by a compound's solubility in lung fluids. The lower the solubility, the lower the removal rate. Thus, it appears that removal of particles from the lungs is largely due to dissolution and vascular transport. Some particles may remain in the alveoli indefinitely.

Minor pathways for elimination of xenobiotics include sweat, saliva, tears, hair and milk. Xenobiotics are excreted into milk by simple diffusion. Because milk is more acidic (pH6.5) than plasma, basic compounds may be concentrated in milk, whereas acidic compounds may attain lower concentrations in milk than in plasma(Findlay, 1983; Wilson, 1983). More important, about 3 to 4 percent of milk consists of lipids, and the lipid content of colostrum after parturition is even higher. Lipid-soluble xenobiotics diffuse along with fats from plasma into the mammary gland and are excreted with milk during lactation. Metals such as lead chemically similar to calcium and chelating agents that form complexes with calcium also form complexes and can be excreted into milk to a considerable extent. The excretion of toxic agents in sweat and saliva is quantitatively of minor importance. Toxic compounds excreted into sweat may produce dermatitis. Substances excreted in saliva enter the mouth, where they are usually swallowed and thus are available for GI absorption.

The liver is the major site of metabolism in a mammalian system, and as such is the primary organ where xenobiotics are converted into more polar compounds. The liver is a target for many toxic xenobiotics due to a first pass effect. Following absorption into systemic circulation, xenobiotics pass through the liver where they may be metabolized. First-pass metabolism increases the solubility of xenobiotics, and also the rate of elimination. This has overall impact of protecting the body from deleterious effects of toxicants and toxins. However, first-pass metabolism has implications on the selection of therapeutic drugs on route of administration and dosages.

1.1.4 Drug metabolism and drug metabolism enzymes

The reactions catalyzed by xenobiotic biotransforming enzymes are generally

categorized into two groups, phase I (Table 3) and phase II (Table 4)(Casarett et al., 2001).

Table 3 The phase I biotransformation of xenobiotics(Casarett et al., 2001)

Reactions	Enzymes	Locations
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Reductive dehalogenation	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes

Table 4 The phase II biotransformation of xenobiotics(Casarett et al., 2001)

Reactions	Enzymes	Locations
Glucuronide conjugation	UDP-glucuronosyltransferases	Microsomes
Sulfate conjugation	sulfotransferase	Cytosol
Glutathione conjugation	glutathione S-transferase	Cytosol, microsomes
Amino acid conjugation	acyl-CoA:amino acid N- acyltransferase	Mitochondria, microsomes
Acylation	arylamine N-acetyltransferase	Mitochondria, cytosol
Methylation	Methytransferase	Cytosol, microsomes, blood

Phase I reactions such as hydrolysis, reduction, and oxidation expose or introduce a functional group (–OH, $–NH_2$, – SH or –COOH) to xenobiotics. Phase I reactions generally render a molecule more polar and more susceptible to phase II metabolism. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis), and conjugation with amino acids (such as glycine, taurine, and glutamic acid). The cofactors for these reactions react with functional groups that are either present on the xenobiotic or are introduced/exposed during phase I biotransformation. Phase I reactions result in only a small increase in hydrophilicity. Most phase II biotransformation reactions result in a large increase in xenobiotic hydrophilicity, hence they greatly facilitate the excretion of foreign chemicals. Phase II biotransformation of xenobiotics may or may not be preceded by phase I biotransformation.

Xenobiotic biotransforming enzymes are widely distributed throughout mammalian systems. The liver has the largest concentration of biotransformation reaction enzymes and thus plays an important role in the first-pass elimination. Enzymes are also located in the skin, lung, nasal mucosa, eye, and gastrointestinal tract, as well as numerous other tissues, including the kidney, adrenal, pancreas, spleen, heart, brain, testis, ovary, placenta, plasma, erythrocytes, platelets, lymphocytes, and aorta(Gram, 1980; Farrell, 1987; Krishna and Klotz, 1994). Intestinal microflora also play an important role in the biotransformation of certain xenobiotics. Although some extrahepatic sites contain high levels of xenobiotic biotransforming enzymes, their overall contribution to the biotransformation of xenobiotics is generally less than that of the liver. For example, the abundance of certain xenobiotic biotransforming enzymes in nasal epithelium rival those found in the liver. The nasal epithelium plays an important role in the biotransformation of inhaled xenobiotics, including odorants, but is quantitatively unimportant in the biotransformation of orally ingested xenobiotics(Brittebo, 1993). Tissues and cells within an organism differ enormously in their capacity to biotransform xenobiotics. This heterogeneity has important biological implications in terms of tissue-specific action of xenobiotics.

Xenobiotic biotransforming enzymes are present in several subcellular compartments. Within the liver and most other organs, the xenobiotic biotransforming enzymes are located primarily in the endoplasmic reticulum (microsomes) or the cytosol, with lesser amounts in mitochondria, nuclei, and lysosomes (see Table 3 and Table 4). The presence of enzymes in the endoplasmic reticulum facilitates the metabolism of lipophilic xenobiotics thus enhancing the rate of urinary or biliary excretion.

Drug-metabolizing enzymes have multiple isoforms in the structure and thus have broad substrate specificities. Enzymes are capable of metabolizing a large variety of endogenous chemicals such as steroid hormones, bilirubin, bile acids, fatty acids, and eicosanoids. The structures, activities and levels of a given biotransforming enzyme may differ heritably among individuals, resulting in the variation in the rate of xenobiotic biotransformation. Most enzymes are expressed constitutively, however, many drug metabolism enzymes are also inducible. This enables xenobiotics to accelerate their own biotransformation and elimination, and therefore, enzyme induction is an adaptive and feedback response to xenobiotic exposure.

Among the phase I biotransforming enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the number of xenobiotics it detoxifies or activates to reactive intermediates(Waterman and Johnson, 1991; Casarett et al., 2001; Johnson and Waterman, 2002). The highest concentration of P450 enzymes involved in xenobiotic biotransformation is found in liver endoplasmic reticulum (microsomes), but P450 enzymes are present in virtually all tissues. The liver microsomal P450 enzymes play a very important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxication of xenobiotics. P450 enzymes in liver and other tissues play important roles in the activation of xenobiotics to toxic and/or carcinogenic metabolites. Microsomal and mitochondrial P450 enzymes play key roles in the biosynthesis or catabolism of steroid hormones, bile acids, fat-soluble vitamins, fatty acids, and eicosanoids, which underscores the catalytic versatility of cytochrome P450.

All P450 enzymes are heme-containing proteins. The heme iron in cytochrome P450 is usually in the ferric (Fe^{3+}) state. When reduced to the ferrous (Fe^{2+}) state, cytochrome

P450 can bind ligands such as O_2 and carbon monoxide (CO). The complex between ferrous cytochrome P450 and CO absorbs light maximally at 450 nm, from which cytochrome P450 derives its name. The absorbance maximum of the CO complex differs slightly among different P450 enzymes and ranges from 447 to 452 nm. All other hemoproteins that bind CO absorb light maximally at 420 nm. The absorbance maximum of cytochrome P450 is due to an unusual fifth ligand to the heme (a cysteine-thiolate). The amino acid sequence around the cysteine residue that forms the thiolate bond with the heme moiety is highly conserved in all P450 enzymes(Negishi et al., 1996). When this thiolate bond is disrupted, cytochrome P450 is converted to a catalytically inactive form called cytochrome P420. By competing with oxygen, CO inhibits cytochrome P450. The inhibitory effect of carbon monoxide can be reversed by irradiation with light at 450 nm, which photodissociates the cytochrome P450–CO complex.

The basic reaction catalyzed by cytochrome P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate, designated RH, and the other is reduced to water with reducing equivalents derived from NADPH, as follows:

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product (ROH)} + H_2O + NADP^+$$

The catalytic cycle of cytochrome P450 has been reviewed by many scientists(Schlichting et al., 2000; Groves, 2003; Denisov et al., 2005). The cycle involves the activation of oxygen and substrate oxidation, which entails the abstraction of a hydrogen atom or an electron from the substrate followed by oxygen rebound (radical recombination). Following the binding of substrate to the P450 enzyme, the heme iron is reduced from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state by the addition of a single electron from NADPH–cytochrome P450 reductase. Oxygen binds to cytochrome P450 in its ferrous state, and the $Fe^{2+}O_2$ complex is converted to an $Fe^{2+}OOH$ complex by the addition of a proton (H^+) and a second electron, which is derived from NADPH–cytochrome P450 reductase or cytochrome b5. Introduction of a second proton cleaves the $Fe^{2+}OOH$ complex to produce water and an $(FeO)^{3+}$ complex, which transfers its oxygen atom to the substrate. Release of the oxidized substrate returns cytochrome P450

to its initial state. If the catalytic cycle is interrupted (uncoupled) following introduction of the first electron, oxygen is released as superoxide anion (O_2^-). If the cycle is interrupted after introduction of the second electron, oxygen is released as hydrogen peroxide (H_2O_2). The final oxygenating species, $(FeO)^{3+}$ can be generated directly by the transfer of an oxygen atom from hydrogen peroxide and certain other hydroperoxides, a process known as the peroxide shunt. For this reason certain P450 reactions can be supported by hydroperoxides in the absence of NADPH–cytochrome P450 reductase and NADPH(Casarett et al., 2001).

Although cytochrome P450 functions as a monooxygenase, the products are not limited to alcohols and phenols due to rearrangement reactions (Guengerich, 1991; Guengerich et al., 1991). Cytochrome P450 catalyzes several types of oxidation reactions, including: 1. Hydroxylation of an aliphatic or aromatic carbon; 2. Epoxidation of a double bond; 3. Heteroatom (S-, N-, and I-) oxygenation and N-hydroxylation; 4. Heteroatom (O-, S-, N- and Si-) dealkylation; 5. Oxidative group transfer; 6. Cleavage of esters; 7. Dehydrogenation(Casarett et al., 2001). The products of cytochrome P450 oxidation are more susceptible to further metabolism and may be acted upon by epoxide hydrolase and other cytochrome P450 enzymes.

1.1.5 Regulation of the drug metabolism

The rate of phase I and phase II metabolism varies greatly between individuals. Metabolic capabilities in an individual are influenced by genetics, age, and prior exposure to xenobiotic inducers.

A number of different genetic factors can affect drug metabolism. The polymorphism of coding sequences of the drug metabolism genes may change the structure of enzyme, thus affecting the binding and catalytic activity of the enzymes. The polymorphism of the regulatory region of the drug metabolism enzyme genes may change the expression levels of the enzymes. Many enzymes are constitutively regulated by endogenous factors and thus express persistently. The genetic variation of these endogenous factors may affect the expression levels of their target drug metabolism enzyme genes.

The environmental factors may regulate drug metabolism by changing the expression level or directly changing the activity of drug metabolism enzymes by interacting with the enzymes. Factors in the environment which are capable of producing such changes include xenobiotics such as diet, diet supplements, alcohol, tobacco and drugs, and the physiological and pathological states such as inflammatory response, fever, liver disease, heart disease, lung disease, kidney disease, mineral and vitamin deficiency pregnancy and aging.

1.1.5.1 Xenobiotics impacts on metabolism

Absorbed xenobiotics can regulate the drug metabolism by direct interaction with drug metabolism enzymes and/or by changing enzyme expression levels. The direct interaction between the drug metabolism enzymes and xenobiotics that function as enzyme inhibitors usually results in a reduction in the activity of drug metabolism enzymes. The competition of multiple drugs as substrates for enzyme binding results in the decrease of the drug metabolism. Some enzyme inhibitors block the catalytic activity of the enzymes.

Xenobiotics modify expression levels of drug metabolism enzymes and drug transporters through the regulation of related gene expression by xenosensors such as various nuclear receptors and transcription factors including the aryl hydrocarbon receptor (AhR), nuclear factor-E2 p45-related factor 2 (Nrf2), hepatocyte nuclear factor 1α (HNF1α), constitutive androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptor α (PPARα), hepatocyte nuclear factor 4α (HNF4α), vitamin D receptor (VDR), liver receptor homolog 1 (LRH1), liver X receptor (LXRα), small heterodimer partner-1 (SHP-1), and glucocorticoid receptor (GR). For example, the expression of CYP1 genes can be induced via the aryl hydrocarbon receptor (AhR) in response to many polycyclic aromatic hydrocarbons (PAHs). Similarly, the steroid family of orphan receptors, the constitutive androstane receptor (CAR) and pregnane X receptors (PXR) 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) has been shown to be activated by lipid lowering agent fibrate-type of compounds leading to transcriptional activation of the promoters on the CYP4A genes. 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(Tirona and Kim, 2005).

Inducers for the phase II drug metabolism enzymes include the phenolic compounds butylated hydroxyanisol (BHA), tert-butylhydroquinone (tBHQ), green tea polyphenol (GTP), (-)-epigallocatechin-3-gallate (EGCG) and the isothiocyanates (PEITC, sulforaphane). They generally possess electrophilic-mediated stress response, resulting in the activation of bZIP transcription factors Nrf2 which dimerizes with Mafs and binds to the antioxidant/electrophile response element (ARE/EpRE) promoter. This promoter which is located in many genes of phase II drug metabolism enzymes 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 including the liver, intestine, kidney, and brain. Theses proteins 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. This implies that phase III transporters may also be regulated in a coordinated fashion, and provides an important means of protecting the body from xenobiotics insults. It appears that in general, exposure to phase I, phase II and phase III gene inducers may trigger a cellular "stress" response leading to the increase in their gene expression, which ultimately enhances the elimination and clearance of these xenobiotics and/or other

"cellular stressors". Consequently, this homeostatic response plays a central role in the protection of the body against "environmental" insults elicited by exposure to xenobiotics(Xu et al., 2005) (Rushmore and Kong, 2002) . These receptors are sensors for the signals of specific xenobiotics such as ethyl alcohol, vegetables containing flavinoids or indoles, enzyme-inducing drugs, polycyclic or halogenated hydrocarbons, and some antibiotics. The magnitude enzyme activity increased by xenobiotic induction may be up to 1000-fold. This enzyme activity alters the rate of biotransformation and may also change efficacy or the toxicity of drugs.

1.1.5.2 Enzyme alterations due to physiological and pathological states

Changes in physiological and pathologic may alter drug metabolism. It is well established that inflammation decreases drug metabolism and clearance of drugs. Fever, liver disease, heart, lung or kidney disease, mineral and vitamin deficiency, pregnancy and aging also affect metabolism. Disease of the liver and other organs responsible for xenobiotic metabolism generally depresses enzyme activity.

1.1.5.2.1 Liver disease

Since drug oxidation occurs predominantly in the liver, acute or chronic disease of this organ has a pronounced effect on metabolism. Liver disease can modify the kinetics of drugs biotransformed by the liver. The capacity of the liver to metabolise drugs depends on hepatic blood flow and liver enzyme activity. Each of these parameters can be affected by liver disease. In addition, liver failure can influence the binding of a drug to plasma proteins. These changes can occur alone or in combination; when they coexist their effect on drug kinetics is generally synergistic, not simply additive. Drugs with a low rate of hepatic extraction are more sensitive to hepatic failure than to changes in liver blood flow. However, drugs having a significant first-pass effect are more sensitive to alterations in hepatic blood flow. Studies on the effects of liver disease on specific isoenzymes of CYP have shown that some isoforms are more susceptible than others to liver disease(Rodighiero, 1999). Drug metabolism may also affected by histological changes in the liver (acute or chronic hepatitis, cirrhosis). The degree of alteration in drug metabolism depends on the severity of liver dysfunction(Paintaud et al., 1996).

Accumulated evidence has demonstrated that liver disease is associated with a reduced metabolic capacity with respect to drugs undergoing oxidative biotransformation, whereas conjugation reactions, especially glucuronidation, seem less affected. Nevertheless, due to the complexity of these activations and gene expressions, condlicting data exist in the literature(Sonne, 1996). Liver disease may lead to a differential alteration of the cytochrome P450s with regard to protein content and activity(Sonne, 1996). The information currently available on specific P450 isoforms involved in drug metabolism has increased tremendously over the latest years(Rodighiero, 1999). In the group of patients with non-primary biliary cirrhosis type liver disease, all enzyme activities measured were impaired relative to the normal group. In the primary biliary cirrhosis group, enzyme activities were altered selectively. (a) Activities of the methyl cholanthrene-inducible forms of cytochrome P-450 were decreased compared to normal controls, whereas the activities of the phenobarbitone-inducible isozymes were relatively unaffected. (b) Sulfotransferase activities were decreased significantly compared to the normal group, whereas sulfatase activities remained unaltered(Iqbal et al., 1990).

Clinical studies on the efficacy of cardiovascular agents, diuretics, psychoactive and anticonvulsant agents, antiemetics, immunosuppressants, naltrexone, tolcapone and toremifene indicated that, the kinetics of drug metabolism is altered by liver disease to an extent that dosages may have to be adjusted(Rodighiero, 1999). Liver disease not only affects pharmacokinetics but also pharmacodynamics(Rodighiero, 1999). A detailed knowledge of the particular isoenzyme involved in the metabolism of a drug and the impact of liver disease on that enzyme can provide a rational basis for dosage adjustment in patients with hepatic impairment(Rodighiero, 1999). From a clinical point of view, these findings may have important implications. However, when treating liver patients, there is no widely accepted model for dose predictions; the best approach should be empiric and based on clinical response. In selected cases, monitoring of plasma drug concentrations and liver function is recommended(Sonne, 1996). Specific probe drugs may be used in order to study the effect of diseases on each enzyme of drug

metabolism. Probe-based assays must be validated during disease, since the pharmacokinetics of the parent drug and/or of its metabolites may be altered. Because of these limitations, therapeutic drug monitoring may currently be the most reliable way to adjust drug dosing(Paintaud et al., 1996).

1.1.5.2.2 Cardiovascular disease

The rate of drug metabolism in the liver may be affected indirectly in patients with heart disease. This may occur either because of reduction in liver blood flow or because of venous congestion in the liver. The pathophysiologic changes occurring in cardiovascular disease can affect the kinetics of drugs in several different ways.

Patients with heart disease can have decreased gastric emptying and intestinal motility, reduced splanchnic blood flow, and bowel edema. These factors could account for a delay and decrease of drug absorption, which could delay the onset of drug action and can result in subtherapeutic plasma drug concentrations.

A reduction in cardiac output due to heart failure can lead to a reduced blood flow to tissues. Thus less drugs can be delivered to poorly perfused organs with possible alterations of the volume of distribution. Another factor that can modify the volume of distribution is presence of edema, with a possible increase in the distribution of water-soluble drugs.

Changes in plasma protein binding of drugs may occur in cardiac patients due to either hypoproteinemia or an increase in α1-acid glycoprotein (AAG). Hyperproteinemia may diminish the binding of drugs, increasing the free drug fraction and the volume of distribution of highly protein-bound drugs. The change in AA, which is an acute-phase reactant to myocardial necrosis, can result in an increase of binding and of total plasma concentrations of drugs bound to this protein. Cardiac failure can reduce renal blood flow and glomerular filtration rate resulting in a decrease in the renal excretion of drugs. Cardiac failure may also impair the active secretion of drugs. Hepatic drug metabolism depends on the intrinsic metabolic capacity of the liver and on the rate of drug delivery to the liver via hepatic blood flow, both of which can be affected by heart disease. Several studies have shown that the presence of cardiovascular disease modifies

metabolic kinetics of drugs. The extent of these alternations is such that in many cases it requires a change in the dosage regimen. A rational basis for a correct therapeutic choice can be provided by adequate knowledge of these modifications(Rodighiero, 1989).

1.1.5.2.3 Kidney disease

The disposition of many drugs is altered in patients with acute and chronic kidney disease. A decline in renal clearance of several drugs has been correlated significantly with residual renal function (ie, creatinine clearance) of subjects. Reductions in nonrenal clearance of some compounds also have been reported and associated with clearance of markers of oxidative and/or conjugative metabolism or P-glycoprotein-mediated transport. The selective modulation of hepatic CYP enzyme activity observed in kidney disease is caused, at least in part, by differentially altered expression of several CYP isoforms. Knowledge of the impact and nature of these alterations associated with kidney disease may facilitate the individualization of medication management in this patient population(Nolin et al., 2003).

1.1.5.2.4 Thyroid function

Thyroid dysfunction can influence the physiological disposition of drugs. Depending on the pharmacokinetic properties of the individual drug, changes in the rate of metabolism ranging from profound to moderate or negligible have been observed. Since renal function is also influenced by thyroid disease, changes in renal elimination of drugs which are excreted in urine is another reason for altered drugs disposition caused by thyroid disease.

In patients with thyrotoxicosis (lower), or myxoedema (higher) altered digitalis plasma levels have been observed. The altered disposition of cardiac glycosides in thyroid dysfunction can be attributed to changes in renal elimination and metabolism. These findings may explain the clinical observation that patients experiencing thyroid toxicity require larger than normal doses of digitalis, while patients with hypothyroid condititions generally require a lower dose. Antipyrene half-lives are shortened in patients experiencing hyperthyroidism and prolonged appreciably in patients experiencing hypothyroidism. The alterations in the disposition of these drugs observed

in patients experiencing thyroid dysfunction can be ascribed to changes in rates of metabolism which is controlled by the levels of circulating thyroid hormones. N-demethylation of aminopyrine is depressed both in hyper- and hypothyroid patients as compared with normal subjects. The physiological disposition of the antithyroid drug propylthiouracil is unchanged during thyrotoxicosis. A decrease in plasma half-life of methimazole is however, observed during hyperthyroidism, whereas half-life is increased in hypothyroid patients. Absorption of paracetamol was faster in patients with untreated thyrotoxicosis than it was following recovery. The peak paracetamol concentration, however, was lower in thyrotoxic patients due to an apparent increase in the total body clearance and a shorter plasma half-life. Both absorption and elimination rates were reduced in hypothyroid patients, but were not significantly different from the euthyroid results. When estimated using a two compartment model the total volume of distribution and the hybrid distribution rate constants were unrelated to thyroid status, although the apparent volume of the central compartment was significantly greater in the thyrotoxic group. These changes in drug disposition may contribute to differences in drug response seen in patients with thyroid disease(Forfar et al., 1980). Glucuronyl transferase activity is increased in hyperthyroidism but is not altered in most patients with hypothyroidism. The extent of increase in glucuronyl transferase activity is similar to that produced by enzyme inducing drugs(Scott et al., 1984). Studies using rat models exhibiting hypothyroid, hyperthyroid and euthyroid condition to determine the effects of thyroid dysfunction on the absorption and disposition characteristics of amiodarone demonstrated that the disposition kinetics of amiodarone are altered in hypo- and hyperthyroidism(Weir and Ueda, 1988). The available data do not allow general predictions of how thyroid disease could alter drug metabolism in man (Eichelbaum, 1976; Forfar et al., 1980; Shenfield, 1981; Scott et al., 1984; Rodighiero, 1985; Ostermann et al., 1988; Weir and Ueda, 1988; Pfeifer, 1991).

1.1.5.2.5 Lung disease

Experimental tuberculosis resulted in decreased microsomal cytosolic protein. The tuberculosis infection produced a decrease in lung cytochrome b5 NADPH-cytochrome

C reductase and microsomal mixed function oxidases (MFO) activities. The pulmonary activity of UDP-glucuronyl transferase was elevated in infected animals. Glutathione S-transferase activity decrease in the lung of tuberculous infected guinea pigs. Some of the changes observed in levels of monooxygenase enzymes in tuberculosis patients were caused by reduced food consumption. In general, tuberculosis infection can be viewed to lower drug metabolizing capacity of the animal, probably due to the damage and disturbed membrane integrity(Batra et al., 1987).

1.1.5.2.6 Mineral and vitamin deficiency

Vitamin A deficiency alone significantly reduced cytochrome P-450 levels in male Sprague-Dawley rats (Hauswirth, 1987). There is increasing evidence that the liver microsomal drug metabolizing system is affected by many different vitamins including ascorbic acid, riboflavin, and α-tocopherol. In organisms experiencing ascorbic acid deficiency there is a decrease in the quantity of hepatic microsomal electron transport components such as cytochrome P450 and NADPH-cytochrome P450 reductase. Vitamin C deficient organisms also exhibit decreases in a variety of drug enzyme reactions such as N-demethylation, O-demethylation, and steroid hydroxylation. In addition, young animals given high supplements of vitamin C have increased quantities of electron transport components and overall drug metabolism activities. Kinetic studies indicate no change in the apparent Km of N-demethylase, O-demethylase or hydroxylase for drug substrates in animals that have vitamin deficiencies or that have been given high amounts of the vitamin. However, there are qualitative changes in both type I and II substrate-cytochrome P-450 binding. Ascorbic acid is not involved in microsomal lipid peroxidation or in any qualitative or quantitative change in phosphatidylcholine. After replenishing vitamin C-deficient animals with ascorbic acid required 3 to 7 days were required for the electron transport components and drug metabolism activities to return to normal levels. Induction with phenobarbital and 3-methylcholanthrene is not impaired in the deficient animal since drug metabolism activities are induced to the same extent as normal controls(Zannoni and Sato, 1976). In a separate study, vitamin C

supplementation had no influence on methacetin metabolism in Gambian men(Powers et al., 1991).

The administration of delta-aminolevulinic acid, a precursor of heme synthesis, to deficient animals caused an increase in the quantity of cytochrome P450. However, The effects of riboflavin deficiency on electron transport components and drug metabolism activities have been noted only in adult animals after prolonged periods of deficiency. Decreases in drug metabolism activities occur with both type I (aminopyrine and ethylmorphine) and type II (aniline) substrates. As was observed in animals with ascorbic acid deficiency, drug enzyme induction occurred to the same extent following administration of phenobarbital in deficient and normal animals. In addition, from 10 to 15 days were required for the drug metabolism activities to return to normal levels when deficient animals were replenished with riboflavin(Zannoni and Sato, 1976).

Vitamin E has been shown to interact with the synthesis and activity of drug metabolizing enzymes. This interaction appears to be based on both on a protection of cytochrome P450 enzymes against oxidative damage and a stabilization of cytochrome P450 associated membrane phospholipids. The enzyme interaction also appears to be based on the gene-regulatory functions of vitamin E. The consequences vitamin-induced alteration of metabolism may be both, beneficial and detrimental: maintenance of an optimum xenobiotic metabolizing system may protect against harmful food ingredients and environmental poisons. However, induction of drug metabolizing enzymes may weaken their therapeutic efficacy (Brigelius-Flohe, 2003).

1.1.5.2.7 Inflammation and drug metabolism

Inflammation and infection have long been known to downregulate the activity and expression of cytochrome P450 (CYP) enzymes involved in hepatic drug clearance. Regulation of hepatic flavin monooxygenases, UDP-glucuronosyltransferases, sulfotransferases, glutathione S-transferases, as well as of hepatic transporters during the inflammatory response, exhibits similarities and differences with regulation of cytochrome P450s (Aitken et al., 2006). However, expression of various cytochrome P450 genes is modulated differentially during inflammation. Whereas the expression of

most P450s in the liver is suppressed, some are induced(Morgan, 1997). Inflammation and infection may lead to a decrease in the capacity of the liver and other organs metabolize xenobiotic chemicals and some endogenous compounds(Renton, 2000). The downregulation of some cytochrome P450s and the induction of others may result from a complex interaction involving inflammatory cytokines, stress hormones, and metabolic perturbations(Iber et al., 1999). The loss in cytochrome P450 enzymes is predominantly an effect at the level of the gene expression and the majority of cytochrome P450s forms examined to date are involved(Renton, 2000). However, it is likely that modulation of RNA and protein turnover, as well as enzyme inhibition, contributes to some of the observed effects. The mechanisms whereby these effects are produced may also vary with both the P450 under study and the time course of the effect(Morgan, 1997).

The loss in drug metabolism is predominantly an effect resulting from the production of cytokines and the modulation of the transcription factors that control the expression of specific cytochrome P450 forms. Many of the effects observed in vivo can be mimicked by pro-inflammatory cytokines and interferons, and P450s are differentially regulated by these agents. Therefore, different cytokine profiles and concentrations in the vicinity of the hepatocyte in different models of inflammation may result in qualitatively and quantitatively different effects on populations of P450s. In addition to cytokines, glucocorticoids may have an important role in P450 regulation in stress conditions, including that caused by inflammatory stimuli(Morgan, 1997).

The complexity of the P450 response to inflammation and infection means many factors must be considered when trying to predict the effect of a given infectious or inflammatory condition on the clinical or toxic response to an administered drug or toxin in humans or animals. The decrease of cytochrome P450 and its dependent drug biotransformation is of concern whenever drugs are used in patients with infections or disease states that induce inflammation. Numerous examples have been reported in clinical studies indicating the occurrence of compromised drug clearance and changes to pharmacokinetics. For any drug that is metabolised by cytochrome P450s and has a narrow therapeutic index, there is a significant risk in placing patients in a position

where an infection or inflammatory response might alter metabolism and an adverse drug response(Carcillo et al., 2003; Lee and Lee, 2005; Prandota, 2005; Renton, 2005) (Renton, 2000) (Morgan, 1997).

The question of whether the down-regulation of the hepatic P450 system to inflammation or infection is a homeostatic or pathological response cannot be answered at present (Morgan, 1997). Environmental factors do not exist in isolation; they interact with each other and with hereditary influences upon drug metabolism. The clinical relevance of altered drug metabolism due to environmental factors depends on the magnitude of the change, the variance in the population administrated the drug, and the safety margin afforded by the drug's therapeutic index. A better understanding of molecular mechanisms underlying regulation of drug metabolism will provide valuable information for prescribing appropriate dosages of therapeutic agents.

1.2 Nuclear Factor-κB and inflammatory responses

1.2.1 NF-κB rel family and functions

NF-κB family members include RELA (p65), RELB, c-REL, NF-κB1 (p50; p105) and NF-κB2 (p52; p100). The N-terminal Rel-homology domain (RHD) contains the dimerization, nuclear-localization and DNA-binding domains. The Rel-homology domain (RHD) is 300-amino acids long and structurally conserved. NF-κB proteins c-REL, RELB and RELA also have a carboxy-terminal non-homologous transactivation domain (TD). RELB has an additional leucine-zipper motif (LZ). p105 and p100 contain ANK repeats at the carboxyl terminus. Proteolytic processing of p105 and p100 at residues 435 and 405, respectively, generates the p50 and p52 NF-κB proteins. The glycine-rich region (GRR) and the carboxy-terminal sites of inducible phosphorylation (in the DSVCDS and EVKEDSAYGS sequences for p105 and p100, respectively) are required for processing. Phosphorylation of RELA at Ser276, Ser529 and Ser536 is important for its transactivation activity.

The carboxy-terminal non-homologous transactivation domains allow c-REL, RELB and RELA proteins to strongly activate transcription of their target genes. The other NF-

κB proteins can still bind to NF-κB consensus sites in DNA and, therefore function as transcriptional repressors. NF-κB proteins p100 and p105 contain 33-amino-acid motif ankyrin repeats which mediate protein-protein interactions. Each member of the NF-κB family, except for RELB, can form homodimers, as well as heterodimers with one another. The main activated form of NF-κB is a heterodimer of the p65 subunit associated with either a p50 or p52 subunit. The p50 (NF-κB1)/p65 (RELA) heterodimer is the most abundant form of NF-κB. p50 and p65 are expressed widely in various cell types. The expression of RELB is restricted to specific regions of the thymus, lymph nodes and Peyer's patches. The expression of c-REL is confined to haematopoietic cells and lymphocytes. The transcription of RELB, c-REL and p105 is regulated by NF-κB.

Activated NF-κB complex translocates into the nucleus and binds DNA at κB-binding motifs GGGRNNYYCC or HGGARNYYCC (where H is A, C, or T; R is an A or G purine; and Y is a C or T pyrimidine). NF-κB can regulate the expression of cytokines, chemokines, growth factors, immunoregulatory molecules, cell adhesion molecules, acute-phase response proteins, stress response genes, cell surface receptors, regulators of apoptosis, viruses, enzymes and others(Kumar et al., 2004). Thus NF-κB has broad physiological functions apart from regulating innate as well as adaptive immune systems. Aberrant activation of the NF-κB pathway is involved in the pathogenesis of a number of human diseases including those related to inflammation, enhanced cellular proliferation, viral infection, and genetic diseases(Kumar et al., 2004).

1.2.2 Regulation of NF-κB activation

NF-κB activity is also regulated by the direct modification of NF-κB proteins through phosphorylation and acetylation. Phosphorylation status determines the association of p65 with CBP/p300 which positively regulates gene expression or HDAC1 which inhibits the expression of NF-κB-regulated genes at both basal and induced levels. The loss of phosphorylation of p65 influences both its DNA-binding and transactivation activities. Phosphorylation of p65 Ser276, Ser529 or Ser536 are required for the transactivation function of p65. Glycogen synthase kinase 3β (GSK3β), TBK1, IKKα

and PKCζ are important for the control of NF-κB transcriptional activity(Li and Verma, 2002; Vermeulen et al., 2002).

Before activation and translocation to the nucleus, the regulatory proteins IκB retain NF-κB proteins in the cytoplasm as an inactive form. IκBα, IκBβ and IκBε are the most common IκB forms. The special IκB member BCL-3 interacts specifically with p50 and p52 homodimers and can induce the expression of NF-κB-regulated genes in contrast to the inhibitory function of the other IκB proteins. IκBα, IκBβ and IκBε act differently in the regulation of NF-κB activation. The cytoplasmic localization of the inactive NF-κB complexes is achieved by balancing continuous movement between the nuclear and cytoplasmic compartments. IκBα masks only one of the two nuclei localization sequences (NLSs) in an NF-κB dimer in an NF-κB–IκBα complex, which allows the complex to shuttle to the nucleus. At the same time, the nuclear-export signal (NES) at the N-terminus of IκBα protein functions to expel the NF-κB–IκBα complex from the nucleus. IκBα regulates transient NF-κB activation. IκBα is degraded rapidly in response to stimuli and quickly resynthesized owing to NF-κB activation. The newly synthesized IκBα has an intrinsic NLS and can enter the nucleus and displace NF-κB from its DNA binding sites and transport NF-κB back to the cytoplasm, thereby carrying out a post-induction repression of NF-κB function. By contrast, IκBβ retains NF-κB–IκBβ complexes in the cytoplasm by masking both NLSs on the NF-κB dimmer. IκBβ maintains persistent NF-κB activation. IκBβ is less sensitive to stimulus-induced degradation than IκBα. The selective interaction between endogenous κB-Ras and IκBβ is crucial for inhibiting IκBβ degradation during NF-κB activation. IκBβ does not have a functional NES and is not NF-κB inducible, so the resynthesized IκBβ is able to interact with NF-κB complexes that are bound to the target promoters but not displace them. The outcome is a sustained NF-κB response. NF-κB–IκBε complexes shuttle actively between the nucleus and cytoplasm(Li and Verma, 2002).

The degradation of IκB is an essential step for releasing NF-κB and its subsequent activation for most known stimuli except ultraviolet radiation and hydrogen peroxide. The inhibitors of NF-κB (IκB) IκBα, IκBβ and IκBε (two transcripts) and BCL-3 contain

ankyrin (ANK) repeats. DSGLDS, DSGLGS and DSGLES induce phosphorylation of IκBα, IκBβ and IκBε for their degradation, respectively. The degradation of IκB is mediated by phosphorylation at its specific serine residues (for example, Ser32 and Ser36 for IκBα) at N-terminus by IκB kinase (IKK) complex. The phosphorylated IκBα is then ubiquitylated at Lys21 and Lys22 by β-transducin repeat-containing protein (β-TRCP) which targets it for degradation by the 26S proteasome, thereby releasing NF-κB dimers from the cytoplasmic NF-κB-IκB complex and allowing them to translocate to the nucleus.

The IκB kinase (IKK) complex consists of two catalytic kinases IKKα and IKKβ, and the regulatory subunit NF-κB essential modulator (NEMO; IKKγ). The IKK complex is a point of convergence for all three signalling pathways in response to the stimuli. IKKα and IKKβ share 52% amino acid homology and have similar functional domains, including an N-terminal catalytic domain, a centrally positioned leucine-zipper motif that is involved in homodimer and heterodimer formation, and a C-terminal helix-loop-helix domain. NEMO/IKKγ interacts with a C-terminal region of IKKβ, designated the NBD, which is critical for the formation of IKK complex. Although NEMO has no catalytic function, it is indispensable for signal-dependent NF-κB activation. IKKβ is mostly required for the classical (canonical) NF-κB pathway that depends on IκB degradation. Despite the structural similarity of IKKα and IKKβ, biological and genetic studies indicate that IKKβ is the dominant kinase involved in the phosphorylation of IκBs. IKKα provides a partially redundant role in stimulus-induced NF-κB activation. Recent studies have shown that IKKα is involved in an alternative (non-canonical) NF-κB pathway that regulates the RelB/p52 dimer. In unstimulated cells, RelB is retained in the cytoplasm as a RelB–p100 precursor complex to repress RelB-mediated transcriptional activity. Upon stimulation, the IκB-like C-terminal domain of p100 is proteolyzed, releasing RelB–p52 dimers that are translocated to the nucleus. The alternative NF-κB pathway is induced in response to only a few members of the TNF family, such as B-cell-activating factor, CD40 ligand, and lymphotoxin-β, and is mostly

involved in lymphoid organ development (Karin and Ben-Neriah, 2000; Verma, 2004; Jimi and Ghosh, 2005; Hayden et al., 2006).

1.2.3 NF-κB signalling pathways

NF-κB is activated rapidly in response to a wide range of stimuli, including pathogens, stress signals and pro-inflammatory cytokines, such as tumour-necrosis factor (TNF) and interleukin-1 (IL-1). NF-κB activity is stimulated by many pathways, including lipopolysaccharide (LPS), tumour-necrosis factor (TNF) and T-cell receptor (TCR) signalling.

LPS binds to TLR4-CD14 and MD-2 complexes on the cell surface and activates an intracellular signalling cascade through the TLR cytoplasmic Toll/IL-1 receptor (TIR)-homology domain, involving the recruitment of MYD88 (myeloid differentiation primary response gene 88) and IRAK (interleukin-1-receptor-associated kinase). Activation of IRAK results in the phosphorylation of TNF-receptor-associated factor 6 (TRAF6), which might relay signals through the TAK1–TAB1–TAB2 complex to IKK complexes to activate the NF-κB pathway.

Cytokine IL-1 activates NF-κB in a similar manner to LPS because of homology between the cytoplasmic signalling domains of the IL-1 receptor (IL-1R) and TLRs. TNF receptors are present on the surface of a wide range of cells. Receptor engagement by TNF results in receptor trimerization and recruitment of the adaptor protein TRADD (TNF receptor associated via death domain) to the cytoplasmic receptor tail. In turn, TRADD interacts with the carboxyl terminus of TRAF2, an adaptor protein that has affinity for various downstream signalling proteins. Mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase kinase 3 (MEKK3) and receptor-interacting serine/threonine kinase (RIP) are likely to have a key role in linking TNF to the activation of IKKs.

T-cell receptor (TCR)-induced activation of NF-κB in peripheral T cells requires a co-stimulatory signal delivered from CD28 in addition to TCR signaling. It also involves the activation of protein kinase Cθ (PKCθ) and IKK2 (IKKβ). PKCθ translocates rapidly

to the plasma membrane of T cells in response to stimulation by antigen-presenting cells or anti-TCR-CD3 antibodies. Although poorly defined, trimolecular complexes of membrane-associated guanylate kinase homologue (MAGUK) and the mucosal-associated lymphoid tissue (MALT)-lymphoma-associated proteins BCL-10 and MALT1 have been implicated in signalling from PKCθ to IKK complexes.

1.2.4 Role of NF-κB in inflammatory response

Inflammation is a response to infection, antigen challenge or tissue injury that is designed to eradicate microbes or irritants and to potentiate tissue repair. Inflammation can be divided into two major categories—acute and chronic—based on timing and pathological features. Chronic inflammatory disorders are characterized by a prolonged duration (weeks to months to years) in which active inflammation, tissue destruction and attempts at tissue repair are occurring simultaneously(Liew, 2003). Infiltration of mononuclear cells and fibrosis are typical histological features of chronic inflammation(Davies et al., 2003). Acute inflammation is typically of relatively short duration (hours to days) and is characterized by vasodilatation, the exudation of protein-rich fluid (plasma) and a migration of cells (primarily neutrophils) into the site of injury. In some cases, activation of the coagulation cascade also occurs(Splettstoesser and Schuff-Werner, 2002; Carraway et al., 2003). Sepsis, severe trauma and major surgery all have acute inflammatory components. A tightly orchestrated process involving numerous soluble and cell associated factors mediates these alterations. The innate immune system plays a critical role in the activation of inflammation. Macrophages produce pro-inflammatory cytokines, chemokines, tissue factor and NO that serve to amplify the proinflammatory response and activate the coagulation cascade. Non-cytokine factors such as the complement system, eicosanoids and PAF are also important. The coagulation cascade is a well-recognized component of the pro-inflammatory response. Recent studies have shown that thrombin is not only important in the induction of fibrin clot formation, but also has direct pro-inflammatory functions. Endogenous

anticoagulant factors such as activated protein C, TFPI and anti-thrombin III serve to control pro-coagulant mechanisms.(Sherwood and Toliver-Kinsky, 2004).

The NF-κB signaling pathway plays a crucial role in the initiation, amplification and resolution of inflammation. NF-κB is highly activated at sites of inflammation in diverse diseases, such as rheumatoid arthritis, inflammatory bowel diseases, MS, psoriasis and asthma. NF-κB is one of the pivotal regulators of pro-inflammatory gene expression and also induces the transcription of pro-inflammatory cytokines and chemokines (such as IL-1, IL-6, IL-8 and TNF), adhesion molecules, matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (Baeuerle and Baichwal, 1997; Lawrence et al., 2001; Tak and Firestein, 2001; Aggarwal, 2004; Karin and Greten, 2005; Xiao and Ghosh, 2005). Cellular responses to pro-inflammatory stimuli such as cytokines IL-1β or TNF-α and control of the expression of dozens of mediators of inflammation is at the center of an amplifying loop that requires subtle adjustments in order to be activated and deactivated at the right time. Accordingly, any dysfunction of the NF-κB activation process may generate chronic inflammation(Makarov, 2000) or to favor cancer development, especially in situations where tumorigenesis is associated with an inflammatory environment(Karin and Ben-Neriah, 2000). The pathogenic effects of NF-κB overactivation in inflammatory diseases are indicated by studies of p50- and c-Rel-knockout mice, which do not develop eosinophilic airway inflammation when sensitized and challenged with allergen ovalbumin(Yang et al., 1998; Donovan et al., 1999). Specific inhibition of NF-κB activity has been shown consistently to be effective at controlling inflammatory diseases in several animal models. Blocking NF-κB activity by the overexpression of IκBα inhibits both the inflammatory response and tissue destruction in rheumatoid synovium(Bondeson et al., 1999). Administration of NF-κB decoys seems to be effective treatments in animal models of rheumatoid arthritis(Miagkov et al., 1998).

1.3 Nuclear receptor as xenosensors

1.3.1 Nuclear receptors

Since the publication in 1985 of a study decribing cloning of the first nuclear receptor cDNA encoding the human glucocorticoid receptor (GR) (Hollenberg et al., 1985; Evans, 2005), the nuclear receptor field has witnessed great progress(Chambon, 2005; Evans, 2005). To date totoal 49 members of a nuclear receptor superfamily have been characterized in mouse and 48 members in humans (Table 5). The Nuclear Receptors Nomenclature Committee (1999) set up a system similar to the nomenclature system of the cytochrome P450 superfamily(Nebert et al., 1987) to connect all known NR sequences based on a phylogenetic tree on the evolution of the two well-conserved domains of NRs (the DNA-binding C domain and the ligand-binding E domain). Each receptor is described by the letters 'NR' (for 'nuclear receptor') and a three-digit identifier: this denotes the subfamily to which a given receptor belongs (indicated by the first digit, an Arabic numeral), the group (denoted by capital letters) and the individual gene (again denoted by Arabic numerals. For example, PXR (NR1I2), NR stands for nuclear receptor superfamily, “1” stands for family, “I” stands for subfamily, and “2” stands for specific individual receptors. Members of the same family in general share at least 80%–90% identity in the DNA-binding domain and at least 40%–60% in the ligand-binding domain. This system is flexible enough to integrate nuclear receptors from invertebrates as well as sequences generated from genome projects for which biological data are not yet available.

Nuclear receptors are one of the most abundant classes of transcriptional regulators in animals (metazoans). Nuclear receptors function as ligand-activated transcription factors, and thus provide a direct link between signaling molecules and transcriptional responses. The superfamily includes receptors for hydrophobic molecules such as steroid hormones (e.g. estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, vitamin D3, ecdysone, oxysterols and bile acids), retinoic acids (all-trans and 9-cis isoforms), thyroid hormones, fatty acids, leukotrienes, prostaglandins, and lipophilic xenobiotics(Robinson-Rechavi et al., 2003).

The mode of action of nuclear receptors (NRs) shares some common features. After diffusion through the cytoplasmic membrane, the ligand can interact with its cognate receptor where it can exert a 'non-genomic effect' by interacting directly such as with kinases. The ratio between cytoplasmic and nuclear location can vary between different receptors and is affected by the nature of a ligand. Ligand binding modulates the interaction of the receptor with a plethora of factors. In the absence of ligand, several nuclear receptors are believed to be bound to the regulatory regions of target genes as a corepressor or histone deacetylase (HDAC) complex. Histone deacetylation is responsible for the chromatin condensation that accounts for the gene-silencing effect of apo receptors. Ligand binding releases the HDAC complex and results in the recruitment of histone acetyltransferase (HAT) and chromatin-remodelling (CRM) complexes. The temporal order and requirement of these complexes can occur in a receptor-, target-gene- and cell-specific manner. In the last step, the polymerase II holoenzyme, which comprises the pol II enzyme, TAF (TATA-binding protein-associated factor) and mediator complexes, is recruited and increases the frequency of transcription initiation.

Table 5 Human nuclear receptor superfamily

Name	Abrevi-ation	Nomen-clature	Ligands	Refseq#
Glucocorticoid receptor	GR	NR3C1	Cortisol, dexamethasone, RU486	NM_000176
Mineralocorticoid receptor	MR	NR3C2	Aldosterone, spirolactone	NM_000901
Progesterone receptor	PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486	NM_000926
Androgen receptor	AR	NR3C4	Testosterone, flutamide	NM_000044
Estrogen receptor	ERα	NR3A1	Estradiol-17β, tamoxifen, raloxifene	NM_000125
Estrogen receptor	Erβ	NR3A2	Estradiol-17β, various synthetic compounds	NM_001437
Retnoid X receptor	RXRα	NR2B1	9-cis-retinoic acid	NM_002957

Table 5 (Continued)

Name	Abrevi-ation	Nomen-clature	Ligands	Refseq#
Retnoid X receptor	RXRβ	NR2B2	9-cis-retinoic acid	NM_021976
Retnoid X receptor	RXRγ	NR2B3	9-cis-retinoic acid	NM_006917
Hepatocyte nuclear factor 4	HNF4α	NR2A1	fatty acids	NM_000457
Hepatocyte nuclear factor 4	HNF4γ	NR2A2	fatty acids	NM_004133
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFα	NR2F1	Orphan	NM_005654
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFβ	NR2F2	Orphan	NM_021005
ErbA2-related gene-2	EAR2	NR2F6	Orphan	XM_373407
Testis receptor	TR2	NR2C1	Orphan	NM_003297
Testis receptor	TR4	NR2C2	Orphan	NM_003298
Germ cell nuclear factor	GCNF	NR6A1	Orphan	NM_033334
Reverse erbA	Rev-erbα	NR1D1	Orphan	NM_021724
Reverse erbA	Rev-erbβ	NR1D2	Orphan	NM_005126
Thyroid hormone receptor	TRα	NR1A1	Thyroid hormone, triiodothyronine	NM_199334
Thyroid hormone receptor	TRβ	NR1A2	Thyroid hormone, triiodothyronine	NM_000461
Retinoic acid receptor	RARα	NR1B1	retinoic acids	NM_000964
Retinoic acid receptor	RARβ	NR1B2	retinoic acids	NM_000965
Retinoic acid receptor	RARγ	NR1B3	retinoic acids	NM_000966
Peroxisome proliferator-activated receptor	PPARα	NR1C1	Fatty acids, leukotriene B4, fibrates	NM_005036
Peroxisome proliferator-activated receptor	PPARβ	NR1C2	fatty acids, eicosanoids	NM_006238
Peroxisome proliferator-activated receptor	PPARγ	NR1C3	Fatty acids, prostaglandin J2,	NM_005037
Vitamin D receptor	VDR	NR1I1	1, 25-dihydroxy vitamin D3, litocholic acid	NM_000376

Table 5 (Continued)

Name	Abrevi-ation	Nomen-clature	Ligands	Refseq#
Liver X receptor	LXRα	NR1H3	Oxysterols, T0901317, GW3965	NM_005693
Liver X receptor	LXRβ	NR1H2	Oxysterols, T0901317, GW3965	NM_007121
Farnesoid X receptor	FXR	NR1H4	Bile acids, Fexaramine	NM_005123
Pregnane X receptor	PXR	NR1I2	xenobiotics, steroids, bile acids	NM_022002
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, henobarbital, steroids	NM_005122
Steroidogenic factor 1	SF-1	NR5A1	Orphan	NM_004959
Liver receptor homologous protein 1	LRH-1	NR5A2	Orphan	NM_003822
Estrogen receptor-related receptor	ERRα	NR3B1	Orphan	NM_004451
Estrogen receptor-related receptor	ERRβ	NR3B2	DES, 4-OH tamoxifen	NM_004452
Estrogen receptor-related receptor	ERRγ	NR3B3	DES, 4-OH tamoxifen	NM_001438
NGF-induced factor B	NGFI-Bα	NR4A1	Orphan	NM_002135
Nur related factor 1	NURR1	NR4A2	Orphan	NM_006186
Neuron-derived orphan receptor 1	NOR1	NR4A3	Orphan	NM_006981
RAR-related orphan receptor	RORα	NR1F1	Cholesterol, cholesteryl sulphate	NM_134261
RAR-related orphan receptor	RORβ	NR1F2	Retinoic acid	NM_006914
RAR-related orphan receptor	RORγ	NR1F3	Retinoic acid	NM_005060
Tailless (Drosophila) homolog	TLX (TLL)	NR2E1	Orphan	NM_003269
Photoreceptor-specific nuclear receptor	PNR	NR2E3	Orphan	NM_016346

Table 5 (Continued)

Name	Abrevi-ation	Nomen-clature	Ligands	Refseq#
DSS-AHC critical region on the DAX1 NR0B1 Orphan chromosome, gene 1	DAX1	NR0B1	Orphan	NM_000475
Short heterodimeric partner	SHP	NR0B2	Orphan	NM_021969

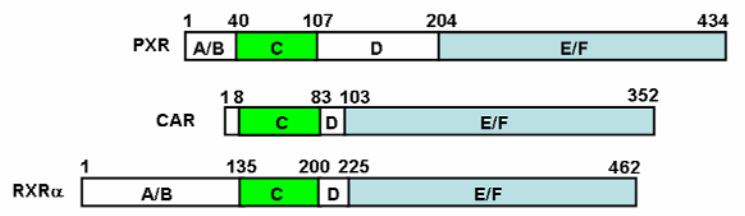


Fig. 1. Functional and structural domains of PXR, CAR and RXRα as examples of nuclear receptors

Domain length was followed reference (Wu et al., 2006).A/B: sequence highly variable; AF-1 transactivation function; 3D-structure is unknown. C: sequence most conserved; DNA-binding to AGGTCA motif; dimerization of nuclear receptors; may contain part of nuclear localization signal (NLS). D: sequence variable; flexible hinge, nuclear localization signal (NLS). E: sequence moderately conserved; the largest domain, secondary structure of 12 -helixes is better conserved than the primary sequence; ligand-binding and induced, AF-2 transactivation function; another NLS, and often a repression function. F: sequence extremely variable; structure and function are unknown(Robinson-Rechavi et al., 2003).

Table 6 Comparison of the domains of PXR and CAR

Peptides	PXR From-To (Length, aa)	CAR From-To (Length, aa)
CHAIN	1-434 (434)	1-352 (352)
DNA_BIND	38-107 (70)	8-83 (76)
ZN_FING NR C4-type	41-61 (21)	11-31 (21)
ZN_FING NR C4-type	77-102 (26)	47-71 (25)
Hinge	108-204 (97)	84-102 (19)
LBD	205-434 (230)	103-352 (250)

The molecules of the nuclear receptor family PXR, CAR and RXRα share common structural traits (Fig. 1, Table 6) except that subfamily NR0 members lack either the DNA or ligand binding domain (Robinson-Rechavi et al., 2003; Gronemeyer et al., 2004; McEwan, 2004; Chambon, 2005; Evans, 2005).

Nuclear receptors can form monomers, homodimers or heterodimers with the promiscuous retinoid X receptor (RXR) (Table 5); the nuclear receptors then bind response elements within the regulatory region (s) of target genes. Nuclear receptor response elements are derivatives of the canonical sequence RGGTCA (in which R is a purine), termed hormone response elements (HREs). Modification, extension and duplication (including alternate relative orientations of the repeat (direct, inverted, everted)) of this sequence generate response elements that are selective for a given receptor (s) or class of receptors(Mangelsdorf and Evans, 1995; Laudet and Gronemeyer, 2002; Gronemeyer et al., 2004). The ability of a specific nuclear receptor to recognize HREs is unique. Data have shown that the most potent of these HREs are direct repeats (DRs) of the core AGGTCA half-site. The model in which DRl serves as an RXR and peroxisome proliferator response element and a DR2 is a second retinoic acid response element, HREs for the vitamin D receptor (VDR), thyroid hormone receptor (TR), and retinoic acid receptor (RAR) are composed of DRs spaced by 3, 4, or 5 nt (i.e., DR3, DR4, and DR5, respectively) has been developed into a 1-5 rule, as reviewed by Mangelsdorf (Sporn et al., 1994) and Leid(Leid et al., 1992).

Nuclear receptors are phosphoproteins, and multiple receptor functions can be affected by phosphorylation in response to various types of effectors. The majority of the nuclear receptor phosphorylation sites lie within the amino-terminal A/B region; they can be complex and comprise up to 13 residues, as in the case of the progesterone receptor. Most of the modified residues are serines surrounded by prolines and therefore correspond to consensus sites for proline-dependent kinases. These include cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs). MAPKs can, independently of hormone, phosphorylate other sites in response to various signals, such as growth factors, stress or cytokines. The N-terminal A/B region also contains

consensus phosphorylation sites for the kinase Akt, which is important in cell survival and proliferation. After translocation into the nucleus, Akt can phosphorylate nuclear receptors, such as estrogen receptor-α (ERα) and the androgen receptor, in their N-terminal A/B region. In addition to the N-terminal domain, the ligand-binding domain (LBD) of nuclear receptors is also a target for ligand-independent phosphorylation, involving the proline-dependent kinases mentioned above. For example, retinoid X receptor α (RXRα) can be targeted by stress kinases (such as c-Jun N-terminal kinases). Other kinases, such as tyrosine kinases, can phosphorylate ERα and RXRα or protein kinase A (PKA) for retinoic acid receptors (RARs). Nuclear receptors can also be phosphorylated in their DNA-binding domain (DBD); examples include PKA acting on ERα, and protein kinase C (PKC) acting on RARα or the vitamin D receptor.

Nuclear receptors regulate diverse functions, such as homeostasis, reproduction, development and metabolism(Robinson-Rechavi et al., 2003; Gronemeyer et al., 2004; McEwan, 2004; Chambon, 2005; Evans, 2005). The physiological function of nuclear receptors was investigated by relating the expression profile of nuclear receptors in multiple tissues with the physiological function of the tissues in mice. The resulting data showed that the regulatory function of nuclear receptors form a hierarchy network governing two physiologic paradigms on an organismal scale: (1) reproduction, development, and growth and (2) nutrient uptake, metabolism, and excretion. Nuclear receptors governing reproduction, development, and growth include: The top regulatory group of SF1, DAX-1, and FXRβ; The secondary group of AR, ERα, ERβ, PR, RARα, RARγ, COUP-TFβ; TLX, COUP-TFα, REV-ERBα and β, RORα and β, ERRβ and γ; and the NR4A orphan receptors (NGFI-B, NOR1, and NURR1), TRα, MR, and LXRβ, RXRβ and RXRγ. Nuclear receptors governing nutrient uptake, metabolism, and excretion are predominantly expressed within the gastro/enterohepatic axis and key metabolic tissues (e.g., adipose and muscle). The uptake, metabolism and excretion receptors include FXRα, liver receptor homolog-1 (LRH-1), SHP, PXR and CAR, VDR, HNF4α and γ, RORγ; PPARα and PPARδ, ERRα, TRβ, RXRα, COUP-TFγ, TR2, and GCNF; PPARγ, LXRα, and GR, and PNR(Bookout et al., 2006). Studies on the temporal

expression profiles of nuclear receptors in adipose tissue, liver, and skeletal muscle of mice showed that of the 45 NRs expressed, 25 are in a rhythmic cycle and 3 exhibit a single transient pulse of expression 4 hr into the light cycle. This finding may may offer a logical explanation for the known cyclic behavior of lipid and glucose metabolism. It also suggests novel roles for endocrine and orphan receptors in coupling the peripheral circadian clock to divergent metabolic outputs(Yang et al., 2006).

Among nuclear receptors, PXR and CAR are responsible for xenobiotic metabolism regulation (Liddle and Goodwin, 2002; Moore et al., 2002; Wei et al., 2002; Willson and Kliewer, 2002; Honkakoski et al., 2003; Moore et al., 2003; Kretschmer and Baldwin, 2005; Wagner et al., 2005). They are restrictly expressed in the metabolic tissues, the liver and intestines. PXR does not show obvious rhythmatic expression while CAR does show rhythmatic expression in mice. As xenosensors, PXR and CAR generally respond to different xenobiotics, and regulate different genes. However, there is also some overlap in the response and regulation of these receptors.

The aryl hydrocarbon receptor (AhR) belongs to the family of bHLH-PAS proteins. However, the AhR behaves much the same as the transcriptional activation of nuclear receptors. The AhR is a ligand-dependent transcription factor that regulates the expression of several drug-metabolizing enzymes and has been implicated in immunosuppression, teratogenesis, cell-specific hyperplasia, and certain types of malignancies and toxicities. AhR regulate xenobiotic metabolism by regulating the expression of genes of the drug metabolism enzymes such as CYP1 gene family.

1.3.2 Pregnane X receptor

PXR is a sensor for endogenous and xenobiotic compounds and a trans-regulator for the expression of many drug metabolism–related genes. The rodent PXR (Kliewer et al., 1998) and its human homolog hPXR (Lehmann et al., 1998), also known as SXR (Blumberg et al., 1998) or hPAR (Bertilsson et al., 1998) were identified as xenobiotic receptors that can be activated by certain xenobiotic and endogenous compounds. The primary drug metabolism gene regulated by PXR is the cyp3a subfamily both in human

and mouse. Other genes that are regulated by PXR include multiple drug resistant genes such as MDR1 (Synold et al., 2001) and MRP2 (Kast et al., 2002) as well as genes involved in metabolism and transport of endogenous molecules. PXR/RXR can also interact with pathways regulated by other nuclear receptors such as the constitutive androstane receptor (CAR)/RXR by mutual binding to the consensus regulatory DNA sequences, thus forming a redundant, compensatory network for the metabolism and disposition of xenobiotic and endogenous compounds(Xie et al., 2000).

PXR is a member of nuclear receptor superfamily. Like most other nuclear receptors, PXR acts in three steps, repression, derepression and transcription activation. At the repression step, apo-PXR recruits a histone-deacetylase-active (HDAC) corepressor complex silencing mediator of retinoid and thyroid hormone receptors (SMRT)(Johnson et al., 2006; Wang et al., 2006). Derepression occurs following ligand binding, holo-PXR dissociates corepressor complex and recruits histone-acetylase-active (HAT) coactivator complex containing peroxisome proliferators-activated receptor gamma co-activator 1α (PGC-1α) and steroid receptor coactivator-1 (SRC-1)(Watkins et al., 2003a; Bhalla et al., 2004; Khan et al., 2006). This complex results in chromatin decondensation, which is believed to be necessary but not sufficient for activation of the target gene. In the third step, the HAT complex dissociates and a second coactivator complex is assembled (TRAP/DRIP/ARC), which is able to establish contact with the basal transcription machinery, and thus results in transcription activation of the target gene(Robinson-Rechavi et al., 2003; Gronemeyer et al., 2004).

Regulation of PXR transcriptional activity can occur in many steps such as ligand binding activation, repression, derepression and transcriptional activation. PXR transcriptional activity is also regulated by other signaling pathways. The interaction between PXR and hepatocyte nuclear factor 4α (HNF4α) enhanced the PXR-activated CYP3A4 gene expression(Abdelrahim et al., 2006). The forkhead transcription factor FOXO1, an activator of gluconeogenic genes, co-activated PXR-mediated transcription(Kodama et al., 2004). The regulation of PXR transcriptional activity may

result in the change of PXR target gene expression and thus the alterations of physiological and pathological states of human individuals.

PXR gene locates at chromosome 3;3q12-q13.3. Dexamethasone and lithocholic acid in primary human hepatocytes(Pascussi et al., 2000a; Moore et al., 2002). Clofibrate, perfluorodecanoic acid, isoniazid, and troleandomycin have been shown to change PXR gene expression level in rat liver(Zhang et al., 1999). In the 5' upstream regulatory sequence of PXR gene, A putative DNA/protein interaction sites for transcription factors such as HNFs, C/EBPα, and Sp1, and VDR, GRα, PRE, and PPARα was identified. This suggests that the PXR gene expression is potentially regulated by many different stimuli including xenobiotics and metabolites(Aouabdi et al., 2006). PXR gene expression is also activated by farnesoid X receptor in response to bile acids(Jung et al., 2006). Three alternatively spliced transcripts of PXR that encode different isoforms have been described, one of which encodes two products through the use of alternative translation initiation codons. Additional transcript variants have been derived from alternative promoter usage, alternative splicing, and/or alternative polyadenylation exist, Although these variants have not been fully described (Bertilsson et al., 1998; Lehmann et al., 1998; Dotzlaw et al., 1999; Gardner-Stephen et al., 2004).

1.3.3 Constitutive androstane receptor

The primary target drug metabolism genes of CAR are in the cyp2b subfamily. The forkhead transcription factor FOXO1 also co-activated CAR-mediated transcription(Kodama et al., 2004). CAR inhibits HNF-4 activity by competing with HNF-4 for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1α. Car also down-regulates key genes in hepatic lipid and glucose metabolism. Through regulation of distal enhancer PBREM and the proximal element OARE car is able to synergistically up-regulate the endogenous CYP2B6 gene in HepG2 cells. In this up-regulation, CAR acts as both a transcription factor and a co-regulator: directly binding to and enhancing PBREM upon activation by xenobiotics such as TCPOBOP and indirectly associating with the OARE in response to okadaic acid. The cohesin protein SMC1 acted

as a CAR binding protein and a negative regulator of OARE activity, thus repressing synergy.

CAR acts in the same steps as PXR in the nucleus. CAR interacts with a number of coactivators and corepressors such as SRC-1, GR-interacting protein 1, Xenopus SRC-3, and PGC-1α. Unlike PXR which is primarily located in the nucleus, CAR is primarily located in the cytoplasm. CAR translocation can be triggered by either direct ligand binding to the receptor, or indirectly, via a partially elucidated signal transduction pathway. CAR exists in a complex with Hsp90, retained in the cytoplasm by the cochaperone CCRP. Indirect activators or the direct binding of ligands to CAR subsequently recruits PP2A to the complex. Once in the nucleus, further activation steps involving calmodulin-dependent kinase and recruitment of coactivators occur before DNA binding and transcriptional activation of target genes. Transcription coactivator PBP either enhances nuclear import or nuclear retention of CAR in hepatocytes, and that PRIP is redundant for CAR function.

1.3.4 Aryl hydrocarbon receptor

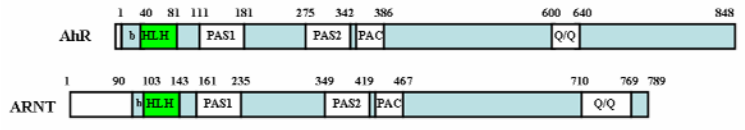
Studies on tissue distribution of the aryl hydrocarbon receptor (AhR) in developing and adult animals demonstrated that the AhR is expressed in a tissue-specific and developmentally specific manner. Also, the expression level of the AhR in culture cells varies more than 50-fold among cell lines. Although the mode of AhR action has been studied extensively, the events that control the expression of the AhR gene itself are poorly understood(Shimba et al., 2003). The DNA sequence coding AhR locates chromosome: 7; 7p15(Le Beau et al., 1994; Micka et al., 1997). The Ah receptor has primary sequence homology to its dimerization partner the AH receptor nuclear translocator, and to the Drosophila proteins Sim and Per. Characterization of the gene encoding the murine AH receptor (Ahr gene) reveals that its structural organization is also conserved with respect to the sim gene, since 6 of 11 Ahr exons are spliced at homologous sites. Interestingly, little splicing homology was observed between the Ahr and per genes. The promoter of the murine Ahr gene is GC-rich and contains no TATA

or CCAAT boxes. However, sequence analysis has shown several binding sites for the transcription factor Sp1 (GC boxes). A potential cAMP response element, AP-1 and E box sites, and two elements demonstrated in other genes to confer placenta-specific expression have been identified(Schmidt et al., 1993). The mouse Ahr gene 5' proximal promoter region contains four potential Sp1 motifs required for efficient basal expression(Fitzgerald et al., 1998). A region between -1431 and -721 represses constitutive promoter activity(Garrison and Denison, 2000). The sequence -378/-359 is core contributor to differentiation-dependent downregulation of AhR promoter activity(Shimba et al., 2003). The 27 bases located between position −197 to −170 of human AhR comprise elements relevant for basal expression. Sequence analysis revealed that this region contains putative binding sites for Sp1 and CREB. The functional relevance of the CREB-site was analyzed using dibutyryl-cAMP. However, the reporter gene activities were not significantly altered after dibutyryl-cAMP-treatment. This result indicates that the CREB-site may have no functional relevance(Racky et al., 2004).

Upon ligand binding, AhR is activated from a dormant state within the cytoplasm in association with a complex of HSP90, XAP2 and p23. This complex translocates into the nucleus and forms a heterodimer with ARNT protein in the nucleus by dissociating from the complex (Perdew, 1988; Pollenz et al., 1994; Hankinson, 1995; Safe and Krishnan, 1995; Safe, 1995; Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998; Kazlauskas et al., 1999; Mimura and Fujii-Kuriyama, 2003; Fujii-Kuriyama and Mimura, 2005). The AhR/ARNT heterodimer can bind to XRE consensus sequence in regulatory region of target genes. This binding may or may not transactivate target gene expression, as our lab has shown Ahr/ARNT-XRE binding is necessary but not sufficient for transactivation(Tian et al., 2003). Transcription elongation is a critical step. Using chromatin immunoprecipitation assays, the 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated recruitment of the aryl hydrocarbon receptor (AhR) and several co-regulators to the CYP1A1 promoter was studied. AhR displayed a time-dependent recruitment, reaching a peak at 75 min and maintaining promoter occupancy for the

remainder of the time course. Recruitment of AhR was followed by TIF2/SRC2, which preceded CBP, histone H3 acetylation, and RNA polymerase II (RNAPII). Simultaneous recruitment to the enhancer and the TATA box region suggests the formation of a large multiprotein complex bridging the two promoter regions(Matthews et al., 2005). The signaling protein complex AhR/ARNT also interacts with other signaling proteins, such as estrogen receptors, COUP-TF(Klinge et al., 2000), retinoblastoma protein(Puga et al., 2000), and crosstalk the other signaling pathways. Induction of CYP1A1 by the AhR/DRE has been considered as a paradigm for transcription, receptor regulation, and expanding biological roles. The AhR mediated transcription is tightly regulated through, at least, two mechanisms: (a) the cytoplasmic AhR interacts with hsp90 and an immunophilin chaperone AIP for proper folding and receptivity, and (b) the agonist-activated, nuclear AhR is degraded through the ubiquitin-26S proteasome mediated protein turnover, such that the transcription by AhR is controlled at a physiologically adequate level(Ma, 2001).

AhR and its nuclear dimmerization partner ARNT belong to bHLH-PAS protein family(Fig. 2).



Protein	AhR(1-848)		ARNT(1-789)	
Domain	Position	Length	Position	Length
Propep	1-10	10		
Basic motif(DNA binding)	13-40	28	90-102	13
Poly-Arg			99-102	4
Helix loop helix motif	41-81	41	103-143	41
Nuclear translocation sequence	42-57	16		
PAS 1	111-181	71	161-235	75
PAS 2	275-342	68	349-419	71
PAC	348-386	39	424-467	44
Poly-Gln			503-507	5
Gln-rich	600-640	41	710-769	60
Poly-Ser			738-741	4

Fig. 2. Functional and structural domains of AhR and ARNT(Wu et al., 2006).

The AhR has been shown to regulate the expression of xenobiotic-metabolizing enzymes such as cytochrome P450. The primary drug metabolism related target genes of AhR include cyp1a1, cyp1a2 and cyp1b1 (Table 7).

Cytochrome P450 1A1 (CYP1A1) is a member of the cytochrome P450 superfamily of enzymes. This protein localizes to the endoplasmic reticulum and its expression is induced by a broad range of xenobiotics including polycyclic aromatic hydrocarbons (PAHs). The enzyme's endogenous substrate is unknown; however, CYP1A1 is able to metabolize some PAHs to carcinogenic intermediates. Overexpression of CYP1A1 has been associated with increased lung cancer risk. A related family member, CYP1A2, is located approximately 25 kb from CYP1A1 on chromosome 15. CYP1A1 is considered to be involved mainly in oxidative metabolism of exogenous chemicals and drugs. Synthesis of this hemoprotein may be induced in liver, lung, and other tissues of experimental animals by the administration of xenobiotic chemicals. At least two kinds of cis-acting regulatory DNA sequences are localized 5' upstream of the gene. One sequence is distributed five times in a relatively wide range from -0.5 to -3.5 kb and functions as an inducible enhancer-designated xenobiotic responsive element or XRE. The other is localized just upstream of the TATA sequence and acts as a regulatory element for the constitutive expression. The two DNA elements are required for a high level of the inducible expression. Their cognate DNA binding factors are recognized in the nuclear extracts of Hepa-1 cells and rat liver cells which exhibit inducible expression of CYP1A1(Fujii-Kuriyama et al., 1992).

Table 7 AhR target genes human cyp1 family

genes	cyp1a1	cyp1a2	cyp1b1
location	15q22-q24	15q24	2p21
Peptide length (aa)	512	516	543
Basal level	low	constitutive	Partly constitutive
Inducibility	~1000 fold	~10 fold	~100 fold

Cytochrome P4501A2 (CYP1A2) is a member of the cytochrome P450 family that is involved in phase I drug metabolism in vertebrates. Using 5'-end deletion analysis, two functionally important cis elements, i.e., a proximal 42-bp DNA from bp -72 to bp -31 and a distal 259-bp DNA from bp -2352 to bp -2094, were identified in its 5' flanking region. The proximal sequence (bp -72 to -31) contained CCAAT and GC boxes, with which well characterized transcription factors such as nuclear factor-1/CCAT transcription factor and simian virus 40 promoter factor-1 could interact. With regard to the 259-bp fragment (bp -2352 to bp -2094), three protein binding sites within the 259-bp fragment were identified by DNase I footprinting analysis; these sites contained activator protein-1, nuclear factor-E1.7, and one-half hepatic nuclear factor-1 (HNF-1) binding consensus sequences. These results suggested that the 259-bp DNA fragment contained positive regulator binding sites and HNF-1 could contribute to the liver-specific expression of human CYP1A2(Chung and Bresnick, 1995). The protein encoded by the CYP1A2 gene localizes to the endoplasmic reticulum and its expression is induced by polycyclic aromatic hydrocarbons (PAHs). Other xenobiotic substrates for this enzyme include caffeine, aflatoxin B1, and acetaminophen. The transcript from this gene contains four Alu sequences flanked by direct repeats in the 3' untranslated region.

CYP1B1 localizes to the endoplasmic reticulum and metabolizes exogenous and endogenous substrates including such as polycyclic aromatic hydrocarbons and 17β-estradiol. Mutations in this gene have been associated with primary congenital glaucoma; therefore it is thought that the enzyme also metabolizes a signaling molecule involved in eye development, possibly a steroid. Cytochrome P450 (CYP) 1B1 is known to be induced by polycyclic aromatic hydrocarbons including 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). The constitutive and TCDD-inducible transcriptional expression of human CYP1B1 is known to be cell-specific. Constitutive expression increased with the regulatory elements that are present at -910 to -852 and -1652 to -1243. Potential enhancer elements for TCDD-induction were located from -1022 to -852 including three XREs, XRE3 at -853, XRE4 at -940, and XRE5 at -989. Gel shift analyses revealed binding of the AhR/ARNT heterodimer to XRE2 at -834, XRE3 at -853, XRE6 at -1024,

and XRE7 at -1490. In addition, the binding of a nuclear transcriptional factor, Sp1, near XRE2 and XRE8 was observed. It was suggested that mutual interaction of XRE2 and XRE3 is important for transcriptional regulation, and that the Sp1 binding to the Sp1-like motif (-824) enhances both the constitutive and inducible transcriptional activities of the human CYP1B1 gene(Tsuchiya et al., 2003).

1.4 Cytochrome P450 3A4

1.4.1 Enzyme characteristics

CYP3A4 is also a member of the cytochrome P450 superfamily of heme-containing enzymes(Negishi et al., 1996; Nelson et al., 1996; Nelson, 1999; Kanamura and Watanabe, 2000; Danielson, 2002; Nebert and Russell, 2002; Estabrook, 2003). The peptide of CYP3A4 enzyme consists of 503 amini acids (NP_059488) and the sequence of first 21 amino acids is thought to be the membrane localizing signaling peptide (NM_017460.3) (EC 1.14.14.1, EC 1.1.1.161). CYP3A4 protein localizes to the endoplasmic reticulum. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The metabolic pathways catalyzed by CYP3A4 enzymes include hydroxylation, dehydration, epoxidation, C- and N-oxidation, sulfooxidation, N-, S- and O-dealkylations, desulfation, deamination, and reduction of azo, nitro, and N-oxide groups. (Li et al., 1995). CYP3A4 is responsible for the metabolism of a large variety of structurally unrelated xenobiotic and endogenous compounds. These substrates include drugs such as acetaminophen, codeine, cyclosporin A, diazepam and erythromycin. The enzyme also metabolizes bile acids, estrogens, environmental contaminants and procarcinogens (Mehmood et al., 1995; Yamazaki et al., 1995; Tsuchiya et al., 2005).

The CYP3A4 enzyme is the most actively expressed member of CYP3A subfamily which is the most abundant group among the cytochrome P450s in the liver. The CYP3A subfamily and consists at least 4 members, CYP3A4, CYP3A5, CYP3A7 and CYP3A43(de Wildt et al., 1999; Wrighton et al., 2000; Lamba et al., 2002; Stevens et al.,

2003). CYP3A4 dominantly expresses and accounts for approximately 30-40% of the total cytochrome P450 content in both liver and intestinal epithelium (Kolars et al., 1994; Koch et al., 2002; von Richter et al., 2004). CYP3A4 is also expressed at lower levels in other extrahepatic tissues including adrenal gland, kidney, lung, duodenal tissue and prostate(Kivisto et al., 1996; Shimada et al., 1996; Anttila et al., 1997; Mace et al., 1998).

1.4.2 Cytochrome P450 3A4 gene

The CYP3A4 gene is part of a cluster of cytochrome P450 genes on chromosome 7q21.1 (Fig. 3). Previously another CYP3A gene, CYP3A3, was thought to exist; however, it is now believed that this sequence represents a transcript variant of CYP3A4.

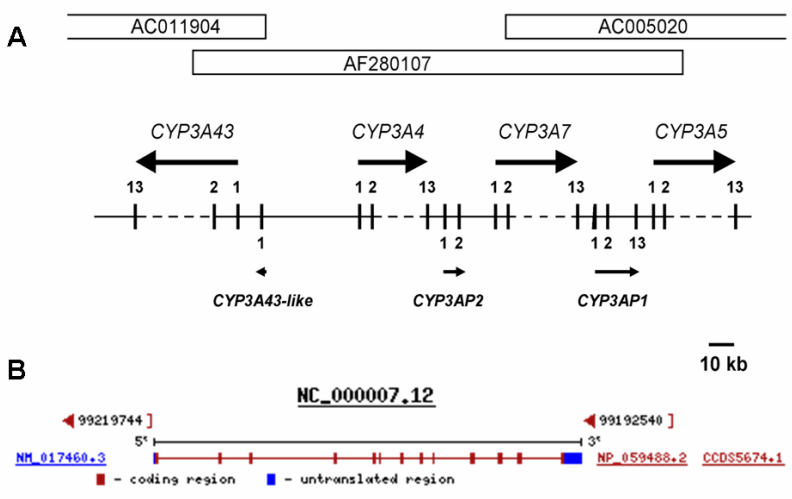


Fig. 3. Schematic structure of the CYP3A locus and CYP3A4 gene. The GenBank™ entries that cover the locus are indicated at the top. Big arrows show direction of CYP3A genes, whereas small arrows denote CYP3A pseudogenes. Note that for the CYP3A genes only exons 1, 2, and 13 are shown (indicated by vertical lines and numbers) ((Finta and Zaphiropoulos, 2000; Finta and Zaphiropoulos, 2002) , GeneBank™.

CYP3A4 gene is a member of the CYP3A locus which is 231 kb long in chromosome 7q21-22. The gene locus sequence contains four CYP3A genes and three

pseudogenes(Gellner et al., 2001). The four known cytochrome P450 3A genes in humans, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, share a high degree of similarity, consist of 13 exons with conserved exon-intron boundaries (Fig. 3). Their intergenic mRNA molecules result from trans-splicing (Finta and Zaphiropoulos, 2000; Finta and Zaphiropoulos, 2002).

1.4.3 Transcriptional regulation gene expression

CYP3A4 gene expression is subjected to regulation by many factors. Many cis-elements in 5'-flanking sequence of CYP3A4 gene have been identified and they are located mainly in four regions (Fig4). They can be bound with diverse transcriptional factors and regulate CYP3A4 gene expression.

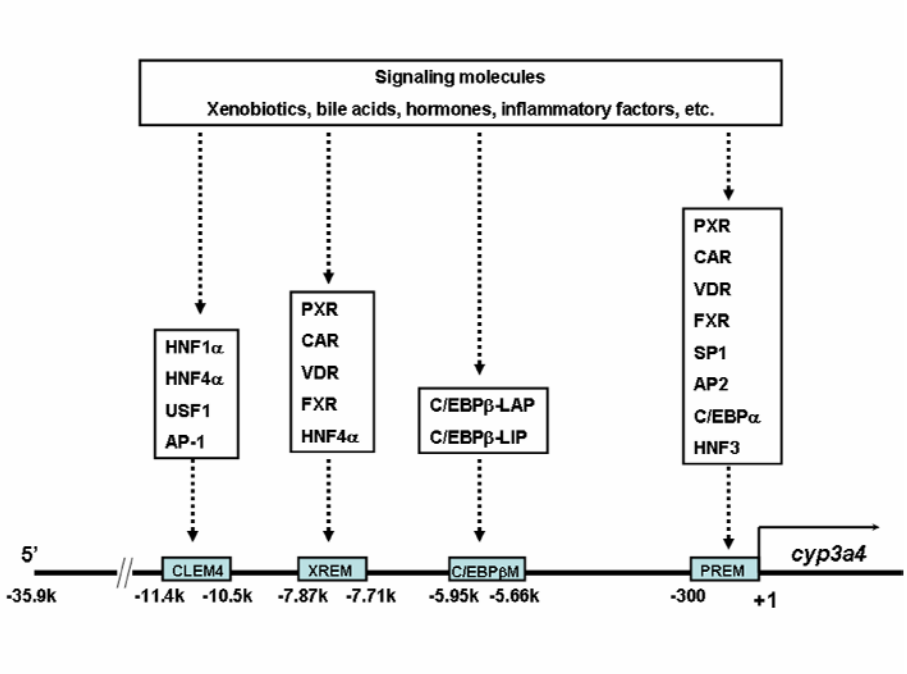


Fig. 4. Factors directly regulate expression of CYP3A4 gene.

In the promoter proximal regulatory element module, there are binding sites for Sp1, AP2, CCAAT/enhancer binding protein (C/EBPα), and hepatic nuclear factor-3 (HNF3), pregnane X receptor (PXR), and HNF3 binding sites, D site-binding protein (DBP), FXR,

VDR (-169 to -152), and CAR (-150bp)(Ourlin et al., 1997; Goodwin et al., 1999; Goodwin et al., 2002; Rodriguez-Antona et al., 2003; Bombail et al., 2004; Gnerre et al., 2004). At -5.95 kb a 288-bp sequence shows maximal response to C/EBPβ-LAP. C/EBPβ-LIP, the truncated form of C/EBPβ-LAP, antagonizes LAP activity and causes gene repression. Site-directed mutagenesis of predicted C/EBPβ binding sites demonstrated the presence of four functional C/EBPβ-responsive motifs within this distal flanking region(Martinez-Jimenez et al., 2005).

From -7.87 kb to -7.60 kb, there are many binding sites for nuclear receptors. -7719- to -7733 (DR3) was identified as functional VDRE (Thompson et al., 2002). HNF4α response element located between positions -7783 and -7771 that confers basal and maximal PXR and CAR-mediated transcriptional activation(Tirona et al., 2003). The region of -7836 to -7607 is responsible for rifampicin-induced CYP3A4 gene induction. Two sites, bases -7738 to -7715 and bases -7698 to -7682, overlapped binding motifs for the orphan human pregnane X receptor (hPXR). There is cooperativity between elements within the distal enhancer region and cis-acting elements in the proximal promoter of CYP3A4. This enhancer module is potent in mediating PXR-activated CYP3A4 gene expression(Goodwin et al., 1999). Region of -7870 to -7720 mediated CAR responsiveness. The human CAR response elements also mediate trans-activation of CYP3A4 by the human pregnane X receptor, suggesting that interplay between these receptors is likely to be an important determinant of CYP3A4 expression(Goodwin et al., 2002). FXR regulates CYP3A4 gene expressionm through a 345-bp element (Gnerre et al., 2004).

The constitutive liver enhancer module of CYP3A4 (CLEM4) is located from -11.4 to -10.5 kb of the CYP3A4 gene. Liver-enriched transcription factors HNF1α and HNF4α, E-box-binding protein USF1, and the Jun family member AP-1 which binds to a cAMP response element region interacted with CLEM4 and were required for the maximal enhancer activity(Matsumura et al., 2004). The CYP3A4 5'-flanking region is 35.8 kb long (Finta and Zaphiropoulos, 2000). There is a region of more than 20 kb upstream to CLEM4 that still needs to be characterized for cis-elements and trans-regulators.

1.4.4 Mediation of CYP3A4 gene expression by PXR

Among many nuclear receptors including PXR, CAR, HNF4α, VDR, GR and FXR which regulate CYP3A4 gene expression, PXR is a key regulator which bridges the signals of environmental and/or physiological molecules and transcriptional response of target genes. PXR is activated by a range of drugs known to induce CYP3A4 expression(Lehmann et al., 1998). Without the mediation of PXR, the induction of CYP3A4 gene expression by xenobiotics is low(Goodwin et al., 1999).PXR is activated by a large number of endogenous compounds. As well as exogenous chemicals(Kliewer et al., 2002). Unlike other CYP3A4-regulating nuclear receptors that interact selectively with their specific ligands, the crystal structures of the PXR ligand binding domain interacting with ligands revealed that PXR has a large, flexible ligand-binding cavity that allows it to interact with a wide range of hydrophobic chemicals(Watkins et al., 2001; Ekins and Schuetz, 2002; Watkins et al., 2002; Watkins et al., 2003b).

1.5 Objectives

The objectives of this research include:

1. To investigate the role of NF-κB in the suppression of CYP3A4 gene expression under inflammatory conditions.

2. To examine the role of the AhR in the regulation of CYP3A4 gene expression.

3. To investigate the ability of seleted xenobiotics to induce CYP3A4 gene expression through PXR.

The primary human hepatocytes were used for the gene expression study model and human heptoma cell line HepG2 was used for the molecular mechanism study model for its consistent and availability. We also used mice as an in vivo model to investigate the drug metabolism gene expression on exposure to xenobiotic mixture. In the studies, quantitative real-time PCR and reporter gene assay were used to determine the gene expression. Techniques including co-immunoprecipitation, GST-pull down, and etc. were used to determine the physical interactions between proteins. Electrophoresis

mobility shift assay and chromatin imunnoprecipitation assay were employed to determine the interaction between nuclear factors and DNA. Other techniques such as small RNA interfering and transient transfection were also used.

1.5.1 Role of NF-κB in the suppression of CYP3A4 gene expression under inflammatory conditions

Inflammatory responses and infections suppress the biotransformation of drugs and decrease the hepatointestinal capacity of drug clearance. This results in alterations of therapeutic indexes and increases the toxicity of certain administered drugs. Inflammatory responses also play important roles in liver pathological conditions such as drug-induced hepatitis and cholestatic diseases (Lehmann et al., 1987; Pirovino et al., 1989). The mechanisms of these clinically important effects have not been well understood. It has been shown that most inflammatory cytokines induced during sepsis and aseptic responses lead to suppression of CYP3A4 gene expression(Abdel-Razzak et al., 1993; Muntane-Relat et al., 1995; Guillen et al., 1998; Jover et al., 2002; Hayney and Muller, 2003; Sunman et al., 2004).

The mechanisms of the suppression of CYP3A4 caused by inflammatory responses and infections have been investigated (Morgan, 1997; Renton, 2004). Several aspects of the transcriptional regulation may be involved including decreases of the PXR and RXR mRNA levels or induction of the C/EBPβ-liver inhibitory protein (LIP) which suppresses CYP3A4 through a distal flanking region (Martinez-Jimenez et al., 2005). It is likely that the modulation of transcriptional activation by several pathways leads to down-regulation of the PXR-regulated gene expression. Jover R, et al (2002) reported that down-regulation of CYP3A4 through translational induction of C/EBPβ-LIP, which competes with and antagonizes constitutive C/EBP transactivators(Jover et al., 2002). Pascussi JM (2000) reported that suppression of CYP3A4 expression resulted from the inhibition of PXR and CAR gene expression(Pascussi et al., 2000b). Beigneux and Feingold have observed that LPS treatment down-regulates the PXR mRNA levels in cells and animals (Beigneux et al., 2002), and this may potentially result in suppression

of CYP3A4 expression. Using PXR and PPARα deficient mice, Richardson and Morgan have shown that endotoxin caused about same levels of suppression of P450 in KO mice as in the wild type, suggesting nuclear receptors PXR and PPARα are not required for regulating the LPS-imposed suppression of the cytochromes P450 including CYP3As (Richardson and Morgan, 2005), at least in the animals whose P450s have not been induced by exogenous agents, suggesting nuclear receptors PXR and PPARα are not required for regulating the LPS-imposed suppression of the cytochromes P450 including CYP3As.

We hypothesize that there may be immediate, early events at transcriptional level where the effects of the proinflammatory responses converge. One of the critical responses to acute infections and inflammations is the activation of NF-κB (Aggarwal, 2004; Karin and Greten, 2005; Xiao and Ghosh, 2005), which has pleiotropic functions and has been shown to down-regulate the transcriptional activity of multiple steroid/nuclear receptors(McKay and Cidlowski, 1999). The NF-κB regulates innate as well as adaptive immune systems. One of the pivotal functions of NF-κB is its swift activation in response to LPS or proinflammatory cytokines, which is an evolutionally conserved defensive mechanism against infections. The classic NF-κB consists of p65 (RelA) and p50 heterodimer and it is activated in response to various stimuli including LPS, TNF-α, dsRNA and UV. We will investigate the role of NF-κB in regulation of the transcriptional activity of PXR/RXRα complex in an attempt to address the mechanism of suppression of the CYP3A4 by LPS and proinflammatory cytokine TNF-α. The preliminary results reveal that NF-κB plays an important role in suppression of the PXR/RXRα-regulated gene expression by interfering with the binding of PXR/RXRα to the regulatory DNA sequences.

1.5.2 Role of aryl hydrocarbon receptor in the regulation of CYP3A4 gene expression

The xenobiotics including environmental chemicals, pharmaceuticals, nutriceuticals, etc are ubiquitous. Human individual is commonly exposed to multiple xenobiotics. Single or multiple xenobiotics may regulate single or multiple gene expression through

single or multiple signaling pathways and modify the physiological or pathological states of the body. Both PXR and AhR are such signaling pathways. They are among major xenosensors, mainly responsing to different spectrum of xenobiotics and transcriptionally regulating different battery of genes, especially drug metabolism enzymes, such as CYP3A and CYP1 gene family, respectively.

Crosstalk between AhR and PXR pathways was reported to take place on the metabolism of omeprazole which is a well-known activator of the aryl hydrocarbon receptor (AhR). When PXR was activated by rifampicin and induced CYP3A4 expression, CYP3A4 enzyme transformed omeprazole into omeprazole-sulphide which is a ligand of AhR, inhibits AhR activation to a DNA-binding form(Gerbal-Chaloin et al., 2006).

To date, there has no report on modulation of PXR transcriptional activity by AhR. Our preliminary experiments showed AhR directly regulated PXR transcriptional activity. We would use methodology of specific ligands, protein-protein interaction and Protein-DNA interaction to investigate the direct regulation of PXR-mediated CYP3A4 gene expression by AhR.

1.5.3 Screening xenobiotics for inducers of CYP3A4 gene expression through PXR

Xenobiotics including drug, dietary supplement, cosmetics, pesticides, pollutants, etc are ubiquitous. The substrate range of CYP3A4 is wide(Li et al., 1995) and the ligand-binding cavity of PXR is promiscuous for xenobiotics(Watkins et al., 2001; Ekins and Schuetz, 2002; Watkins et al., 2002; Watkins et al., 2003b), thus the ubiquitous chemicals might induce CYP3A4 through activating PXR and cause adverse interaction between xenobiotics(Dresser and Bailey, 2002; Mannel, 2004; Pal and Mitra, 2006). The knowledge of the inducibility of CYP3A4 gene expression will help protect individuals from harm caused by adverse xenobiotics interactions. To obtain such knowledge, we will use a cell-based PXR-mediated CYP3A4 gene expression model system to screen xenobiotics for CYP3A4 inducers through PXR.

CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and reagents

Oligonucleotides as the PCR primers, ER6, XRE, κB EMSA probe, the DNA modifying enzymes, and LipofectAMINE were from Invitrogen (Calsbad, CA). DULBECO'S modified Eagle's medium were purchased from Invitrogen or HyClone (Logan, Utah), fetal bovine serum was from Atlanta Biologicals (Lawrenceville, Ga). Plasmid DNA purification kits were purchased from Sigma Chemical (St. Louis, Mo) and Qiagen (Valencia, CA). Rifampicin, lipopolysaccharide, hyperforin, α-naphthaflavone, β-Naphthaflavone, 5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN), resveratrol were from Sigma Chemical. TCDD was a gift from Professor Stephen Safe (Texas A&M University, College Station). Recombinant human TNF-α was obtained from Roche Applied Science (Indianapolis, IN). The human HepG2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA).

2.2 Plasmids

pGL3-3A4-Luc. The reporter plasmid pGL3-3A4-Luc was constructed based on the literature via the following steps(Goodwin et al., 1999). First, the promoter module (-362/+53)-containing DNA fragment was generated by PCR amplification using human genomic DNA as the template with the primers (5-CATTGCTGGCTGAGGTGGTT-3' and 5'-CATAAGCTTTGTTGCTCTTTGCTGGGCTATGTGC-3'). The 1.13 kb PCR product was restricted with Bgl II and Hind III and the resultant 415 bp fragment was cloned into pGL3-basic vector (Promega) to yield pGL 3A4 (-362/+53). The DNA fragment corresponding to the XREM region (-7836 to –7208) (Goodwin et al., 1999) was generated by PCR with the Cyp3A4-3 oligonucleotides primers (5'-GGGGTACCAT -TCTAGAGAGATGGTTCATTCC-3') and Cyp3A4-4 (5' CCGCTCGAGATCTTCGT

CAACAGGTTAAAGGAG-3'), 5' Kpn I site and 3' Bgl II sites were created by restriction digestion. The Kpn I and Bgl II fragment was then inserted into the Kpn I and Bgl II-restricted pGL-3A4 plasmid to yield pGL3-3A4-Luc reporter gene.

pCI–hPXR and Flag-hPXR. The expression vector for hPXR, pCI–hPXR and Flag-hPXR, were generated from the DNA fragment corresponding to the coding region of hPXR (amino acids 1 to 434) was generated by RT-PCR using total RNA from HepG2 cells. For pCI-PXR, the PCR primers were 5-GGGAATTCCCACCAGGAGGTGAGAC CCAAAGAAAGCTGG-3' and 5'-GGGGTCGACGCGGCCGCTCAGCTACCTGTGA -TGCCGAACA-3'; for Flag-PXR, the PCR primers were 5'-ATAAGAATGCGGCCGC CTGGAGGTGAGACCCAAAGA-3', and 5'-CGGGATCCTCAGCTACCTGTGATGC CG-3', were based on the published hPXR sequence(Lehmann et al., 1998). The PCR products was modified with EcoR I and Not I, or Not I and BamH I, and cloned into the pCI-neo vector (Promega) or p3XFLAG-myc -CMV-26 vector (Sigma).

Expression plasmids for hPXR and its domain deletion mutants: The expression plasmids for hPXR and its domain deletion mutants were constructed by subcloning the specific inserts into p3XFLAG-myc-CMV-26 vector (Sigma) with EcoRI/BamHI sites for overexpression and reporter gene assay, and subcloning into pGEX-5X-3 (Amersham Bioscience, GE Healthcare) with EcoRI/XhoI sites for preparing and purifying GST-PXR fusion proteins.

Expression plasmids for hRXRα and its domain deletion mutants: The expression plasmids for hRXRα and its domain deletion mutants were constructed by subcloning the specific inserts into pGEX-5X-3 (Amersham Bioscience, GE Healthcare) with EcoRI/XhoI sites for preparing and purifying GST-RXRα fusion proteins.

Expression plasmids for hAhR and its domain deletion mutants: The expression plasmids for hAhR and its domain deletion mutants were cloned into p3XFLAG-myc-CMV-26 vector (Sigma) with HindIII/BglII sites for overexpression and reporter gene assays.

The information on the domain boundaries of PXR, RXRα and AhR has been described(Wu et al., 2006).

The expression plasmids for human p65 (1-551aa) and p50 (1-433aa) were subcloned into pcDNA3.1/HisA (Invitrogen).

The reporter plasmid pGUD-luc 6.1 was from Professor Mike Denison (Davis, CA).

2.3 Cell culture

Cell suspension of primary human hepatocytes was purchased from Cambrex BioScience (Walkersville, MD). Two 12-well plate of human primary hepatocytes came from two donors. The donor of the human hepatocytes for the study described in 3.1 was a 26 yr old male without heart disease or hypertension. Serological tests were negative for HIV-1/2, HBsAg, HCV, HTLV-1/II and RPR/Syphilis.

Upon arrival the cells were resuspended in DMEM containing 5% FBS , antibiotics, 4 μg/ml insulin, and 1 μmol/L dexamethasone and plated in collagen-coated plate, then maintained in WEM containing ITS+ , 0.1 μmol/L dexamethasone and antibiotics overnight. Cells were then treated with DMSO, RIF, RIF+LPS and RIF+TNF-α. Twenty-four hours after the treatment, cells were harvested, total RNA was then isolated and mRNA expression was determined by real time RT-PCR.

HepG2 and Hep1c1c7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) in 5% CO_2 at 37 °C.

2.4 Experiments on mice

The dosing regimen in this study was similar to that described by Maher and coworkers(Maher et al., 2005) with modification. Eight-week old male mice (25 ± 2 g) were divided into 6 groups with 6 mice in each group. Mice were administered with chemicals in 0.5 ml corn oil (Sigma) intraperitoneal injection as indicated in Table 8 for 4 consecutive days at 2:00pm. Livers were collected and flash frozen in liquid nitrogen and stored -80 °C.

Table 8 Dosing regimen for intraperitoneal administration

No.	Compound	Dosing Regimen
1	DMSO	50 µl/day (25-30g/per capita)
2	PCN	50 mg/kg CO i.p. per capita/day
3	TCDD	10 µg/kg CO i.p. per capita/day
4	PCN+TCDD	50 mg/kg CO i.p. per capita/day
		10 µg/kg CO i.p. per capita/day
5	β–NF	50 mg/kg CO i.p. per capita/day
6	β–NF+ PCN	10 µg/kg CO i.p. per capita/day
		50 mg/kg CO i.p. per capita/day

Eight-week old male C57BL/6 mice (25 ± 2 g) were divided into 4 groups with 5 mice in each group. The groups of mice were administered with chemicals (Table 8) in 0.25 ml corn oil (Sigma) via intraperitoneal (ip) injection. Treatments 1-4 were administrered at 2:00pm each day for 4 consecutive days. Livers were collected and weighed, then flash frozened in liquid nitrogen and store at -80 °C.

2.5 Transient transfection and stabilization of transfectants in mammalian cells

Transient transfection: HepG2 or Hep1c1c7 cells were seeded in 12-well plates at approximately 40% confluence and after 24 hr cells were transfected using LipofectAMINE (Invitrogen) according to the protocol provided by Invitrogen. After 6 hr transfected cells were treated with chemicals for 24 or 48 hr and cells were then collected and assayed for luciferase activity.

Stably-transfected cells: After transient transfection with 3xFlag-hPXR or –hAhR into HepG2 or Cos-7 cells in 6-well plates, media were added and cells were incubated for 24 hr. Cells were transferred to 10-cm plates and incubated for 24-48 hr, then digested with trypsin to make cells suspending and selected (neomycin 1mg/ml) for 7 days. The resistant cells were incubated with non-selecting media for 3-4 days, then incubated with selecting media for an additional 2-3 wk. Colonies were tested for expression and function of target genes using Western blot or immunocytochemistry and reporter gene assays.

2.6 Immunocytochemistry

Primary human hepatocytes was cultured in 24-well plates and treated with 5 μg/ml LPS or 2 ng/ml TNF-α for 1 hr. Cells were washed with cold PBS(3X), then fixed with fresh 4% formaldehyde in PBS for 10 min at room temperature. After washing with PBS(3X), cells were permeablized with 0.2% Triton X-100 for 10 min at room temperature. After again washing with PBS for 5 min (3X), cells were blocked with 5% bovine serum albumin in PBS/Tween20 for 1 hr at room temperature. Then primary antibody against p65 (Santa Cruz, sc-109X) diluted (1:500) in PBS/Tween20 was added and the reaction was incubated at room temperature for 1 h. After washing with PBS/Tween20 for 10 min(3X), secondary antibody conjugated with Alexa Fluo-568 (Molecular Probe, A11011) diluted in PBS/Tween20 (1:1000) was added and incubated for 1 hr at room temperature. The cells were washed with PBS/Tween20 for 10 min(3X) and DAPI (Vector Laboratories, Inc. Burlingame, CA) was added to stain the cells. The images were visualized and representative views of the cells were recorded by fluorescence microscopy with an Olympus IX71 microscope.

2.7 Reporter gene assays

After cells were treated with the desired chemicals the cells were then harvested to determine the activity using the luciferase Assay System (Promega). Results are expressed as means±SD or SE for at least 3 replicated experiments for each treatment group.

2.8 Western blot analysis

Western blot analysis was performed on whole cell lysates from HepG2 cells. Cell lysates were prepared in 2xSDS loading buffer and heated at 95ºC for 5-10 min. Then centrifuged for 5 min at 16000 g. Proteins were resolved by electrophoresis in a denaturing SDS-PAGE 10% polyacrylamide and transferred to nitrocellulose (Bio Rad). Protein blots were blocked overnight at room temperature in 5% low-fat skim milk in

TBST buffer (Tris-buffered saline with 0.1% Tween 20). Immunoblot analysis was perform using primary antibodies (1ug:10000ul TBST) to the target proteins and the corresponding secondary antibodies (1 ul:10000ul TBST). Detection was performed using the chemiluminescence method (Perkin Elmer) or alkaline phosphatase substrate depending on the type of the conjugate on the secondary antibodies.

2.9 GST pull-down

For studies on the physical interaction between RXRα and p65(Section 3.1), the GST-pulldown assay was used essentially as described (Tian et al., 2003). [^{35}S] -labeled full-length p65 protein was generated with a TNT-coupled Reticulocyte Lysate System (Promega) using the T7 promoter-driven cDNA plasmid as the template. PCR-generated cDNA fragments of RXRα corresponding to the domains of RXRα were inserted into pGEX-5X-3, yielding the expression plasmids for GST-RXRα fusion peptides. The plasmids were expressed in *E. coli* (BL21), and fusion polypeptides were purified with the Glutathione-Sepharose 4B affinity matrix (Amersham Biosciences) according to the manufacturer's instructions. Ten micrograms of each fusion polypeptides (estimated by comparison with BSA in an SDS-PAGE gel with Coomassie staining) was incubated with 10 μl of radiolabeled p65 in a total of 250 μl binding reaction buffer (20 mM Hepes (pH 7.9), 1% Triton X-100, 20 mM DTT, 0.5% bovine serum albumin, and 100 mM KCl) for 2 h at 4°C. After incubation, the beads were washed five times with the same buffer lacking BSA. The bound proteins were eluted by boiling in the SDS-PAGE sample buffer, and resolved by 8% SDS-PAGE gel electrophoresis. The signals were detected by autoradiography.

For the studies on the physical interactions between PXR and AhR, RXRα and AhR (Section 3.2), the procedure was modified. [^{35}S]-labeled full-length human AhR and its domain deletion mutants were generated with a TNT-coupled Reticulocyte Lysate System (Promega) using the T7 promoter-driven PCR products as the templates. Ethidium bromide (200 μg/ml) was added to the binding buffer as previously described (Lai and Herr, 1992). The serial washing buffers contained 20 mM Hepes (pH 7.9), 10%

glycerol, 200 mM KCl , 1% Triton X-100 (or 1% NP40 wash twice, 0.2% NP40 or none), 3 mM EDTA, 10 mM DTT, 2 mM PMSF, ethidium bromide 200 μg/ml (first wash step).

2.10 Co-immunoprecipitation

The coimmunoprecipitation assays were based on a published procedure with some modifications (Tian et al., 1999; Tian et al., 2003). HepG2 stably expressing 3XFlag-PXR were treated with DMSO (control), rifampicin (5 μM) and TCDD (10 nM) for 4 hr, and cells were washed with ice-cold phosphate-buffered saline (2X) and collected. Co-IP cell lysis buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 2 mM Na_3VO_4, 50 mM NaF, 20 mM $ZnCl_2$, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mm DTT, 5 μg/ml leupeptin) was added and incubate at 0°C for 10-15 min. After centrifugation for 15 min at 12, 000 x *g*, supernatant fractions were collected and incubated with Staph A cells for 10 min. After centrifugation for 3-5 min at 12000 g, the supernatant was incubated with G-plus Sepharose beads for 1 hr at 4°C. An aliquot of the supernatant was centrifuged for 3 min at 5000 g and proper antibody pre-bound to 30 μl of G-plus Sepharose beads were added and incubated with gentle rocking at 4 °C for 2 hr on a rotary shaker. The beads were washed in lysis buffer (3X) and then boiled in 2x SDS sample buffer. The proteins were separated by 8% SDS-polyacrylamide gel. Proteins on the gel were transferred to nitrocellulose membranes (BioRad) and the membranes were blocked with 5% bovine serum albumin in TBST buffer (20 mM Tris-HCL, pH 7.6, 137 mM NaCl, 2.68 mM KCl, 0.05% Tween 20), and incubated with appropriate primary antibodies at 37 °C for 60 min. Blots were washed with TBST (3X), then incubated with a 1:10000 dilution of immunoaffinity-purified goat anti-rabbit IgG linked to alkaline phosphatase. Blots were washed with TBST (3X) and subsequently developed using Western Blue substrate (Promega).

2.11 Small interfering RNA

Small interfering RNAs were synthesized using an siRNA Construction Kit (Ambion,

Austin) using the sequences summarized in Table 9.

Table 9 Oligonucleotide sequences for small interfering RNAs

Oligos	Sequences
hAhR-R1/R2	AAATCCTTCCAAGCGGCATAGCCTGTCTC
	AACTATGCCGCTTGGAAGGATCCTGTCTC
hAhR-R3/R4	AAGGAGAATTCTTATTACAGGCCTGTCTC
	AACCTGTAATAAGAATTCTCCCCTGTCTC
hAhR-R5/R6	AAGACATCAGACACATGCAGACCTGTCTC
	AATCTGCATGTGTCTGATGTCCCTGTCTC
hAhR-R7/R8	AATGTCTTTACAGACTTACATCCTGTCTC
	AAATGTAAGTCTGTAAAGACACCTGTCTC
mh-NGC-R1/R2	AATACTACGAGCTCGATACGCCCTGTCTC
	AAGCGTATCGAGCTCGTAGTACCTGTCTC
S-GL2-R1/R2*	AACGTACGCGGAATACTTCGACCTGTCTC
	AATCGAAGTATTCCGCGTACGCCTGTCTC
S-AhR-R1/R2*	AATACTTCCACCTCAGTTGGCCCTGTCTC
	AAGCCAACTGAGGTGGAAGTACCTGTCTC
S-ARNT-R1/R2*	AACCATCTTACGCATGGCAGTCCTGTCTC
	AAACTGCCATGCGTAAGATGGCCTGTCTC

* The sequences were described previously(Abdelrahim et al., 2003).

2.12 Real-time PCR

For qPCR, total RNA samples were reverse-transcribed by using MMLV-reverse transcriptase (Invitrogen) and the cDNA samples were used for quantification by PCR. Amplifications were performed in the ABI Prism 7900HT (Applied Biosystems) by using SYBR Green Master Mix (Applied Biosystems). The PCR primers used are summarized in Table 10.

2.13 Microarray assay

Six CodeLink Human Whole Genome chips were used in this study. Total RNA was extracted from primary human hepatocytes treated with DMSO, RIF (10μM), RIF (10μM)

+TNF-α (2ng), RIF (10μM)+LPS (5μg), TNF-α (2ng), LPS (5μg)for 24 hr using RNAeasy Kit (Qiagen). The RNA quality testing, hybridization and data acquisition were performed in The CERH Genomic Facility Core (Texas A&M University). Data clustering was performed by the CERH Biostatistics and Bioinformatics Facility Core (Texas A&M University).

Table 10 Primer pairs for quantative real-time PCR

Target	Forward	Reverse
hPXR*	GGCCACTGGCTATCACTTCAA	TTCATGGCCCTCCTGAAAA
hRXRα*	TCAATGGCGTCCTCAAGGTC	TTGCCTGAGGAGCGGTCC
hCYP3A4**	CCACAAAGCTCTGTCCGATCT	GAACACTGCTCGTGGTTTCACA
hCYP1A1***	CAAATGCAGCTGCGCTCTT	CCCAACCAGACCAGGTAGACA
h β-actin**	CCATCGAGCACGGCATC	ATTGTAGAAGGTGTGGTGCCAGA

* primer pairs were previously described(Nishimura et al., 2004);

** primer pairs were previously described (Gu et al., 2006);

*** primer pair were previously described (McFadyen et al., 2003).

2.14 EMSA assay

Human PXR and human RXRα polypeptides were generated by in vitro transcription coupled to translation using TNT coupled Reticulocyte Lysate System (Promega, Madison, I). Oligonucleotides used for EMSA were the ER-6 consensus sequences in *CYP3A4* promoter region as described (Xie et al., 2000) taTGAACTcaaaggAGGTCAgt with 5' overhang gg. The double-stranded oligonucleotide was labeled with [α-^{32}P] dCTP using Klenow enzyme (USB, Cleveland, Ohio). For EMSA assays, PXR and RXRα, recombinant p50 (Progema) and p65 (produced by baculoviral expressions) in various combinations were incubated for 30 min in a reaction mixture containing 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 20 mM Hepes, pH 7.9, and 4% Ficoll (400 K) and ~30000 cpm of radiolabeled double-stranded oligonucleotide probe. After incubation for 30 min, at room temperature, the reaction mixtures were separated by electrophoresis in 4.5% nondenaturing polyacrylamide gel using ¼ TBE buffer. The results were recorded by autoradiography.

2.15 Chromatin immunoprecipitation assay

The ChIP assay was based on a published procedure with modifications(Tian et al., 2003). HepG2 cells were transfected with 3XFlag-tagged PXR and pGL3-3A4-Luc and were maintained in 10-cm plates under standard cell culture conditions. At 95% confluence formaldehyde was added directly to tissue culture media to a final concentration of 1% for cross-linking, and the plates were incubated for 15 min at room temperature on a rocker. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The plates were incubated at room temperature for 5 min. The plates were then rinsed with ice-cold PBS (2X). The cells were scraped off the plates and collected into 50-ml conical tubes by centrifugation (600 g for 5 min at 4 °C), and the pellet was washed with phosphate-buffered saline containing 1 mM PMSF and resuspended in 2 ml of cell lysis buffer (5 mM PIPES, (pH 8), 1 mM EDTA, 0.5 mM EGTA, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM DTT, and 5 µg/ml each of leupeptin and aprotinin), and incubated for on ice for 10 min. The cells were homogenized on ice using a B type pestle by douncing 200 times to aid the release of nuclei. The nuclei were collected by centrifugation (5000 x g for 10 min at 4 °C) and then resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 0.5 mM EGTA, 1% SDS, 1 mM PMSF, 1 mM DTT, 5 µg/ml each of leupeptin and aprotinin) and incubated again on ice for 10 min. The samples were sonicated into DNA fragments of 0.5–1.5 kbp (checked by agarose gel electrophoresis/ethidium bromide staining) and microcentrifuged at 16000 x g for 10 min at 4 °C. The supernatant was cleared by incubation with Staph A cells (2.5 µg/per sample, Roche Applied Science) for 15 min and AG beads for 30-60 min sequentially at 4 °C on a rotating platform. The supernate was aliquoted after centrifugation at 12, 000 x g for 5 min to the clean tubes. Appropriate antibodies (1 µg each) were added to the aliquots and then 25 µl of precleared 50% protein A/G beads (Amersham Biosciences) was added. The final volume of each sample was adjusted to no more than 500 µl with the same amount of IP dilution buffer (0.01% SDS, 1.1% Trition X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 167 mM NaCl, 100

µg/ml sonicated salmon sperm DNA) as the nuclei lysis buffer. The mixtures were incubated on the rotating platform at 4 °C, overnight. After incubation and collecting the beads by centrifugation at 5000 g for 1 min in a microcentrifuge, and pellets were washed with 1 ml of 1x Dialysis buffer (2 mM EDTA; 50 mM Tris-Cl, pH 8.0;) with 100 µg/ml sonicated salmon sperm DNA, with 1x dialysis buffer (2X) and with 1 ml of IP Wash buffer (100 mM Tris-Cl, pH 9.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) (3X) for 10 min. After washing, 200 µl of PK digestion buffer (50 mM Tris, pH8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 mg/ml proteinase K) was added to each sample, and the reaction mixture was incubated at 55 ^{o}C for 3 hr and then at 65 ^{o}C for 6 hr to reverse the cross-linking. The sample was extracted once with phenol-chloroform-isoamyl alcohol and precipitated with ethanol in the presence of 20 µg of glycogen overnight. The precipitated pellets were collected by centrifugation at 14000×g in a microcentrifuge and the pellets were resuspended in 20 µl of TE buffer. Aliquots from each tube were amplified by PCR and PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR primer pairs were 5'-TTGGACTCCCCAGTAACATTG-3' and 5'-TGCATGGAGCTTTCCTGC-3', for amplifying the *CYP3A4* promoter region, and 5'-ACTCATGTCCCAATTAAAGGTC-3' and 5'-TGTTCTTGTCAGAAGTTCAGC-3', amplifying the enhancer module.

2.16 Statistics

Discriptive statistics such as mean, standard deviation, standard error, and analytic statistics such as student t test were performed.

CHAPTER III
RESULTS

3.1 Role of NF-κB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P450 3A4 by proinflammatory agents*

3.1.1 Suppression of PXR-mediated gene activation by LPS and TNF-α in human liver cells

The effects of LPS and TNF-α on the expression of PXR, RXRα and CYP3A4 were investigated in primary human hepatocytes by quantitative real time PCR. Treatment of hepatocytes with the prototypical human PXR agonist RIF resulted in a 34-fold increase in CYP3A4 mRNA, and the RIF-induced CYP3A4 mRNA levels were suppressed by more than 50% and 90% after co-treatment with either TNF-α (2 ng/ml, 24 hr) or LPS (5 μg/ml, 24 hr), respectively (Fig. 5A). In contrast, PXR mRNA levels were unchanged by TNF-α treatment, and there was approximately a 30% decrease in hPXR mRNA in LPS treated samples (Fig. 5B). RXRα mRNA levels were not significantly changed after treatments with either LPS or TNF-α alone (Fig. 5C). Activation of NF-κB by LPS or TNF-α was confirmed by immunocytochemistry for p65 nuclear translocation (Fig. 5D). The RNA samples were also analyzed by microarray profiling and the results were consistent with those obtained by quantitative PCR, with respect to the changes of PXR, RXRα levels and the suppression of *CYP3A4* by LPS or TNF-α (data not shown).

To further investigate the effects of proinflammatory agents on the transcriptional activity of PXR and to avoid the donor variability in PXR-regulated genes in the human primary hepatocytes, we constructed the luciferase reporter gene driven by PXR responsive enhancer modules for determining the PXR-regulated gene expression in a

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human hepatoma cell line (HepG2) (Fig. 6A) (Goodwin et al., 1999). HepG2 cells were transiently co-transfected with pGL3-3A4-Luc and hPXR expression plasmids pCI-hPXR. The transfected cells were then co-treated with RIF and LPS or RIF and TNF-α.

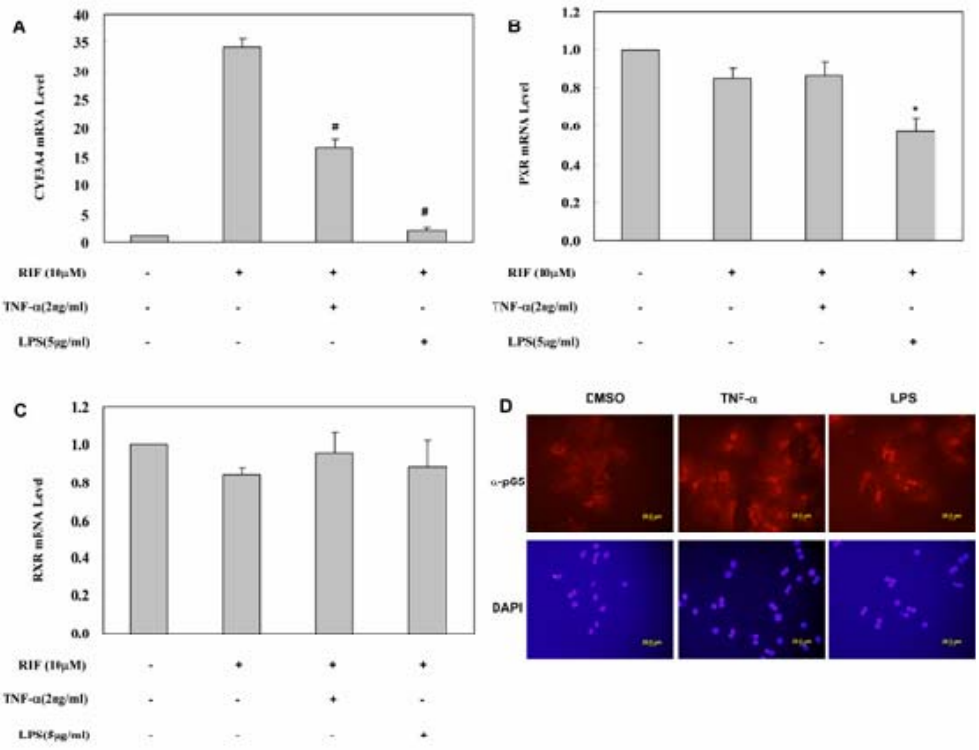

Fig. 5. The effects of LPS and TNF-α on RIF-induced CYP3A4, and PXR and RXRα mRNA levels in primary human hepatocytes. Primary human hepatocytes were treated with either RIF or cotreated with LPS (5 μg/ml) or TNF-α (2 ng/ml) for 24 hr. The total RNA was isolated and relative mRNA levels of CYP3A4, PXR, and RXRα were quantified by real time RT-PCR. Changes of CYP3A4, PXR and RXRα mRNA were normalized with β-actin (housekeeping gene) and presented in (A), (B), and (C), respectively. # and *, statistically significant difference ($p<0.01$ and $p<0.05$, respectively) compared with RIF treatment. The data are given as means ± S.D. of three independent real-time PCR experiments. (D) Nuclear translocation of p65 indicated activation of NF-κB as determined by immunocytochemical staining with an antibody against p65.

TNF-α and LPS caused significant suppression of the induced luciferase activity (Fig. 6B), and this is consistent with results obtained from the primary human hepatocytes

(Fig. 5). These data also confirmed the utility of the HepG2 cell culture model for analysis of PXR-regulated transcription.

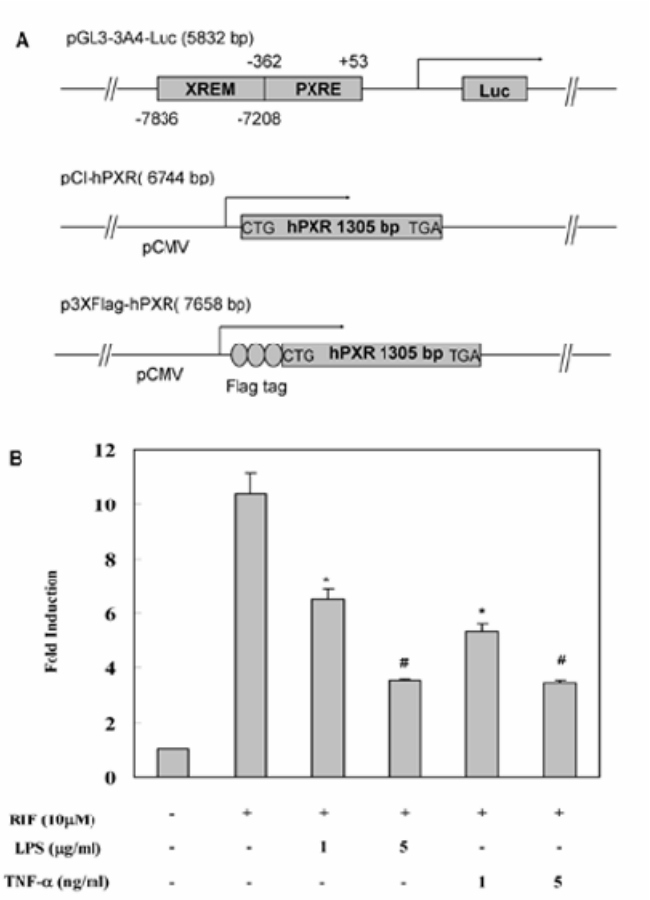

Fig. 6. Suppression of PXR-mediated CYP3A4 reporter gene expression by LPS and TNF-α. (A) Luciferase reporter gene pGL3-3A4-Luc and human PXR expression plasmids used for analysis of PXR-regulated gene expression. The pGL3-3A4-Luc contained the distal upstream enhancer module XREM and was constructed as previously described (Goodwin et al., 1999). (B) Suppression of PXR-mediated pGL3-3A4-Luc luciferase reporter gene activity by TNF-α and LPS. HepG2 cells were transiently co-transfected with pCI-hPXR and pGL3-3A4-Luc reporter plasmids, and after 6 hr, the transfected cells were co-treated with either RIF + TNF-α or RIF + LPS for 24 hr, respectively. The luciferase activity was assayed 48 hr after the treatments. # and *, statistically significant differences ($p<0.01$ and $p<0.05$, respectively) compared with RIF treatment are indicated. All data are given as means ± S.D. of three independent experiments.

3.1.2 NF-κB plays a critical role in down-regulation of CYP3A4 gene expression by inflammatory mediators

NF-κB is an immediate early gene, which is activated in response to infection, and various stress stimuli. NF-κB plays a pivotal role in mediating the pathological effects of TNF-α and LPS. It has been demonstrated that NF-κB regulates several nuclear/steroid hormone receptors through physical and function interactions, resulting in transrepression of the genes regulated by these receptors (refs). To test the role of NF-κB in mediating the suppression of PXR-dependent transcriptional activity, we first transiently co-transfected NF-κB p65 with PXR-driven luciferase reporter gene in HepG2 cells. Co-expression of NF-κB p65 potently suppressed PXR-driven luciferase reporter gene activity, suggesting a role for NF-κB in mediating the suppressive response (Fig. 7). To further demonstrate that NF-κB is specifically involved in the suppression of *CYP3A4* expression, we co-expressed the NF-κB super repressor, SRIκBα, in transient transfection assays and analyzed the the effects of TNF-α and LPS on NF-κB-dependent inhibitory effects in the treated cells. SRIκBα is a mutant of IκBα with a serine to alanine mutation at residues 32 and 36. These mutations render IκBα risitant to phosphorylation at serines 32 and 36, and to degradation by the proteosome pathway, thus causing constitutive inhibition of NF-κB. In transient transfection assays, HepG2 cells were co-transfected with plasmids that express PXR, pGL3-3A4-Luc reporter genes and increasing amounts of SRIκBα expression plasmid. As expected, activation of NF-κB by either TNF-α or LPS caused suppression of the reporter gene activity. However, LPS or TNF-α induced suppression of pGL3-3A4-Luc was reversed by coexpression of SRIκBα (Fig. 7), indicating that NF-κB activation is directly responsible for the suppression of the PXR-regulated gene expression.

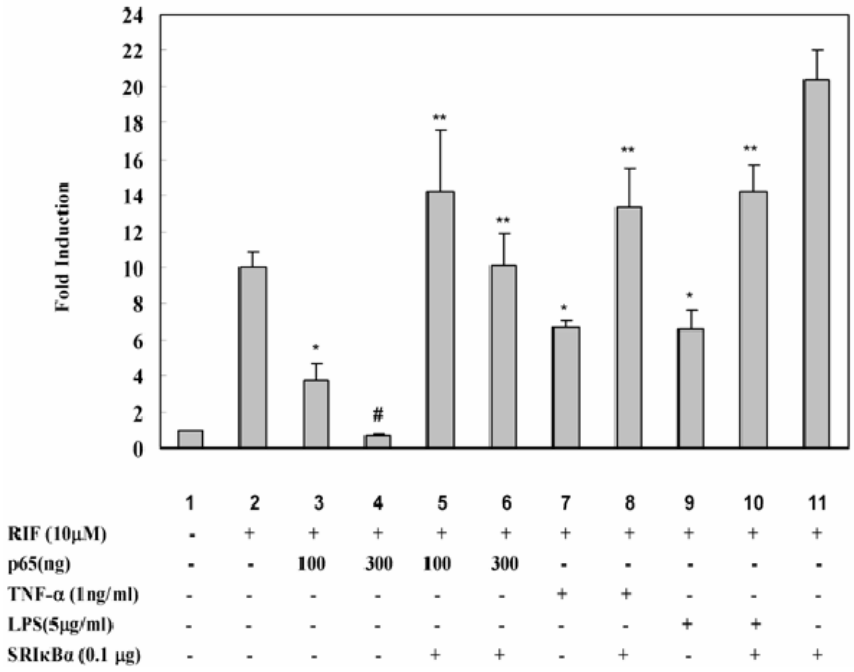

Fig. 7. NF-κB activation is responsible for the suppression of PXR-regulated gene expression by inflammatory agents. HepG2 cells were seeded in 12 well plates, and the cells were transiently co-transfected with plasmids of pCI-hPXR (μg), pGL3-3A4-Luc (μg). The transcriptional activity of PXR was either suppressed by co-expression with p65 or treated with LPS or TNF-α. HepG2 cells were co-transfected with SRIκBα as indicated. The cells were harvested 48 hr after the TNF-α or LPS treatment for determination of luciferase activity. # and*, statistically significant difference ($p<0.01$ and $p<0.05$, respectively) compared with RIF treatment; **, statistically significant difference ($p<0.05$) compared with corresponding treatment without transfection of SRIκBα . All data are given as means ± S.D. of three independent experiments.

3.1.3 NF-κB regulates PXR transcriptional activity by disrupting the association between PXR/RXRα complex and DNA regulatory sequences

It has been shown that NF-κB regulates the transcriptional activity of steroid hormone receptors through direct protein-protein interaction. Na *et al*. reported that NF-κB directly interacted with RXR (Na et al., 1999). The association of NF-κB with nuclear receptors may potentially have a functional impact on the transcriptional activity of the PXR/RXR complex. One possible effect is that the binding of p65 with RXRα may

interfere with the formation of the enhancersome consisting of the PXR/RXR complex and consensus promoter DNA sequences. To test this hypothesis, we performed electrophoretic mobility shift assay (EMSA). PXR and RXRα proteins were generated through in vitro transcription coupled to translation. PXR and RXRα individually did not bind the ER6 probe, however, a combination of both proteins bound the radiolabeled ER6 probe (Fig. 4, compare lanes 2, 3 with lanes 5 and 6). Addition of the recombinant p65 protein, disrupted the PXR/RXRα binding to the consensus ER6 sequence (Fig. 8, compare lane 5 with lanes 10 and 11). Interestingly, disruption of DNA binding by p65 could be reversed upon addition of p50 protein which is a cognate p65 partner and is known to negatively regulate p65 activity (Fig. 8, compare lane 10 with lane 12). As expected, addition of BSA had no effects (compare lanes 5 with 7) on retarded band formation, suggesting the p65 disrupted PXR/RXRα complex binding to DNA in this assay.

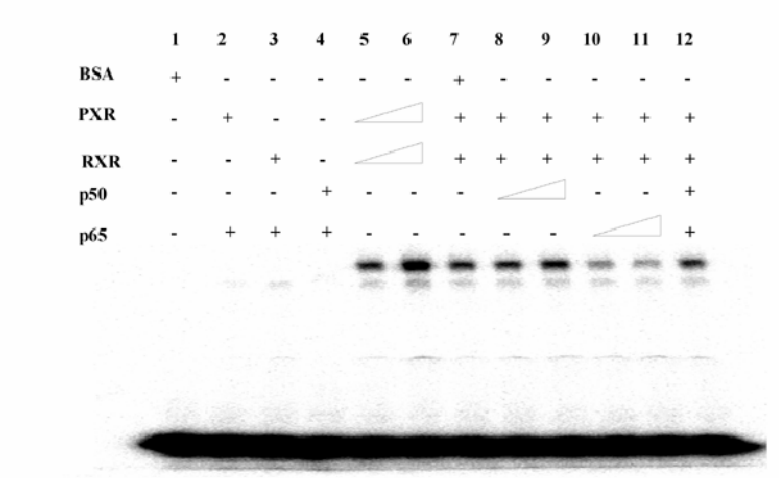

Fig. 8. The effects of NF-κB proteins on the association of PXR/RXRα complex with the consensus DNA sequence determined by EMSA. PXR and RXRα proteins were generated by in vitro transcription coupled with translation and 1 µl each (lane 5) or 2 µl each (lane 6) of the translated products was incubated with 30000 cpm radiolabeled consensus DNA sequence (ER6). The effects of p65 on the PXR/RXRα complex were tested by co-incubation of PXR/RXR complex with increasing amount of recombinant p65 (1 and 3 µl, containing approximately 5 ng/µl) for 30 min (lanes 10 and 11). The disruptive effects of p65 as indicated in lane10 could be reversed by co-incubation with 1 µl p50 (Promega CAT # E3770) (lane 12). BSA was used as the negative control (lanes 7 and 8). The protein-DNA complexes were separated by non-denaturing 4% polyacrylamide gel electrophoresis and results were determined by autoradiography.

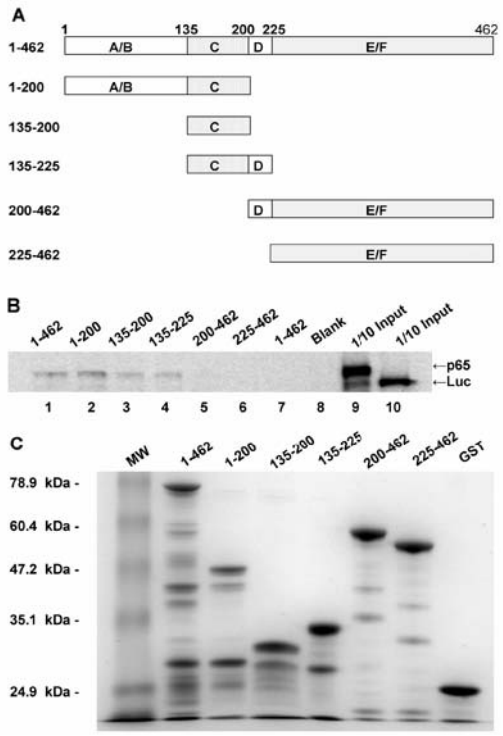

Fig. 9. NF-κB p65 interacts directly with the RXRα DNA binding domain. A. schematic illustration of the domains of RXRα (Bairoch et al., 2005) and deletion mutants used for GST pulldown assays. B. GST pull-down analysis of the domains of RXRα that interact with p65. In lanes 1-6 the radiolabeled p65 was incubated with various recombinant GST-RXRα fusion peptides as indicated in A. Lanes 9 and 10 are the input controls with 1/10 of the radiolabeled p65 and luciferase, respectively. Lane 7 was the full length GST-RXRα pull-downed luciferase (negative control). After washing the p65 associated with the GST-RXRα fusion peptides was separated by SDS-PAGE and radioactivity was detected by autoradiography.

The results of EMSA are consistent with the hypothesis that association between RXRα and p65 prevents RXRα binding to DNA sequences. To further analyze the interaction between RXRα and p65, we mapped the domains of RXRα responsible for association with p65 using a GST-pulldown assay. The known functional modular domains were fused with GST in various combinations (Fig. 9A) and p65 was radiolabeled by in vitro transcription coupled translation in the presence of [^{35}S]-methionine. Interestingly, the DNA binding domain (domain C, amino acid 135-200) is

critical for mediating RXRα-p65 interactions, since domain C and fusion peptides that contain domain C all interacted with the radiolabeled p65, whereas fragments that do not contain domain C did not associate with p65. The p65 association with the RXRα DNA binding domain may generate steric hindrance, that inhibits RXRα binding to DNA, thus resulting in inhibition of gene expression.

To further test this hypothesis in vivo, we performed a ChIP assay on HepG2 cells transfected with Flag-tagged PXR. LPS and TNF-α treatments as well as transient co-expression of p65 significantly decreased the association of RXRα with the regulatory regions of the *CYP3A4* supporting the hypothesis that NF-κB interfered with binding of the PXR/RXRα complex to *CYP3A4* regulatory sequences, thereby inhibiting the PXR-dependent gene expression (Fig. 10).

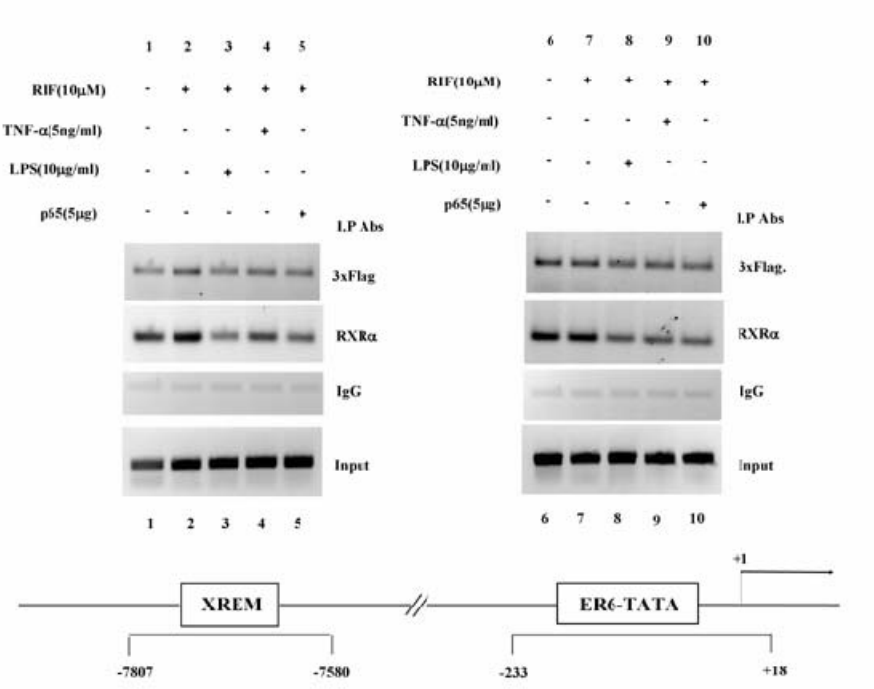

Fig. 10. Effects of NF-κB activation on the associations of PXR and RXRα with the regulatory regions of *CYP3A4* determined by the chromatin immunoprecipitation (ChIP) assay. HepG2 cells were co-tansfected with 3xFlag-tagged hPXR (Fig. 5A) and pGL3-3A4-Luc. NF-κB was activated by either cotransfection with p65 (0.5 μg/ml) or treatment of the cells with LPS or TNF-α. The cells were cross-linked with formaldehyde and association of Flag-tagged PXR, RXRα with DNA sequences were determined by ChIP assay. The regions of PCR amplification were indicated in the lower panel.

3.1.4 Other PXR-regulated genes suppressed by proinflammatory agents

We also showed that TNF-α and LPS inhibited expression of on the other PXR-mediated genes in primary human hepatocytes using a microarray assay and confirmation of induced mRNAs was determined by quantitative real-time PCR. TNF-α and LPS both suppressed basal and PXR-induced gene expression of MDR1, CYP2B6, CYP2C8, CYP2C9 and EPHX1 (Table 11). Results suggested that

Table 11 PXR-induced genes are suppressed by proinflammatory agents (fold induction)

Genes	DMSO	RIF	RIF +TNF-α	RIF +LPS	TNF-α	LPS
MDR1	1.00	2.70	2.38	1.99	0.94	0.77
CYP2B6	1.00	6.73	5.81	1.54	0.46	0.40
CYP2C8	1.00	2.30	1.57	0.13	0.11	0.02
CYP2C9	1.00	3.22	2.84	0.74	0.42	0.28
EPHX1	1.00	2.38	1.55	0.49	0.73	0.40

3.1.5 Role of PXR in regulation of NF-κB transcriptional activity

Using a p65-activated NF-κB responsive pHIV-Luc reporter gene, HepG2 cells were cotransfected with PXR and treated with the PXR ligand rifampicin. We observed that PXR suppressed NF-κB p65 transcriptional activity in a ligand-dependent manner (Fig. 11).

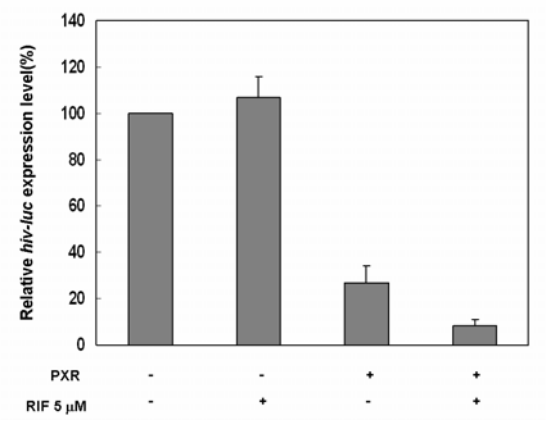

Fig. 11. PXR regulated NF-κB transcriptional activity

We further detected physical interactions between PXR and p65 using GST pull-down assay. Result of this assay showed that C, D and E/F domain interacted with p65 (Fig. 12).

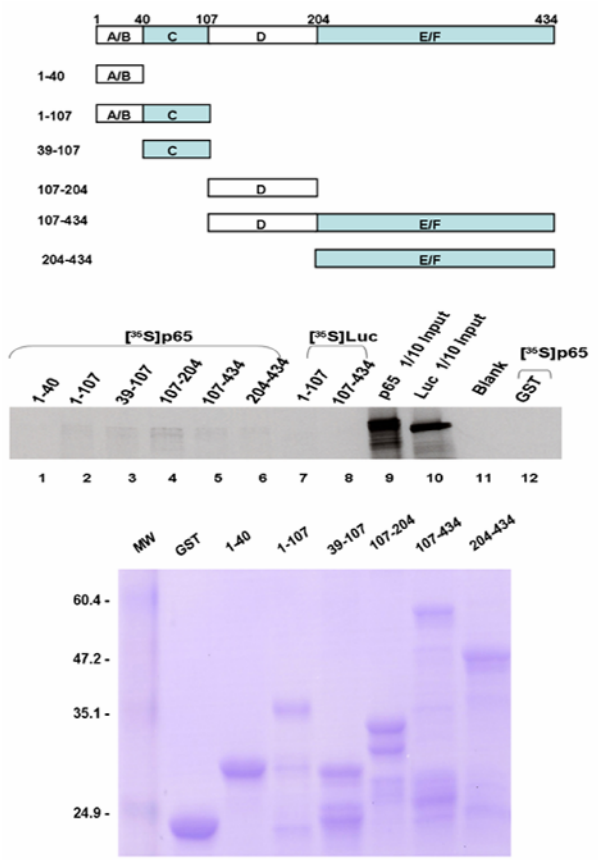

Fig. 12. PXR physically interacts with p65 as determined in a GST pull-down assay. A. schematic illustration of the domains of PXR (Bairoch et al., 2005) and deletion mutants used for GST pulldown assays. B. GST pull-down analysis of the domains of PXR that interact with p65. In lanes 1-6 the radiolabeled p65 was incubated with various recombinant GST-PXR fusion peptides as indicated in A. Lanes 9 and 10 are the input controls with 1/10 of the radiolabeled p65 and luciferase, respectively. Lanes 7 and 8 showed the negative control by using the N-terminal and C-terminal of GST-PXR pull-downed luciferase. Lane 12 was the control for GST protein. After washes the p65 association with the GST-PXR fusion peptides was determined from separation by SDS-PAGE and by autoradiography to detect the radiolabeled peptide.

3.2 Cross talk between AhR and PXR pathways at transcriptional level

3.2.1 Crosstalk between AhR- and PXR-dependent gene expressions in mice

3.2.1.1 AhR agonists enhanced PCN-induced PXR target gene expression

Mice were treated with a combination of PCN, TCDD, β-NF as indicated (Figs. 13-17) and the results showed that PCN induced Cyp3a11 gene expression by about 2-fold and both TCDD and β-NF suppressed basal Cyp3a11 gene expression. TCDD significantly enhanced PCN-induced Cyp3a11 gene expression by 26% ($P<0.05$), whereas β-NF had no significant effect on PCN-induced Cyp3a11 gene expression (Fig. 13).

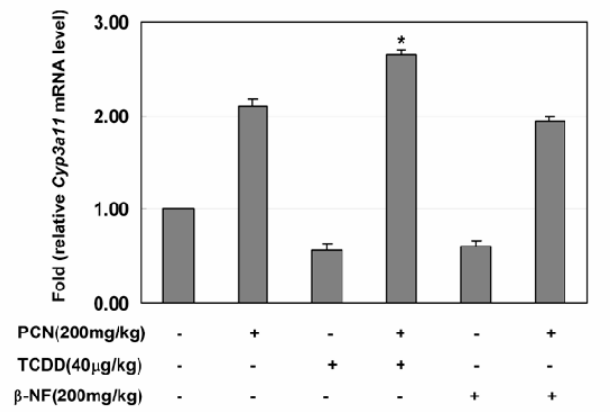

Fig. 13. Effects of PCN, TCDD, β-NF and their combinations on Cyp3a11 gene expression in mice. Chemicals at the indicated doses were administered i.p. injection daily for four days (i.e. PCN 50mg/kg/day) and 24 hr after the last dose, animals were sacrificed, the livers were removed and frozen in liquid nitrogen and stored in -80^{o}C. Total RNA was extracted with Trizol. The relative mRNA levels were tested by quantitative real-time PCR. *, $p<0.05$, compared with the group of PCN treatment.

Table 12 Induction of Gsta1 gene expression by ligands.

Treatment	Mean ± SE
DMSO	1.00
PCN	11.8 ± 2.2
TCDD	2.8 ± 0.7
PCN+TCDD	42.9 ± 11.1*
β-NF	3.7± 0.9
PCN+β-NF	16.9 ± 2.1

* $P<0.05$, compare with the sum of the fold induction by PCN and TCDD treatment.

PCN, TCDD and β-NF alone induced mouse Gsta1 gene expression, thus PCN is a major inducer, and TCDD or β-NF is minor inducer for Gsta1. co-treatment with PCN and TCDD cause synergistic induction of Gsta1 ($P<0.05$), compared with the sum of the induction of Gsta1 by PCN and TCDD alone; co treatment with PCN and β-NF cause enhanced induction, compared with the level of the induction of Gsta1 by PCN (Table 12, Fig. 14A). Compare with the induction of Gsta1 gene expression by the combination of PCN, TCDD and β-NF, the pattern of induction and the induction level of Ugt1a1 is slight, although co-treatment of PCN and TCDD or β-NF has a raised induction by comparison of treatment of single chemical (Fig. 14 B).

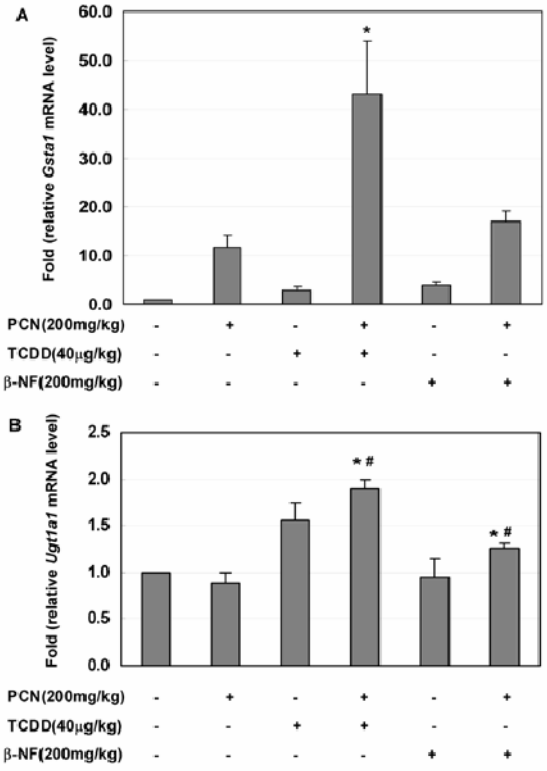

Fig. 14. Effects of combination of PCN, TCDD and β-NF on Gsta1 and Ugt1a1 gene expression in mice. Chemicals with indicated dosage were administrated i.p. in four days (i.e.PCN 50mg/kg/day). Twenty-four hours after last dose, the livers were collected snap-freezed in liquid nitrogen, stored in -80C. Total RNA was extracted with Trizol. The relative mRNA levels were tested by quantitative real-time PCR. *, $p<0.05$ (A), 0.01 (B), compared with the group of PCN treatment. #, $p<0.05$, compared with the group of TCDD or β-NF treatment.

Treatment of PCN, TCDD and β-NF alone suppressed transporter Mdr1 and Oatp2 gene basal expression. Co-treatment of PCN and TCDD or β-NF raised the induction level, compared with single chemical treatment (Fig. 15).

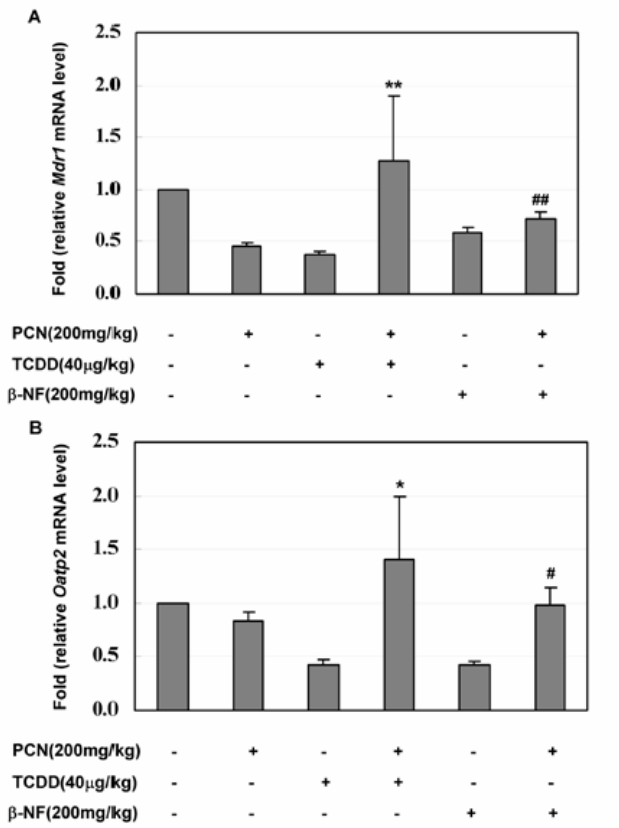

Fig. 15. Effects of combination of PCN, TCDD and β-NF on Mdr1 and Oatp2 gene expression in mice. Chemicals with indicated dosage were administrated i.p. in four days (i.e.PCN 50mg/kg/day). Twenty-four hours after last dose, the livers were collected snap-freezed in liquid nitrogen, stored in -80C. Total RNA was extracted with Trizol. The relative mRNA levels were tested by quantitative real-time PCR. *, **, $p<0.05$, compared with the group of TCDD treatment. #, ##, $p<0.05$, compared with the group of β-NF treatment.

3.2.1.2 PXR agonists suppressed TCDD- and β-NF-induced AhR target gene expression

When treat mice with combination of PCN, TCDD, β-NF as indicated (Figs.13-17), we checked the expression levels of AhR target genes Cyp1a1, Cyp1a2 and Cyp1b1.

Results showed that TCDD induced the Cyp1a1, Cyp1a2 and Cyp1b1 gene expression, co-treatment of PCN suppressed insignificantly TCDD-induced Cyp1a1 gene expression by 9%, significantly enhanced TCDD-induced Cyp1a2 gene expression in 1.3 fold ($P<0.001$), and significantly suppressed TCDD-induced Cyp1b1 gene expression by about 20% ($P<0.05$) (Table 13) (Fig. 16).

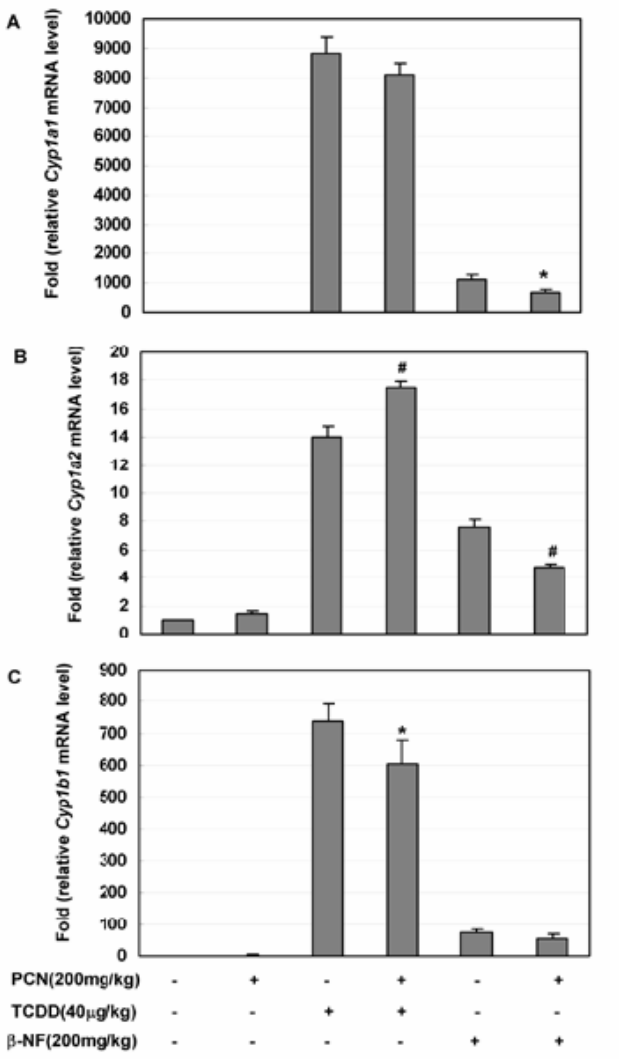

Fig. 16. Effect of the combination of PCN, TCDD, β-NF on AhR target genes in mice. Chemicals with indicated dosage were administrated i.p. in four days (i.e.PCN 50mg/kg/day). Twenty-four hours after last dose, the livers were collected snap-freezed in liquid nitrogen, stored in -80^{o}C. Total RNA was extracted with Trizol. The relative mRNA levels were tested by quantitative real-time PCR. *, $p<0.05$, compared with the group of TCDD or β-NF treatment. #, $p<0.01$, compared with the group of TCDD or β-NF treatment.

Beta-NF induced the Cyp1a1, Cyp1a2 and Cyp1b1 gene expression, co-treatment of PCN suppressed significantly β-NF-induced Cyp1a1 and Cyp1a2 gene expression by 42% ($P<0.05$) and 36% ($P<0.001$), respectively, and suppressed β-NF-induced Cyp1b1 gene expression by about 26% (Table 13) (Fig. 16).

Table 13 Effect of the combination of PCN, TCDD, β-NF on AhR target genes (Mean±SE, n=6)

Treatment	Cyp1a1	Cyp1a2	Cyp1b1
DMSO	1.0	1.0	1.0
PCN	0.7±0	1.4±0.2	1.9±0.5
TCDD	8817.6±556.8	14.0±0.8	739.6±50.6
PCN+TCDD	8097.1±383.8	17.5±0.4	603.8±73.8
β-NF	1120.9±170.1	7.6±0.6	73.2±11.5
PCN+β-NF	655.1±131.7	4.8±0.2	54.5±14.0

However, compare with vehicle control, PCN, TCDD and β-NF or the chemical combination treatment didn't significantly change the level of Pxr mRNA level. PCN didn't significantly the mRNA level of Ahr, while TCDD and β-NF increased the level of Ahr gene expression. PCN enhanced the induction of Ahr gene expression by TCDD (Fig. 17).

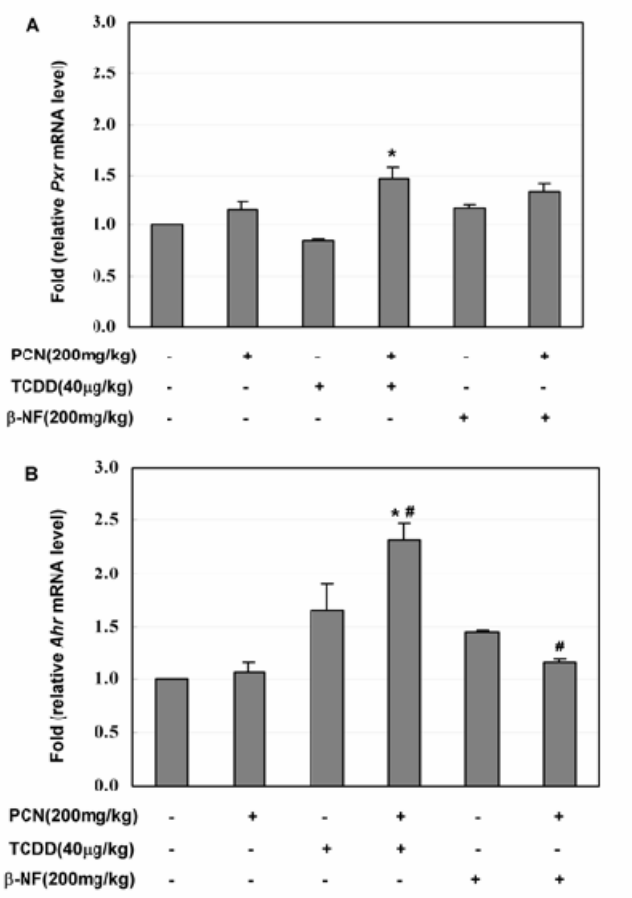

Fig. 17. Effects of the combination of PCN, TCDD and β-NF administration on Pxr and Ahr gene expression in mice. Chemicals with indicated dosage were administrated i.p. in four days (i.e.PCN 50mg/kg/day). Twenty-four hours after last dose, the livers were collected snap-freezed in liquid nitrogen, stored in -80^{0}C. Total RNA was extracted with Trizol. The relative mRNA levels were tested by quantitative real-time PCR. *, $p<0.01$ (A), compared with the group of TCDD treatment. #, $p<0.05$, compared with the group of TCDD or β-NF treatment. * $p<0.01$ (B), compared with the group of PCN treatment.

3.2.2 AhR agonists and PXR agonists mutually regulated their target gene expression in human and mouse cell lines

3.2.2.1 AhR agonists enhanced RIF-induced PXR target gene expression

In a pilot study, we treated primary human hepatocyte by the combination of RIF and TCDD, the results showed that co-treatment of RIF and TCDD enhanced CYP3A4 and

mdr1 gene expression in 1.7- and 1.4- fold respectively (Fig. 18 A, B), irrelated to the expression level of the gene of PXR, RXRα, AhR and ARNT (Fig. 18C, D, E, F).

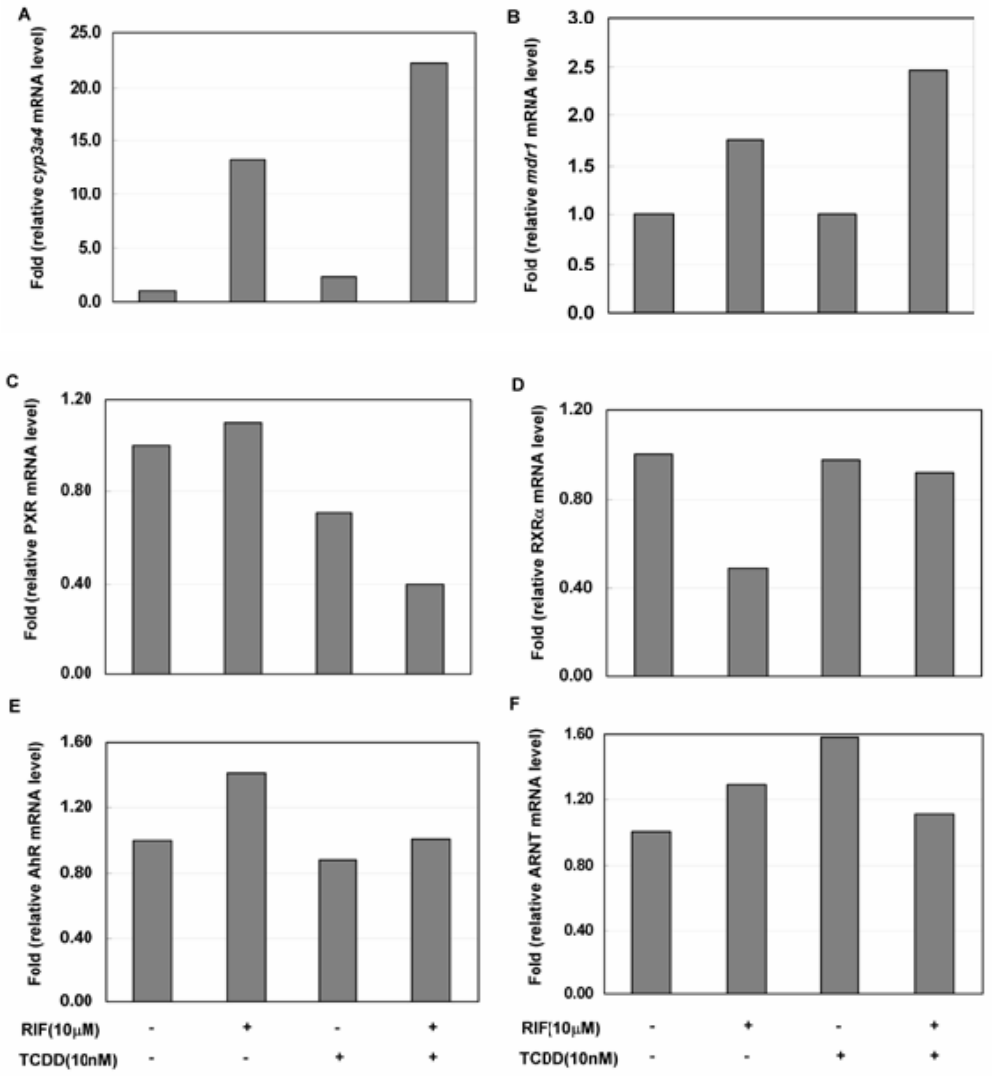

Fig. 18. TCDD enhanced the induction of CYP3A4 and mdr gene expression by rifampicin in primary human hepatocytes. Primary human hepatocytes was plated in 12-well plates, treated with chemicals indicated for 24 hr. Total RNA were extracted with RNAeasy from Qiagen. Relative mRNA levels were tested by quantative real-time PCR. CYP3A4 and MDR1 relative mRNA levels were shown in A and B respectively. Relative mRNA levels were shown as in C PXR , D RXRα , E AhR , F ARNT.

In view that AhR agonist TCDD enhanced PCN-induced gene expression in mice and RIF-induced gene expression in primary human hepatocytes, we further use PXR-mediated CYP3A4-Luc gene expression in HepG2 cell line as a model system to investigate the mechanism. To facilitate the testing, we constructed a stably-transfected HepG2 cell line with PXR and CYP3A4-Luc and named it as S95 cell line. We treated S95 cell line with the combination of human PXR agonists RIF, hyperforin, clotrimazole, human AhR agonists TCDD, BaP, 3-MC, β-NF and human AhR antagonists resveratrol, α-NF as indicated (Figs. 19-20).

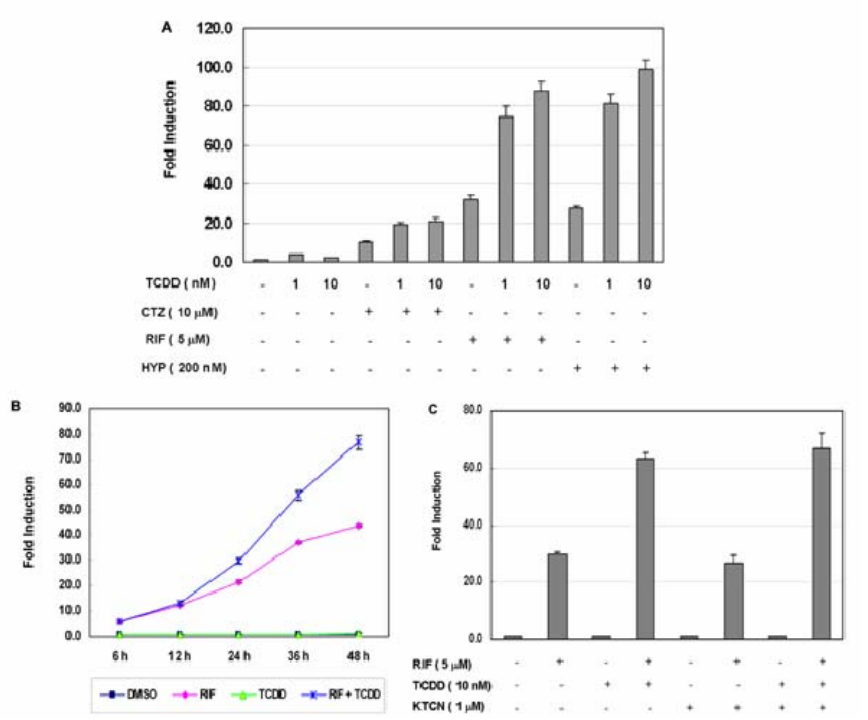

Fig. 19. Effect of TCDD on PXR ligands-induced pGL3-3A4-Luc gene expression in S95 cell line. (A) Combinations of clotrimazole (CTZ), rifampicin (RIF) or hyperforin (HYP) and TCDD were added to cell cultures, 48 hr later, cells were collected and relative luciferase activity were tested. Fold induction were calculated by normalizing to vehicle controls. (B) Combination of TCDD (10 nM) and rifampicin (RIF, 5 μM) were added to S95 cell line at certain time points as indicated and cells were collected at the same time to test the luciferase activity. (C) Combinations of chemicals indicted were added to S95 cell cultures, 48 hr later, cells were collected and relative luciferase activity were tested. Media were changed every 24 hr. Fold induction were calculated by normalizing to vehicle controls.

Results showed that TCDD enhanced human PXR agonists RIF, hyperforin and clotrimazole induced CYP3A4-Luc gene expression in S95 cells (Fig19A, B), co-treatment of ketoconazole, the inhibitor of cytochrome P450, had no significant effect on the enhanced RIF-induced CYP3A4-Luc gene expression by TCDD, excluding the possibility that the enhancement was caused by the metabolite with more potency (Fig19 C).

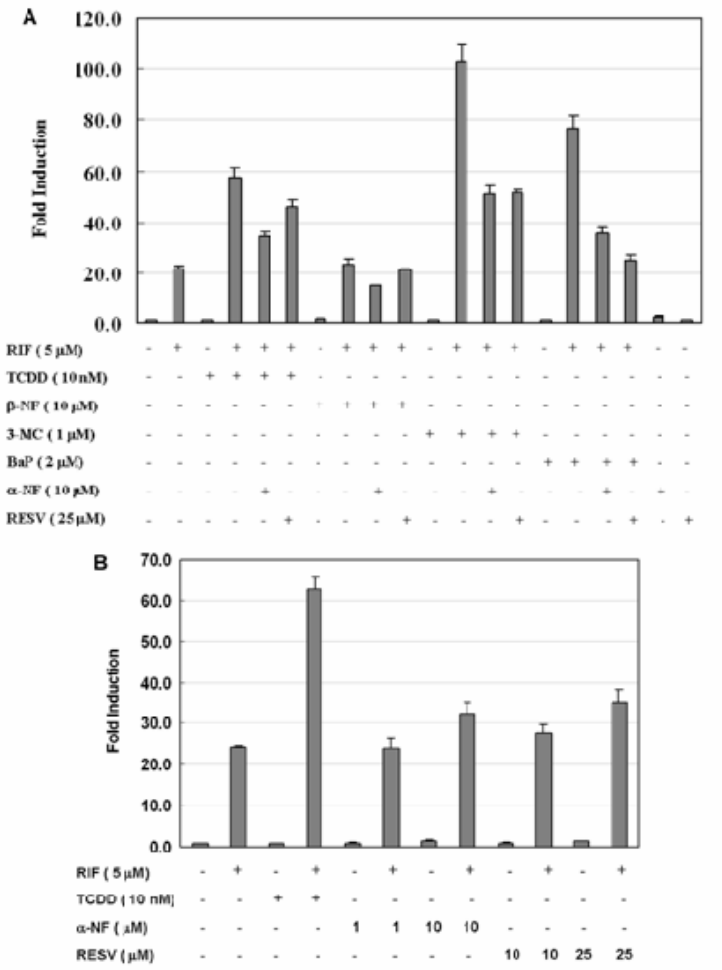

Fig. 20. Effects of AhR agonists TCDD, β-NF, 3-MC, BaP and antagonists α-NF, resveratrol (RESV) on RIF-induced pGL3-3A4-Luc gene expression in S95 cells. (A) (B) Combinations of chemicals indicted were added to cell cultures, 48 hr later, cells were collected and relative luciferase activity were tested. Media were changed every 24 hr. Fold induction were calculated by normalizing to vehicle controls.

As the enhancing effect of TCDD treatment on the RIF-induced CYP3A4-Luc gene expression, AhR agonists 3-MC, BaP and β-NF enhanced RIF-induced CYP3A4-Luc gene expression. The enhancement was knocked down by AhR antagonists α-NF or resveratrol (Fig. 20A). Interestingly, co-treatment of α-NF or resveratrol showed relatively low enhancement of the induction of CYP3A4-Luc gene expression by RIF (Fig. 20B).

In considering that TCDD, β-NF, 3-MC and BaP are AhR agonists, and α-NF and resveratrol are AhR antagonists, causing AhR translocation when binding, these data suggested that AhR was involved in the regulation of PXR-transcriptional activity.

3.2.2.2 PXR agonists suppressed TCDD- and β-NF- induced AhR target gene expression

In the pilot study using primary human hepatocytes treated with the combination of RIF and TCDD, TCDD induced cyp1a1 gene expression by 176 fold, RIF suppressed TCDD-induced cyp1a1 gene expression by 10% (Fig. 21A). Using reporter gene assay, co-transfecting HepG2 cells with human PXR and pGud-luc6.1 which is the reporter for AhR transcriptional activity and treating with the combination of RIF and TCDD, the result showed that RIF suppressed basal pGud-luc6.1 gene expression by 80% and TCDD induced pGud-luc6.1 gene expression by 15% ($P<0.05$) (Fig. 21B).

When co-transfecting Hep1c1c7 cells with mouse PXR and pGud-luc6.1 treating with the combination of PCN, hyperforin, TCDD and β-NF as indicated in Fig. 21 C, the results showed that PCN induced pGud-luc6.1 gene expression in about 2.5 fold ($P<0.01$), but suppressed TCDD- and β-NF- induced pGud-luc6.1 gene expression 15% ($P<0.01$) and 17% ($P<0.05$) respectively, (Fig. 21), hyperforin suppressed TCDD-induced pGud-luc6.1 gene expression by 28% ($P<0.01$). Hyperforin has no significant effect on the pGud-luc6.1 gene basal expression or β-NF-induced expression.

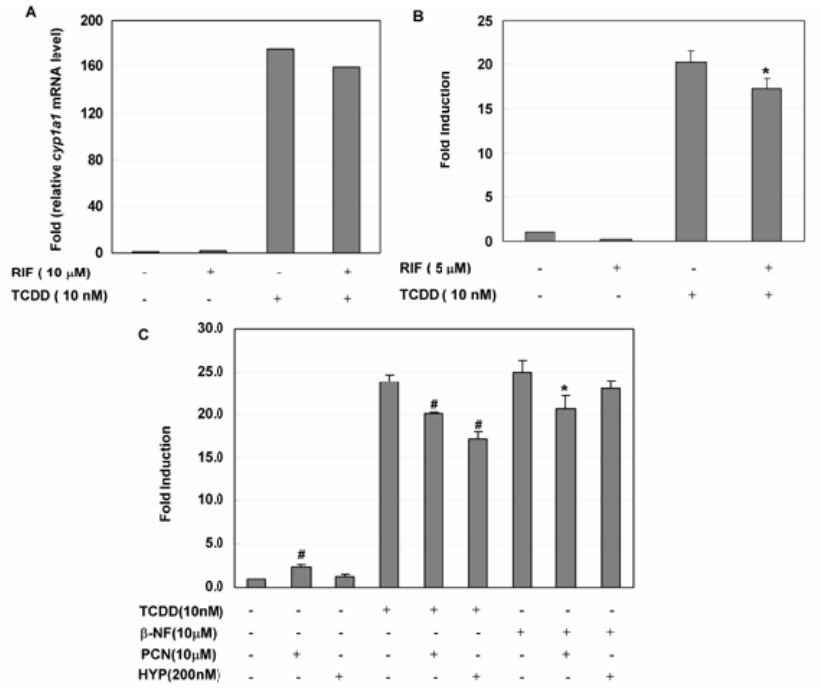

Fig. 21. Effects of PXR ligand on AhR-mediated gene expression. (A)Primary human hepatocyte was treated with the combination of RIF and TCDD as indicated for 24 hr. Total RNA was isolated and reverse-transcribed into one-strand cDNA with random primer and tested with quantitative real-time PCR. The level of cyp1a1 mRNA was normalized with the level of b-actin level. (B) Co-transfected 0.5 μg pGud-luc6.1 into HepG2 cells which stably expressed hPXR. 6 hr later treat with RIF and TCDD for additional 36 hr. Collect cells and test the relative luciferase activity. (C) Co-transfected Hep1c1c7 cells with 0.1 μg mouse PXR expression plasmid and 0.5 μg pGud-luc6.1. 6 hr later treat with RIF and TCDD for additional 24 hr. Collect cells and test the relative luciferase activity. The data shown is the representative of three independent experiments except (A) with one donor.

3.2.3 AhR was involved in the PXR-mediated CYP3A gene expression

To further investigate if AhR was involved in the regulation of PXR-transcriptional activity, we co-transfect AhR plasmids in several doses and several small interfering RNAs targeting AhR and ARNT. The results showed that overexpression of the AhR enhanced PXR-induced CYP3A4-Luc reporter gene expression in a dose dependent manner (Fig22A). Interestingly, in this transient transfection system, TCDD induced

CYP3A4-Luc reporter gene expression the way different from stably-transfected CYP3A4-Luc reporter gene by comparing the results in Fig22A and Fig. 19 and 20.

Small interfering RNAs targeting AhR and ARNT knock down the induction of CYP3A4-Luc reporter gene expression. These data confirmed that AhR was involved in the regulation of PXR transcriptional activity to induce gene expression (Fig22B).

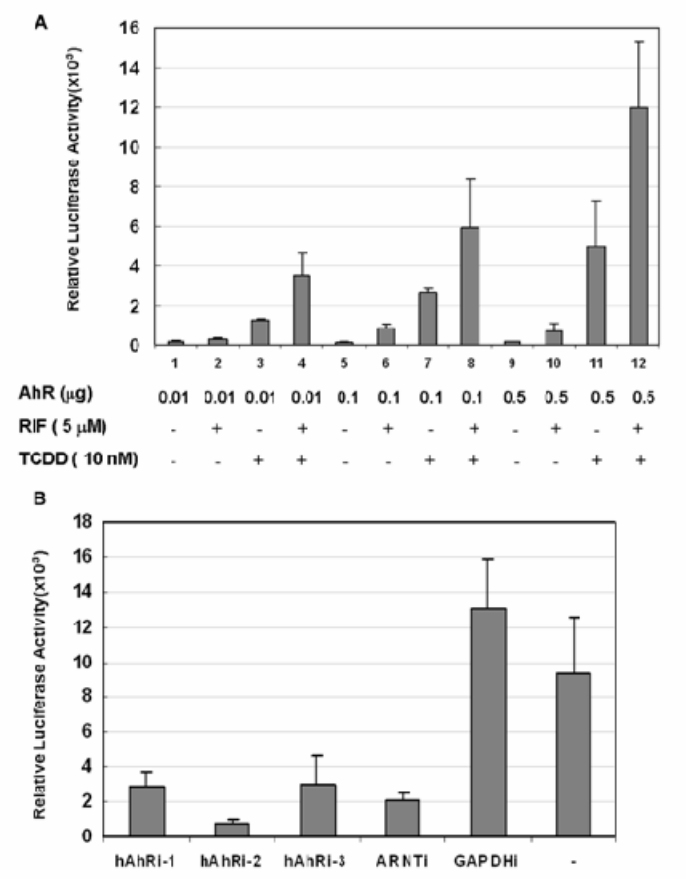

Fig. 22. Effect of AhR expression level on PXR-mediated CYP3A4-Luc gene expression. (A) Co-transfected different amount of AhR expression plasmid with 0.5 μg pGL3-3A4-Luc into HepG2 cells which stably expressed hPXR. 6 hr later treat with RIF and TCDD for additional 48 hr. Collect cells and test the relative luciferase activity. (B) Transfect 10nM of siRNAs indicated into HepG2 cells which stably expressed hPXR for 6 hr and incubate with regular media for 30 hr. Transfect 0.5 μg pGL3-3A4-Luc for 6 hr and treat with RIF and TCDD for additional 48 hr. Collect cells and test the relative luciferase activity. The data shown is the representative of three independent experiments.

3.2.4 PXR was involved in AhR-mediated target gene expression

RIF, PCN and hyperforin are PXR agonists, the suppressed TCDD-induced gene expression, suggesting PXR was involved in the AhR target gene expression. We used reporter gene assay and cotransfected mouse PXR expression plasmid in Hep1c1c7 cell lines in which PXR expression was lower that normal mouse liver cells. Results showed that overexpression of PXR suppressed TCDD- or β-NF- induced pGud-luc6.1 gene expression in a dose-dependent manner (Fig. 23). Taken together, PXR was involved in the AhR-transcriptional activity in a ligand-dependent manner.

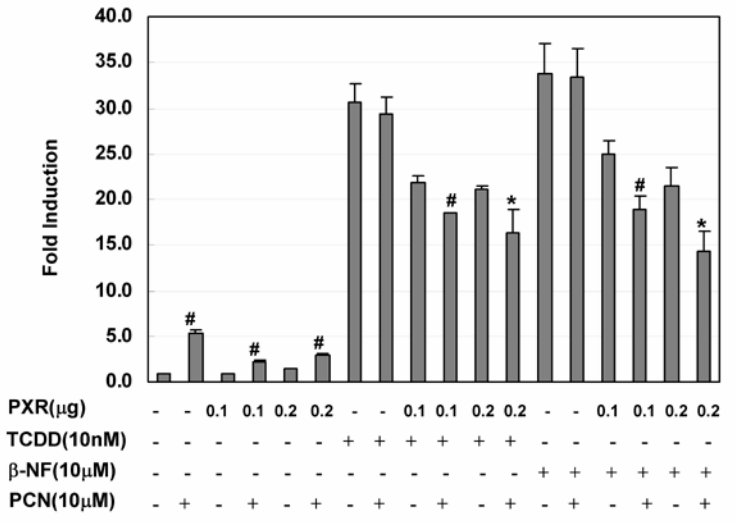

Fig. 23. Effects of dosage of PXR and its ligands on AhR-mediated gene expression. Co-transfected Hep1c1c7 cells with various amount of mouse PXR expression plasmid as indicated and 0.5 μg pGud-luc6.1. 6 hr later treat with RIF and TCDD for additional 24 hr. Collect cells and test the relative luciferase activity. The data shown is the representative of three independent experiments.

3.2.5 AhR and PXR interact in vivo and in vitro

To investigate how PXR and AhR are involved in the mutual regulation of their transcriptional activity and target gene expression, we checked if AhR and PXR were associated in vivo. We performed co-immunoprecipitation in HepG2 cells which stably expressed 3xFlag-PXR to detect if AhR is associated with PXR or its partner RXRα in a complex in vivo (Fig. 24). Our results showed that AhR was associated with PXR and

RXRα. To further confirm the association between AhR and PXR or RXRα, we performed GST-pull down assay to detect if AhR interacted directly with PXR or RXRα in vitro. The data showed that the basic domain of AhR is required for AhR N-terminus to interact with A/B/C domain of PXR, the C-terminal of AhR interact with D/E/F domain of PXR. There is no interaction between PXR and ARNT (Fig. 25).

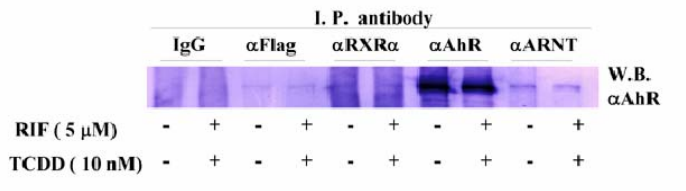

Fig. 24. AhR is associated with PXR and RXRα in HepG2 cells. Co-immunoprecipitation assay was performed on the 3XFlag-hPXR-enhanced HepG2 cells treated with RIF and TCDD, DMSO as control. The data shown is the representative of three independent experiments.

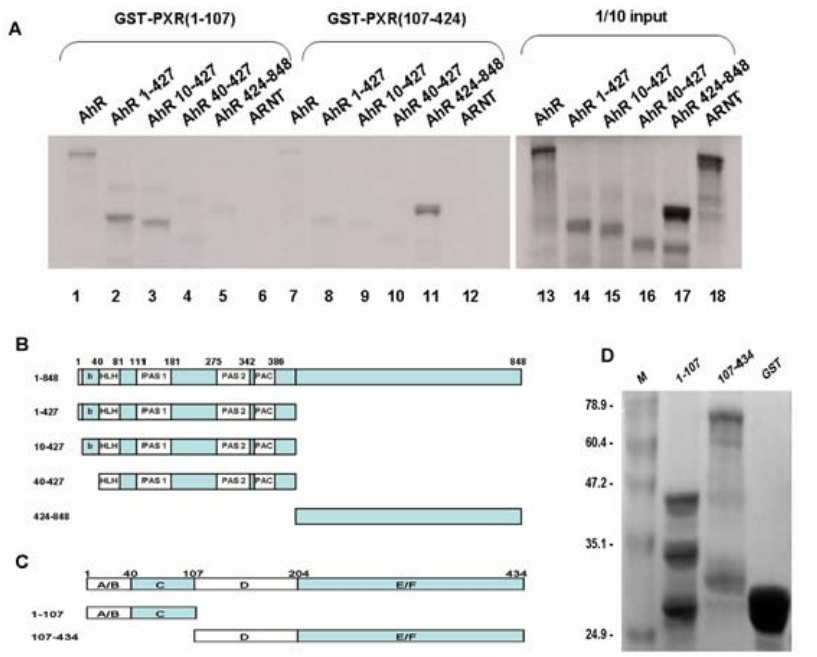

Fig. 25. AhR interacts physically with PXR in a domain-preference manner. GST pull-down assay was performed as regular procedure using TNT translated different fragments of AhR as indicated which was incorporated S-35, GST-PXR (1-107) and GST-PXR (107-434) purified from *E. coli*. The data shown is the representative of three independent experiments.

The basic domain of AhR is required for AhR N-terminus to interact with A/B/C domain of RXRα and the C-terminal of AhR interacts with A/B/C domain of RXRα. There is no interaction between RXRα and ARNT (Fig. 26).

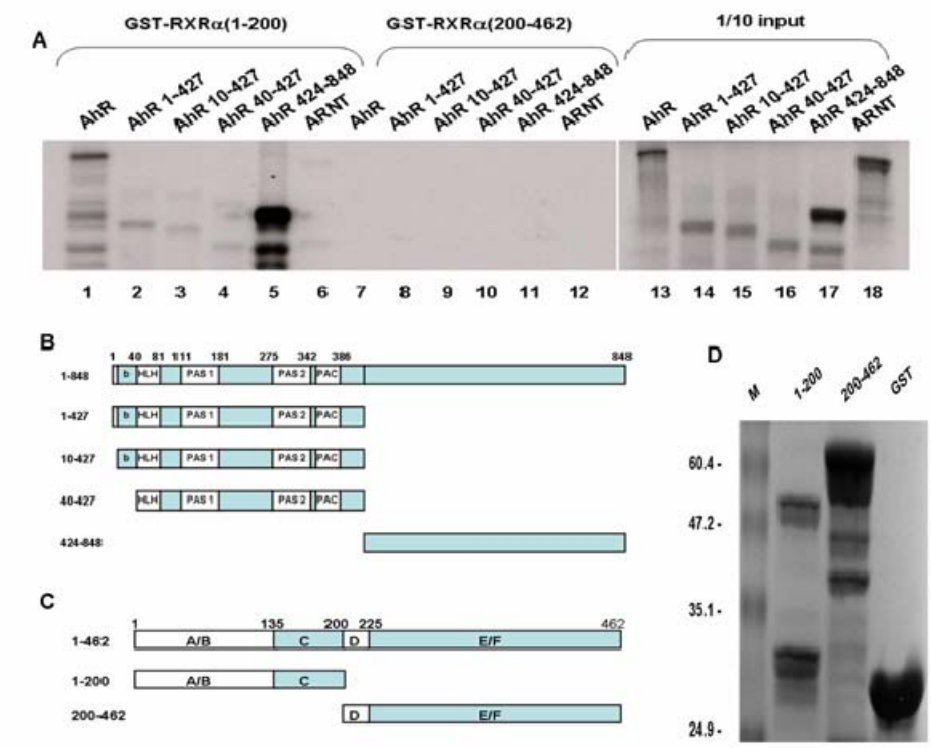

Fig. 26. AhR interacts physically with RXRα in a domain-preference manner. GST pull-down assay was performed as regular procedure using TNT translated different fragments of AhR as indicated which was incorporated S-35, GST-RXRα (1-200) and GST-RXRα (200-462) purified from *E. coli*. The data shown is the representative of three independent experiments.

To test the function of the association between PXR and AhR, we constructed AhR expression plasmids with deleted domains and co-transfected the HepG2 cells which stably expressed PXR with pGL3-3a4-luc gene (Fig. 27). AhR (1-848) and AhR (11-848) with Pro domain deletion of AhR enhanced the expression of the reporter gene, while AhR (40-848) with Pro and basic region deletion of AhR has no significant effect on the expression the reporter gene. The result suggested that basic domain is required for the

enhancement of PXR-regulated gene expression by AhR. In contrast to the effect of AhR (1-848) and AhR (11-848) respectively, AhR (1-427) and AhR (11-427) with C-terminal deletion of AhR repressed the enhanceability of endogenous AhR, the result suggested that C-terminal of AhR is also required to enhance the expression of PXR-regulated gene. The lack of either basic region or C-terminal resulted in the loss of the enhanceability of AhR and the repress of the activity of endogenous AhR, by comparing AhR (40-848), AhR (1-427) and AhR (11-427) with AhR (1-848), except for AhR (424-848) which is unable to translocate to nuclear and interact with PXR. The lack of both basic region and C-terminal resulted in the derepression of the effects of AhR (40-848) and AhR (1-427) by comparing with AhR (40-427).

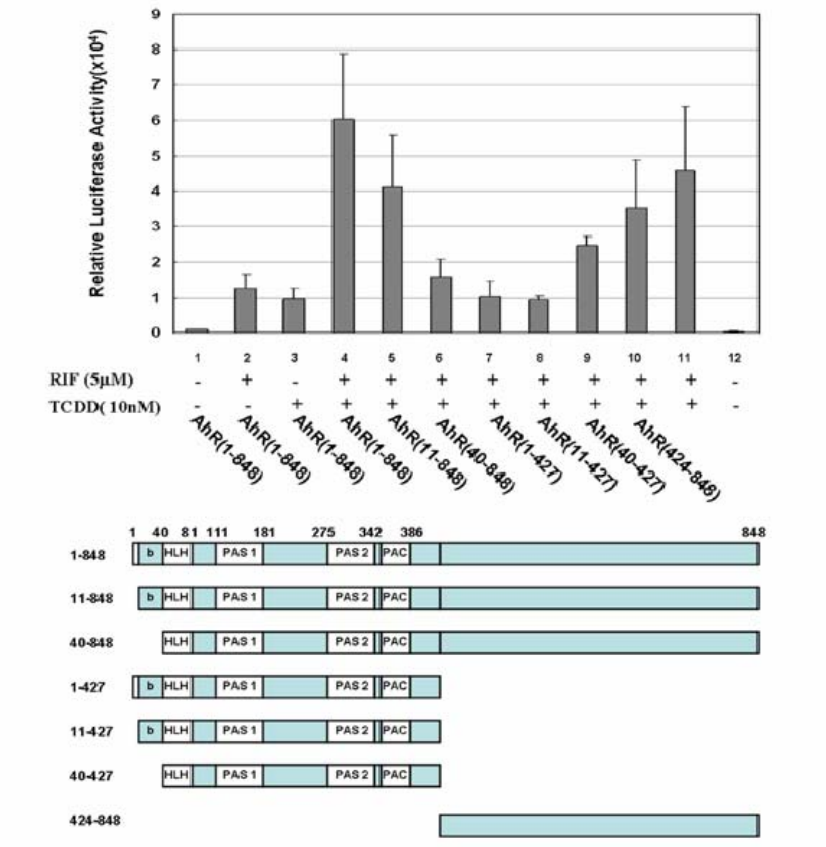

Fig. 27. AhR modulates PXR transcriptional activity in a domain-dependent manner. Cotransfect HepG2 cells which stably expressed PXR with pGL3-3A4-Luc and AhR of different domain deletions for 6 hr. Treat with chemicals as indicated for 48 hr, then test luciferase activity. The data shown is the representative of three independent experiments.

The result that both basic region and C-terminal of AhR were required for enhancement of induction of PXR-mediated CYP3A4 gene expression by activated AhR (Fig. 27) was in accordance with that PXR and AhR interact domain-preferently in GST pull-down assay.

3.3 Screening xenobiotics for PXR-mediated CYP3A4 inducers through cell-based assay system

3.3.1 Function of PXR domains on PXR-transcriptional activity in response to rifampicin

The result of the reporter gene assay using pGL3-3A4-Luc and PXR domains in HepG2 cells showed that the full PXR (A/B/C/D/E/F) was activated by ligand rifampicin to induce pGL3-3a4-luc gene expression up to 4 fold (Fig. 28). In response to the treatment of rifampicin and compared with vehicle control, the pGL3-3a4-luc gene expression obviously decreased with the A/B domain, the pGL3-3a4-luc gene expression slightly decreased with the A/B/C, A/B/C/D and C domain or domain combinations, the pGL3-3a4-luc gene expression didn't change with the C/D domain combination, the pGL3-3a4-luc gene expression slightly increased with the C/D/E/F domain combination, the pGL3-3a4-luc gene expression obviously increased with the D, D/E/F and E/F domain or domain combinations. The C/D/E/F domain combination, which is lack of the A/B domain, had no significant change in response to rifampicin. The result suggests that A/B domain in PXR palys an important role in ligand-dependent activation of PXR transcription activity. The mechanisms accounting for other changes remain to be further illucidated.

The basal levels of the pGL3-3a4-luc gene expression with the PXR domains or domain combinations in HepG2 cells were divided into three groups. The pGL3-3a4-luc gene expression with the A/B, A/B/C and C domain or domain combinations had the highest level; The pGL3-3a4-luc gene expression with the A/B/C/D, A/B/C/D/E/F, C/D and C/D/E/F domain combinations had the intermediate level; The pGL3-3a4-luc gene

expression with the D, D/E/F and E/F domain or domain combinations had the lowest level. By comparison with the pGL3-3a4-luc gene expressions among A/B/C, A/B/C/D and A/B/C/D/E/F domain combinations, and among C, C/D and C/D/E/F domain ordomain combinations, the results showed that D is a transcription-suppressive domain in PXR.

In response to the treatment of rifampicin, the levels of pGL3-3a4-luc gene expression in HepG2 cells differentiated with the PXR domains or domain combinations. The results by comparisons between A/B/C/D and A/B/C/D/E/F, and between C/D and C/D/E/F showed that E/F domain is required for PXR to be activated in response to the ligands. This is consistant with the known findings on the nuclear receptors. The results by comparisons between A/B/C and A/B/C/D, and between C and C/D showed that D is a suppressive domain for PXR ranscription activity. This is consistent with the basal activity of PXR. The underlying mechanisms remain to be illucidated.

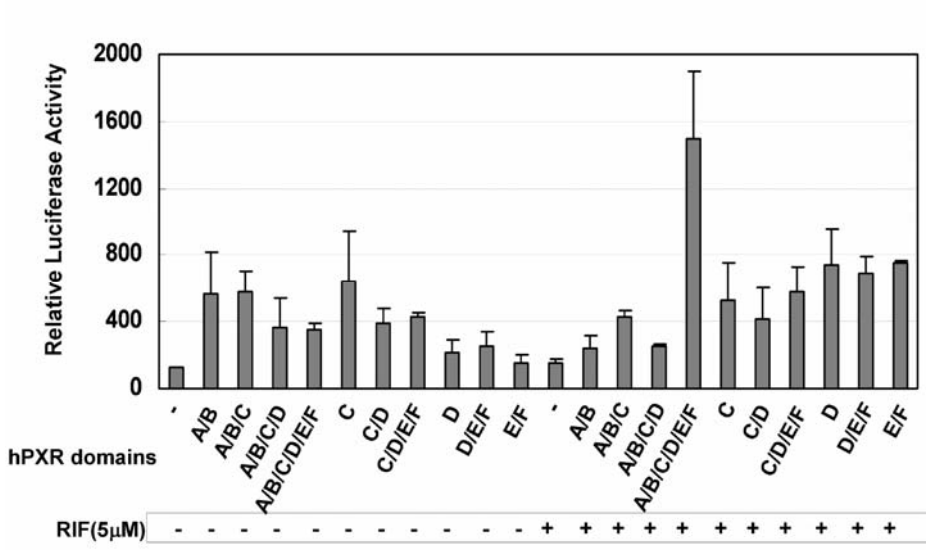

Fig. 28. Role of PXR domains in PXR transcriptional activity. HepG2 cells were transiently co-transfected with reporter gene pGL3-3A4-Luc 0.5 μg/well and PXR domains-containing expression plasmid 0.2 μg/well as indicated for 6 hr, and then were treated with rifampicin for 48 hr. Relative luciferase activity assay were performed.

3.3.2 Green tea polyphenols (GTPs) enhance PXR-induced CYP3A4-Luc gene expression in stably-transfected HepG2 cells

Green tea polyphenols extract alone didn't induce PXR-mediated CYP3A4-Luc gene expression. They enhanced RIF-induced CYP3A4-Luc gene expression in a dose dependent manner (Fig. 29).

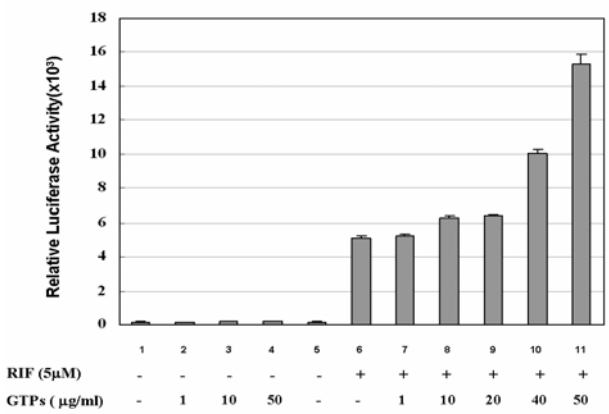

Fig. 29. Green tea polyphenols enhanced activated-PXR-induced CYP3A4-Luc gene expression. Measure 500 mg GTPs and dissolve to H_2O to make stock solutions. Treat cell-based reporter gene assay with chemicals as indicated every 24 hr for 48 hr.

3.3.3 Dietary supplements induce PXR-mediated CYP3A4-Luc gene expression in stably-transfected HepG2 cells

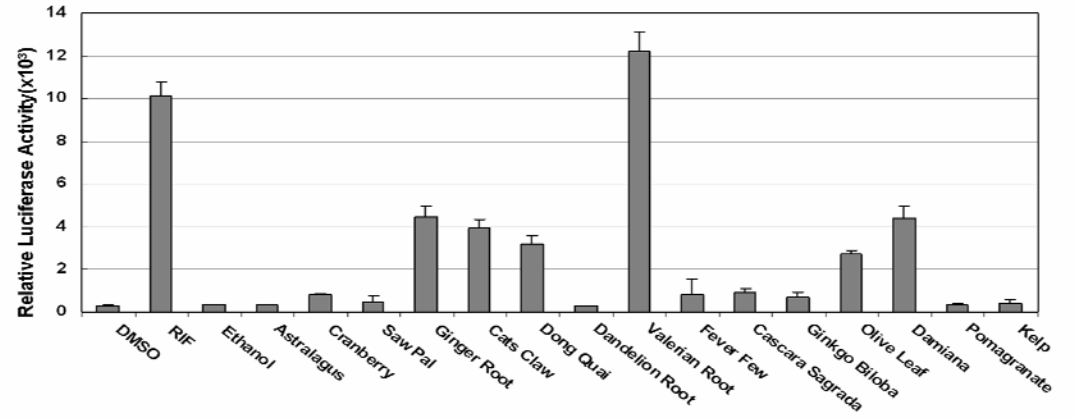

Fig. 30. Effects of dietary supplements on PXR-mediated CYP3A4 gene expression. Add 1 ml ethanol to 500 mg powder of one capsule of dietary supplement. Vortex 5 min and centrifuge at top speed for 5 min. Treat HepG2 cells which stably expressed PXR and pGL3-3A4-Luc with 1 μl supernatant in 1 ml medium for 48 hr. Test the activity of luciferase.

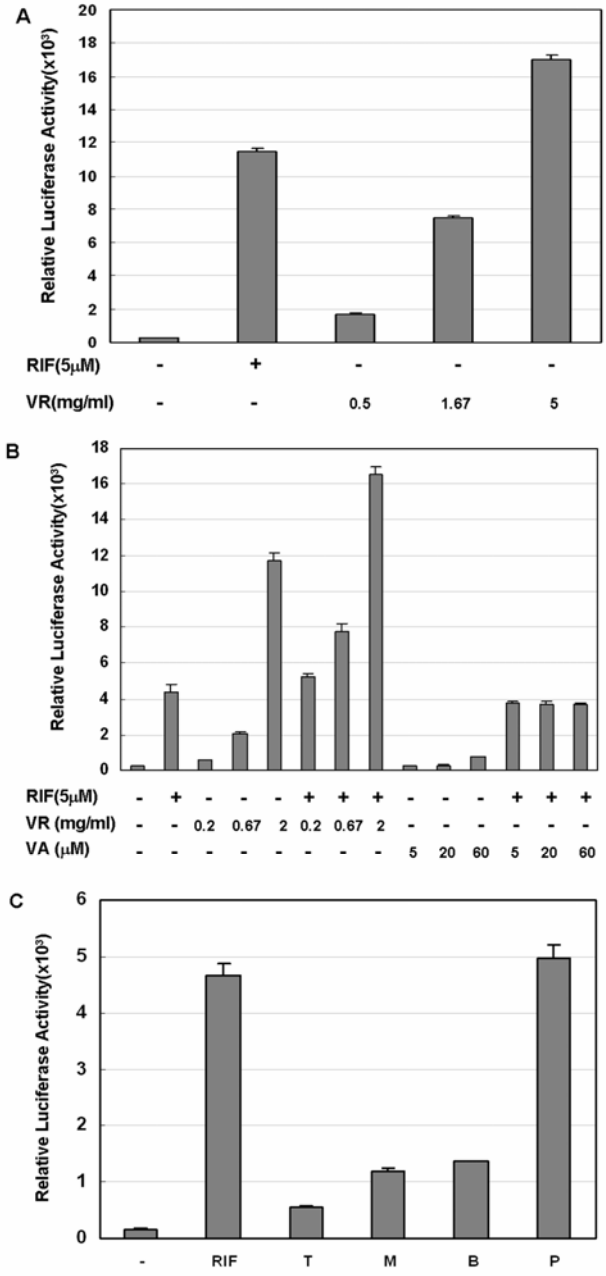

Fig. 31. Effects of the components of Valerian Root on PXR-mediated CYP3A4 gene expression. Valerian Root capsules were purchased from GNC store in Post Oak Mall (College Station). Use hexane to extract the powder in capsule of Valerian Root. Valerianic acid was purchased through VWR international. Treat HepG2 cells which stably expressed PXR and pGL3-3A4-Luc with extracts or chemicals for 48 hr. Test the activity of luciferase. Media were changed every 24 hr.

Dietary supplements were purchased from GNC store in Post Oak Mall (College Station). Among extracts surveyed, extracts of Ginger Root, Cats Claw, Dong Quai, Valerian Root, Olive Leaf and Damiana obviously induced PXR-mediated CYP3A4-Luc gene expression. Extracts of Cranberry, Fever Few, Cascara Sagrada and Ginkgo Biloba slightly induced PXR-mediated CYP3A4-Luc gene expression. The Valerian Root extract had the most potency of the induction (Fig. 30). We further investigated the component of Valerian Root responsible for the induction. The valerianic acid has little effect on luciferase gene expression by comparing with rifampicin (Fig. 31B). The bottom component of TLC has the most potent induction power (Fig. 31C).

3.3.4 Aflatoxin B1 enhanced activated-PXR-induced CYP3A4-Luc gene expression

In the treatment with the cell-based assay system with AFB1 alone, the induction of luciferase expression was marginal. While the cotreatment of AFB1 enhanced rifampicin-induced luciferase activity in a dose-dependent manner (Fig. 32).

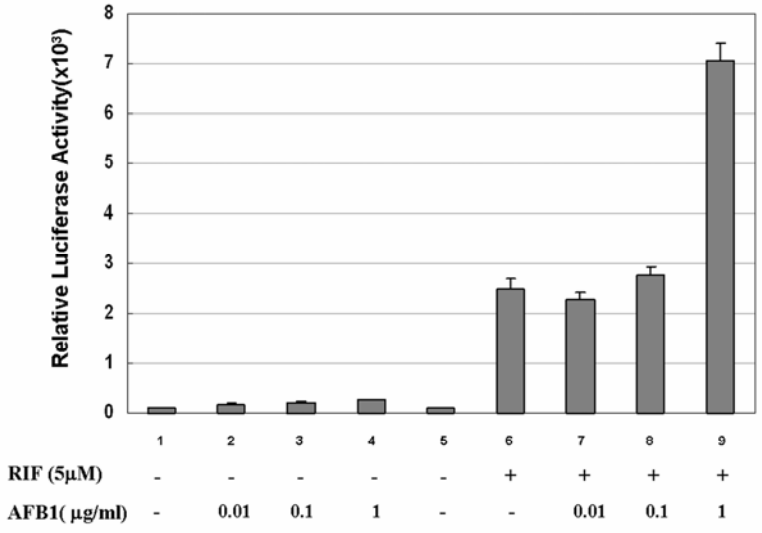

Fig. 32. Aflatoxin B1 enhanced activated-PXR-induced CYP3A4-Luc gene expression. Treat HepG2 cells which stably expressed PXR and pGL3-3A4-Luc with aflatoxin B1 with or without RIF as indicated for 48 hr. Test the activity of luciferase. Media were changed every 24 hr.

3.3.5 Effects of metal ions on PXR-induced CYP3A4 gene expression

Co-treatment of metal ions indicated in Fig. 33 with rifampicin resulted differentiated effects on the PXR-mediated CYP3A4-Luc gene expression. $NaAsO_2$ suppressed rifampicin induced luciferase ecpression. $CuSO_4$ and $MnCl_2$ enhanced rifampicin induced luciferase expression (Fig. 33A, B). The $CuSO_4$ and $MnCl_2$ alone had little effects on the PXR-mediated CYP3A4-Luc gene expression (Fig. 33B).

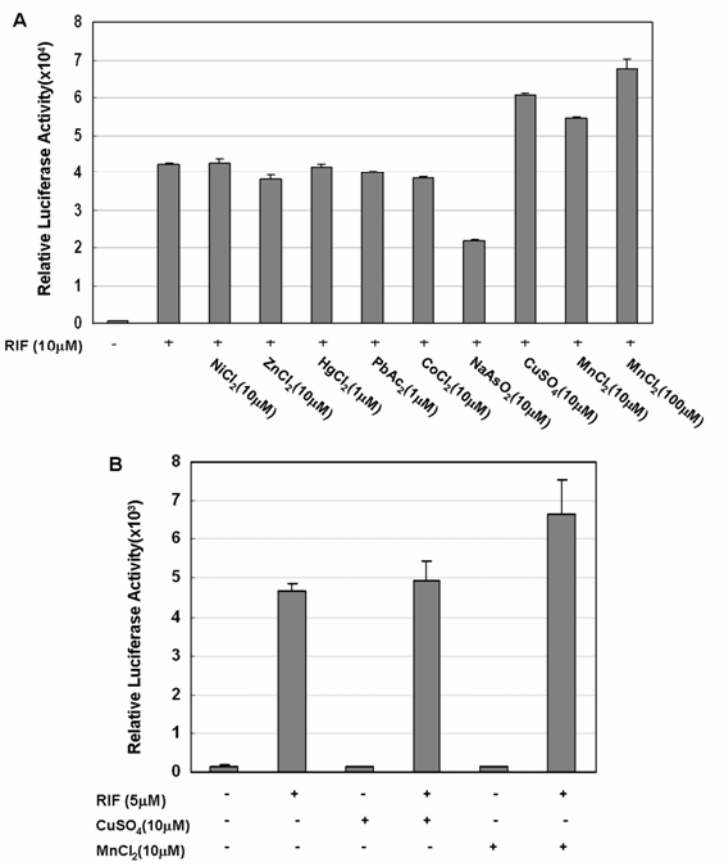

Fig. 33. Effects of metal ions on PXR-induced CYP3A4 gene expression. Treat HepG2 cells which stably expressed PXR and pGL3-3A4-Luc with metal ions with or without RIF as indicated for 48 hr. Test the activity of luciferase.

CHAPTER IV
DISCUSSION AND SUMMARY

4.1 NF-κB plays a key role in the suppression of PXR-mediated CYP3A4 gene expression

CYP3A4 is a predominant human liver monooxygenase metabolizing more than half of the drugs in use today. Transcriptional and post transcriptional regulations of the expression of this enzyme are of great importance in therapeutic application as well as development of therapeutics. Recent studies have demonstrated that the ligand-dependent transcription factor hPXR plays a pivotal role in coordinated regulation of *CYP3A4*, conjugation enzymes as well as transporters at the transcriptional level (reviewed in ref (Handschin and Meyer, 2003; Honkakoski et al., 2003), therefore, it is important to analyze physiological and pathological conditions that may impact the PXR activity.

Infections and inflammatory responses have long been observed to suppress hepato - intestinal cytochromes P450 as well as phase II enzymes, resulting in reduced capacity of drug clearance in both human and experimental animals (reviewed in (Morgan et al., 2002; Renton, 2004)). These clinically important phenomena have been investigated extensively. Several mechanisms have been proposed to explain the infection- and inflammation-induced suppression of *CYP3A4* expression. For example, it has been observed that LPS treatment down-regulates the PXR mRNA levels in cells and animals (Beigneux et al., 2002), and this may potentially result in suppression of *CYP3A4* expression. However, the levels of the nuclear receptors may not be an accurate gauge in evaluating their transcriptional activity. Using real time quantitative PCR and microarray profiling with LPS and TNF-α treated primary human hepatocytes, we found a slight decrease of PXR mRNA and RXRα mRNA level was essentially unchanged (Fig. 5). The marginal decrease in PXR mRNA may not account for the dramatic suppression of the CYP3A4 mRNA by LPS and TNF-α (Fig. 5). Using PXR and PPARα deficient mice, Richardson and Morgan have shown that endotoxin caused about same levels of

suppression of P450 in KO mice as in the wild type, suggesting nuclear receptors PXR and PPARα are not required for regulating the LPS-imposed suppression of the cytochromes P450 including CYP3As (Richardson and Morgan, 2005), at least in the animals whose P450s have not been induced by exogenous agents. However, since there have been extensive cross-talks between nuclear receptors, the compensatory roles of other nuclear receptors in mediating the LPS-induced suppression remains to be investigated. This is especially true in view of the current finding that the NF-κB-mediated suppression of the nuclear receptors may be through a general mechanism where the functions of a common partner (RXR) for nuclear receptors is being compromised upon NF-κB activation.

Recent studies have shown that the DNA sequences around -5.95 kbp at *CYP3A4* regulatory region contains CCAAT/enhancer sequences in the promoter regions of *CYP3A4* can be regulated by LIP (Martinez-Jimenez et al., 2005), thereby causing suppression. Liver enriched transcription factor has also been shown to mediate the LPS suppressive effects of the organic anion transporting peptide 4 (Li and Klaassen, 2004). In our current studies, we found that PXR-directed luciferase reporter gene without the CCAAT/enhancer sequences were also suppressed by NF-κB activation (Fig. 6), and inhibition of NF-κB alleviated the suppression (Fig. 7), suggesting disruption of the binding of PXR/RXRα complex to the consensus sequences (Figs 8-10) is an important mechanism in addition to the regulation by LIP. It is highly likely that more than one mechanism may be responsible for the suppression of *CYP3A4* gene expression.

A common transcriptional response to the challenges of infection and inflammation is the induction of immediate early genes. One of these genes is the pleiotropic transcription factor NF-κB, which is activated in response to various proinflammatory stimuli. NF-κB has been shown to interact with nuclear/steroid receptor, Ah receptor (Tian et al., 1999; Ke et al., 2001) and modulates the transcriptional activity of these receptors (reviewed in ref (McKay and Cidlowski, 1999; Tian et al., 2002). In mouse LPS-induced CNS inflammation model, it has been shown that Toll-like receptor regulates the suppression of the hepatic cytochromes P450 by LPS (Abdulla et al., 2005;

Goralski et al., 2005). These studies suggested NF-κB is involved in regulation of the hepatic P450. Although it is unknown if NF-κB activation plays a role in the transcriptional activity of PXR, it has been found that the common dimerization partner RXR for the nuclear receptors interacted with NF-κB (Na et al., 1999). We hypothesized that NF-κB may play a direct role in suppression of *CYP3A4* expression, and developed a PXR-driven luciferase assay using HepG2 cell culture model for the analysis of transcriptional regulation of PXR by the proinflammatory agents. In comparison with human primary hepatocyte culture, the magnitudes of PXR activation by rifampicin or transrepression of PXR by NF-κB activation were lower, which may be due to the clonal nature of the immortalized cell line, as it is well-known that hepatocytes loss certain aspects of xenobiotic responses in ex vivo culture conditions. These quantitative differences notwithstanding, the HepG2-based culture model has allowed us to analyze the transcriptional regulation by PXR and overcome certain drawbacks associated with using human primary hepatocyte culture, such as the donor variability and cost.

In this study, the important role of NF-κB in suppression of *CYP3A4* is demonstrated based on the following results: (i) TNF-α and LPS treatments of human primary hepatocytes resulted in activation of the NF-κB and coincided with the down-regulation of the *CYP3A4* and in luciferase reporter gene assay, and activation of NF-κB suppressed the PXR-driven luciferase reporter gene activity; (ii) TNF-α and LPS-imposed repression of *CYP3A4* promoter activity could be reversed by the NF-κB super repressor (SRIκBα), thus demonstrating the specific involvement of NF-κB.

To further elucidate the mechanism underlying the suppression of *CYP3A4* by NF-κB, we performed EMSA, GST-pulldown as well as ChIP assays to test the interaction between NF-κB and PXR/RXRα complexes. Using EMSA assay, we found that binding of PXR/RXRα heterodimer to the ER6 consensus sequences was inhibited by p65. The inhibitory effects of p65 on the binding of PXR/RXRα to ER6 could be alleviated by p50, which is the cognate partner for p65, suggesting that the inhibitory effect of p65 could be competitively decreased by p50, which is consistent with the hypothesis that p65 interferes with the association of PXR/RXRα with DNA sequences (Fig. 8). This

notion was further strengthened by the observation that the association between RXR and NF-κB p65 was mediated through the RXR DNA binding domain as determined by GST-pulldown assay (Fig. 9). We also detected that NF-κB p65 interacted with PXR DNA binding, D domain and E/F domain (Fig. 12).

Furthermore, using the ChIP assay, we found that the association of RXRα with the regulatory regions of *CYP3A4* was disrupted upon activation of NF-κB by either by LPS treatment or transient expression of p65, suggesting that the association between PXR/RXRα complex with DNA sequences was disrupted by NF-κB in vivo (Fig. 10).

Transcriptional activation of gene expression consists of multiple interconnected, yet distinct steps involving a constellation of transcriptional factors at different steps. For example, in regulation of *cyp1a1* gene expression, the regulatory steps that have been investigated include histone remodeling and modifications (Ke et al., 2001; Wang and Hankinson, 2002), recruitments of co-activator (Beischlag et al., 2002) and mediator complexes(Wang et al., 2004), recruitment of the positive transcriptional elongation factor (P-TEFb) which leads to phosphorylation of the C-terminal domain of the large subunit of RNA Pol II (Tian et al., 2003). It is highly likely that transcriptional regulation of *CYP3A4* is also subjected to the regulation at these critical steps by various signaling mechanisms including NF-κB activation.

Taken together, these in vitro and in vivo results suggest that activation of NF-κB results in disruption of the interaction of the PXR/RXRα complex with the consensus DNA sequences in the regulatory regions of *CYP3A4*, thus providing a mechanistic explanation for the observed suppression *CYP3A4* by LPS, proinflammatory cytokines and other stress signals that are known to induce NF-κB. The mechanism is depicted showing that NF-κB activation by physiological and pathological stimuli leads to its translocation into the nucleus where it interrupts the binding of PXR/RXRα complex to the cognate consensus DNA sequences thereby causing transcriptional suppression (Fig. 34). Since RXRα binding is interfered with by NF-κB, this mechanism of suppression by NF-κB may be extended to other nuclear receptor-regulated systems where RXRα is a dimmerization partner.

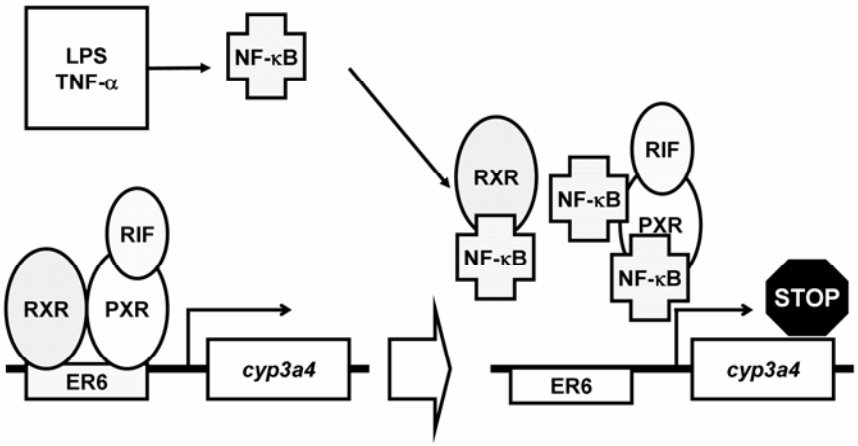

Fig. 34. Schematic illustration of the suppression of PXR transcription activity by NF-κB. Activation of NF-κB in response to LPS, proinflammatory cytokines and other stress signals results in disruption of the association of the PXR/RXRα complex with the consensus DNA sequences in the regulatory regions of *CYP3A4*, thus providing a mechanistic explanation for the observed suppression of *CYP3A4* by proinflammatory agents.

We also detected that other PXR-regulated gene MDR1, CYP2B6, CYP2C8, CYP2C9, EPHX1 had the same expression profiles as that of CYP3A4 gene in primary human hepatocytes in response to the proinflammatory agents. The results suggested that mechanism of NF-κB regulating PXR transcriptional activity could be extended to other PXR-regulated genes.

The interaction between NF-κB and PXR, RXRα may reciprocally interfere NF-κB p65 transcriptional activity, as seen in Fig. 11. The mechanism of how PXR suppresses NF-κB p65 transcriptional activity remains to be illucidated.

4.2 Interactions between AhR and PXR and/or RXRα enhanced PXR transcription activity and suppressed AhR transcription activity

In nature, we are typically exposed to xenobiotic mixtures, such as mixed environmental contaminants as well as co-administered multiple therapeutic drugs, which contain both PXR and AhR ligands. Pregnane X Receptor (PXR) and Aryl Hydrocarbon Receptor (AhR) are ligand-dependent transcription factors that regulate the

gene expressions of phase I, II drug metabolism enzymes and membrane-bound transporters. They are known to be regulated by distinct sets of ligands. Rifampicin (RIF), hyperforin (HYP) and clotrimazole (CTZ) are well characterized PXR ligands and 2, 3, 7, 8-Tetrachlorodibenzo-*p*-Dioxin (TCDD), β-naphthoflavone (b-NF), 3-methylcholanthrene (3-MC) and Benzo[a]pyrene (BaP) are known ligands for the Ah receptor.

In the current study, we found that activation of the AhR significantly or synergistically enhances the expression of PXR-regulated PXR/*CYP3A4-Luc*iferase reporter gene. We also observed the same profiles with the *CYP3A4* in primary human hepatocytes, *Cyp3a11* and *mGsta1* in the mouse studies. Activation of PXR represses the expression of AhR-activated luciferase reporter gene. We also observed the same profiles with *cyp1a1* in primary human hepatocytes, *mCyp1a1*, *mCyp1a2* (with the exception of AhR activation by TCDD) and *mCyp1b1* in the mouse studies. The results from the reporter gene assays suggest that the crosstalk between PXR and AhR pathway may happen at the transcriptional level. We further detected the increased enhancement of PXR-regulated reporter gene expression with the increases of overexpression of AhR, and the lowered enhancement of PXR-regulated reporter gene expression with the decreases of the level of AhR or ARNT mRNA with the targetted small interfering RNAs. The repression of the expression level of AhR-regulated luciferase gene comes with the increases of the PXR expression level in a ligand-dependent manner. The results suggest the PXR and AhR are directly involved in the crosstalk (Fig. 35). We detected that AhR interacted physically with PXR and RXRα in vivo and in vitro in a domain-specific manner, which may account for the cross-regulation of AhR and PXR target gene expression. How the interaction between AhR and PXR or RXRα regulates transcription activity of AhR and PXR bidirectioally is not clear and need further studies including looking at the recruitment of interaction partner to the promoter of the target genes.

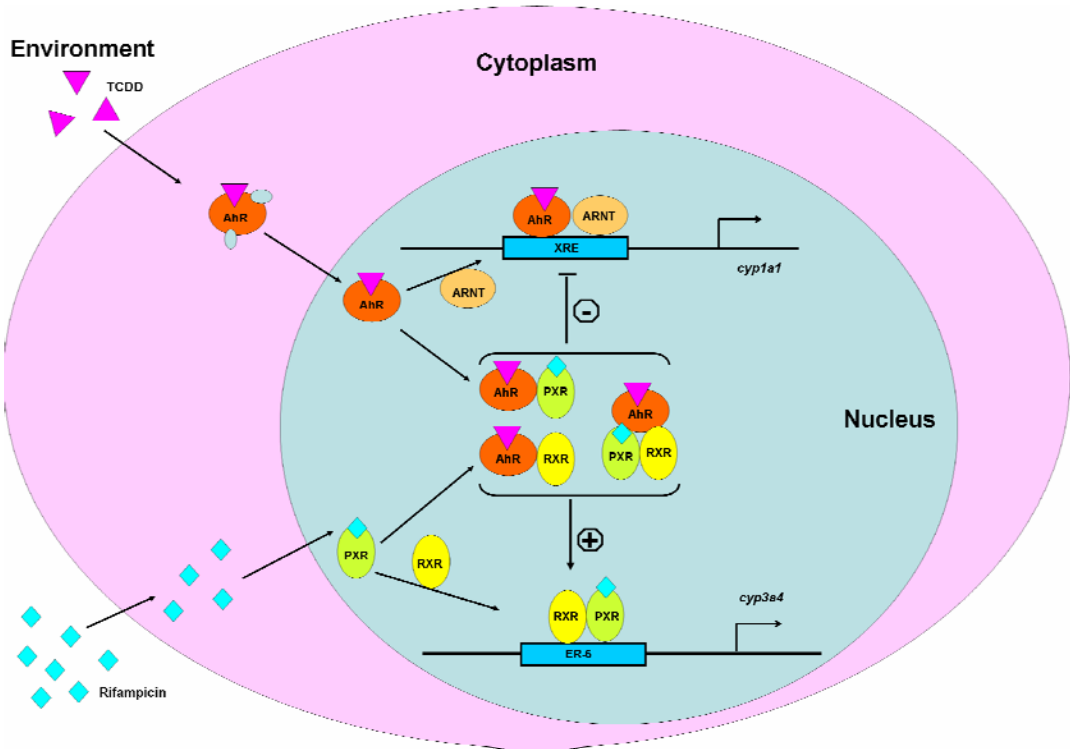

Fig. 35. Crosstalk between PXR and AhR pathways (Schematic model). Upon activation, AhR translocates to the nucleus and binds to XRE sequences with ARNT to activate the expression of its target genes. Upon activation, PXR activated the expression of its target genes. AhR interacts with PXR and/or RXRα and/or both to form complex (es), resulting in the enhancement of trancriptional activity of PXR and compromise of trancriptional activity of AhR.

We detected the functional and physical interaction between AhR and PXR. Both basic region and C-terminal of AhR are required to enhance the transcriptional activity of PXR by AhR (Fig. 27). However, how the basic region and C-terminal of AhR contribute to the enhanceability of AhR is unclear. We detected that PXR expression and activation repressed the transcription activity of AhR (Fig. 23). How the physical interaction between AhR and PXR impacts the transcription activity of AhR remains to be investigated.

Crosstalk betweem AhR and nuclear receptors may be a ubiquitous phenomena.

Crosstalk and the mechanism of the crosstalk between AhR and estrogen receptor pathways have been well documented. AhR signaling pathway interferes ER signaling

pathway. TCDD-induced degradation of ERα may contribute to the antiestrogenic activity of AhR agonists and this pathway may be involved in AhR-mediated disruption of other endocrine responses(Wormke et al., 2000). Both AhR and ER α may share ligands. Classical AhR ligands PCB and 3-MC directly activate ER α-dependent transactivation(Wormke et al., 2003; Abdelrahim et al., 2006). The ligand status of the AhR modulates activation of the BRCA-1 promoter by estrogen(Hockings et al., 2006). For the CAD gene, ERα/Sp1 and the AhR are constitutively bound to the CAD gene promoter, E2 enhances ERα-Sp1 interactions which are decreased by cotreatment with TCDD, thus the inhibitory AhR-ERα/Sp1 cross talk might be partly due to enhanced association of AhR and ERα, which coordinately dissociates ER and Sp1 and decreases ERα/Sp1-mediated transactivation, whereas remaining associated with the CAD promoter(Khan et al., 2006). Ligand-activated AhR/Arnt may modulate ER-mediated estrogen signaling, resulting in adverse estrogen-related actions of dioxin-type environmental contaminants. The modulation is through the direct association between agonist-activated AhR/Arnt heterodimer and estrogen receptors ERα or ERβ, and the association results in the recruitment of unliganded ER and the co-activator p300 to estrogen-responsive gene promoters, leading to activation of transcription and estrogenic effects. The function of liganded ER is attenuated. Estrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in AhR-/- or ERα-/- ovariectomized mice (Ohtake et al., 2003).

On the other hand, ER can also regulate AhR signal transduction. ER competites with AhR and for the rate-limiting co-regulators ERAP140 and SMRT or for nuclear factor-1 (NF-1). Competitive binding of NF-1 by estrogen-activated ER resulted in diminished TCDD-mediated CYP1A1 transcriptional activation (Reviewed in the reference (Pocar et al., 2005)). ERα is recruited to AhR/ARNT and work as a transcriptional co-repressor. AhR interacts directly with ERα, however, the reports on the interaction between ARNT and ERα are inconsistent(Klinge et al., 2000; Beischlag and Perdew, 2005). ERα also was reported to enhance the activity of AhR when being recruited to AhR in HuH7

human liver cells. ERa also plays a role in TCDD-dependent CYP1A1 expression(Matthews et al., 2005).

There are cross-talks between AhR and other steroid hormone receptors. Ligand-activated AhR has anti-androgenic effect in LNCaP prostate cancer cells. Interaction of the AhR ligand complex with AP-1 proteins resulted in diminished induction of prostate-specific-antigen (PSA) by testosterone. However, this was not caused by a decrease in intracellular levels of the androgen receptor (AR) or concentrations of intracellular dihydrotestosterone (DHT). The presence of AR within the ovary and endometrium would suggest the presence of a potential AhR/AR cross-talk to also be effective within female reproductive organs (Reviewed in the reference (Pocar et al., 2005)). A unidirectional inhibitory progesterone receptor (PR)/AhR cross-talk involves both PR isoforms, PR-A and PR-B, and repression of AhR–ARNT transcriptional activity requires the active progesterone responsive element (PRE)-binding form of PR-B, but not PR-A (Reviewed in the reference (Pocar et al., 2005)).

AhR signaling pathway also crosstalks with COUP-TF. AhR interacts directly with COUP-TF in a ligand-specific manner in vitro and in transfected CV-1 cells. In contrast, the AhR nuclear translocator protein (ARNT) did not interact with COUP-TF. Purified COUP-TFI bound the consensus XRE, suggesting a role for COUP-TF as a AhR/ARNT competitor for XRE binding. In transiently transfected MCF-7 human breast cancer cells, overexpression of COUP-TFI inhibited TCDD-activated reporter gene activity from the CYP1A1 promoter and COUP-TFI did not block the antiestrogenic activity of TCDD. The specific interaction of COUP-TF with XREs and AhR together with the inhibition of TCDD-induced gene expression by COUP-TF suggests that COUP-TF may regulate AhR action both by direct DNA binding competition and through protein-protein interactions(Klinge et al., 2000).

Crosstalk between AhR and PPARα has been documented. The negative cross-talk between AhR and PPARα took place on CYP4A and CYP1A. In HepG2 cells, PPARα and RXRα protein expression was decreased by AhR ligand Sudan III (S.III) treatment in a dose dependent manner. AhR has an inhibitory effect on PPARα function and a new

pathway by which AhR ligands could disturb lipid metabolism(Shaban et al., 2004). S.III treatment decreased basal and PPARα ligand clofibric acid (CA)-induced CYP2B, CYP3A and CYP2C11 protein, activities and mRNA expression in the livers of male Wistar rats(Shaban et al., 2005). Coexposure with 3MC and the PPARα ligand WY leads to an additive or potentiating effect on CYP1A1 inducibility, depending on the WY concentration. Furthermore, at high concentration (200 μM), WY induced AhR expression, which could explain the potentiating effect on CYP1A1 inducibility observed after addition of an AhR ligand (3MC). This phenomenon should be taken into account for risk assessment involving CYP1A1 induction(Fallone et al., 2005)

Crosstalk also takes place between SHP (short heterodimer partner) and AhR. SHP is an orphan nuclear receptor lacking a DNA binding domain that interacts with nuclear receptors (NR) including thyroid receptor (TR), retinoic acid receptors (RAR and RXR), and estrogen receptors α and β (ERα and ERβ). SHP acts as a negative regulator of these receptors by inhibiting DNA binding and transcriptional activation. In human endometrial carcinoma cells RL95-2, SHP inhibited TCDD-stimulated reporter activity from the AhR-responsive CYP1A1 and UGT1A6 gene promoters in a concentration-dependent manner. ARNT interacted directly with SHP in vitro, but AhR did not interact with GST-SHP. SHP inhibited AhR/ARNT-DNA binding in vitro. SHP may play a role for in the suppression of agonist-activated AhR/ARNT activity(Klinge et al., 2001).

Both the AhR and RA pathways regulate transcription of a variety of genes that are critical for the physiological effects mediated by these pathways (Murphy et al., 2007). However, crosstalk between AhR and Retinoids pathways has also been documented. There are several levels of molecular interactions between AhR and RA pathways, including ligand switch, direct inhibition of gene expression, alteration of receptor availability, and competition for transcriptional cofactors.

The interaction between AhR and Retinoids pathways results in some physiological effects. RA and RAR/RXR are required for the embryonic development of blood vessel and bone. The embryonic organogenesis of medaka fish was specifically inhibited by an inhibitor of RA synthesis (diethylaminobenzaldehyde), antagonists of RAR (Ro41-5253)

and RXR (Ro71-4595), agonist (β-naphthoflavone) and antagonist (α-naphthoflavone) of AhR, and excess RA(Hayashida et al., 2004). Livers from AhR -/- mice fed the vitamin A-deficient diet showed a decrease in collagen deposition, consistent with the absence of liver fibrosis(Andreola et al., 2004). Disruption of thyroid hormone and retinoid homeostasis is mediated entirely via AhR after exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Induction of UGT1A6 is thought to be responsible at least partly for reduced serum thyroid hormone levels in TCDD-exposed mice(Nishimura et al., 2005). Supplementation of vitamin A might attenuate the liver damage caused by TCDD(Yang et al., 2005b).

AhR and the AhR signaling pathway may be activated by pharmacological doses of some synthetic retinoids, which are activators for the retinoic acid receptor/retinoid X receptor pathway(Gambone et al., 2002; Soprano and Soprano, 2003). However, in SCC12Y cells TCDD treatment results in a decreased binding of all-trans RA to RARα without any change in *RAR*α gene expression(Lorick et al., 1998). The activated AhR exerts complex effects on the metabolism of estrogens and retinoids.

AhR and RAR/RXR mutually regulate their gene expression and thus alter the receptor level. The expression of the AhR is affected by retinoic acid(Wanner et al., 1995). RA and RAR/RXR are required for expression of AhR mRNA (Wanner et al., 1996; Hayashida et al., 2004). On the other hand, TCDD and the AhR pathway are known to alter the expression of the RARs and RXRs, although the effect of TCDD on *RAR* and *RXR* gene expression is receptor and cell-type dependent. RARβ expression is inhibited by TCDD exposure of embryonic palate mesenchymal cells(Weston et al., 1995). In normal human keratinocytes, TCDD treatment results in an increase in RARγ and RXRα mRNA levels(Murphy et al., 2004). Therefore, one way in which TCDD may alter all-trans RA target gene expression in some cell types may be through alterations of the receptor availability.

The activated AhR and retinoids pathways exert complex effects on the regulation of cognate genes. Exposure of TCDD, methylcholanthrene or benzo[*a*]pyrene inhibits all-trans RA-induced activation of transglutaminase in SCC-4 keratinocytes(Rubin and Rice,

1988). TCDD also demonstrates an inhibitory action toward other all-trans RA target genes, including RDH9(Tijet et al., 2006) and CRABPII(Weston et al., 1995). TCDD activates expression of retinal oxidase, the enzyme that catalyzes the conversion of retinal to RA. However, cotreatment with all-trans RA and TCDD results in the downregulation of retinal oxidase expression and activity(Yang et al., 2005a). Although the majority of data indicate that TCDD/AhR inhibit RA-mediated gene expression, there is growing evidence indicating that the interaction is more complex and may be tissue and cell-type specific.

Data from AhR knockout mice support the hypothesis that the AhR pathway interferes with expression of RA pathway target genes. For example, the expression of CRBPI is higher in the livers of AhR null animals than in their wild-type counterparts. In addition, all-trans RA levels are elevated in the livers of AhR null mice in comparison to wild-type mice(Andreola et al., 1997), which is coupled to a downregulation in CYP2C39 mRNA expression in the AhR null animals(Andreola et al., 2004). These data suggest that the AhR pathway, in the absence of exogenous ligand, is inhibitory toward the basal expression of genes that encode for proteins critical for retinoid homeostasis. Conversely, the RA pathway also has an inhibitory effect on AhR-mediated transcription, and one of the most extensively studied is the effect of all-trans RA on expression of CYP1A1. RARα$^{-/-}$ null animals display an increase in hepatic CYP1A1 activity after TCDD treatment compared to wild-type mice, suggesting that RARα may play an inhibitory role in TCDD-mediated CYP1A1 gene regulation (Hoegberg et al., 2005). Vitamin A inhibits CYP1A1 activity and mRNA expression of TCDD-induced Cyp1a1 and AhR in mice(Yang et al., 2005b).

AhR signaling pathway and retinoids signaling pathway interacted at transcriptional level. All-trans RA exposure is inhibitory to xenobiotic-induced CYP1A1 expression and activity, and that this inhibition is mediated through the RARE in the promoter(Wanner et al., 1996). TCDD exposure results in increased MMP-1 (matrix metalloproteinase-1, interstitial collagenase) expression in keratinocytes that is further enhanced by co-treatment with all-trans retinoic acid. TCDD-induced expression of

MMP-1 appears to be mediated through two AP-1 elements in the proximal promoter of the MMP-1 gene. However, retinoic acid-mediated induction of keratinocyte MMP-1 is a result of both promoter activation and increased mRNA stability. These findings are the first to demonstrate TCDD-induced expression of MMP-1 and to demonstrate interactions between the TCDD/AhR and retinoic acid pathways on MMP-1 expression(Murphy et al., 2004). The coactivation of all-trans RA and TCDD was also observed for PAI-2, a regulator of matrix remodeling, indicating that all-trans RA/TCDD coactivation is not limited to MMPs. TCDD inhibition of transglutaminase in the SCC-4 cells is mediated primarily at the level of transcription, and does not result from a change in mRNA stability(Krig and Rice, 2000). However, the mechanism of TCDD-induced interference of all-trans RA-induced transglutaminase expression is still unknown. Although it is clear that there are interactions between these pathways at the level of transcriptional activation, it is unclear how these interactions are accomplished.

The involvement of the corepressor as well as the coactivator proteins may provide a molecular pathway for the transcriptional cross talk between the AhR and RA pathways. TCDD exerted a dose-dependent effect on a retinoic acid-dependent reporter gene expressed in MCF-7 cells. AhR was shown to be involved in a mutual antagonism with RARα corepressor SMRT (silencing mediator of retinoid and thyroid receptors). This, and the documented physical interaction between AhR and SMRT suggested that SMRT sequestration by AhR might activate RARα in the absence of ligand. Concurring with this interpretation, an interaction in vitro between AhR and the PML protein (the core component of nuclear bodies) was observed. (Widerak et al., 2006). Recent evidence indicates that corepressors may be a link between the AhR and RA expression pathways. The SMRT corepressor is known to interact with both the AhR and RARs and modulate their transactivating function (Nguyen et al., 1999; Rushing and Denison, 2002; Widerak et al., 2006) . Further, SMRT may also be involved in TCDD-mediated effects on RAR binding and transactivation through the RARE.

In the study presented in this dissertation, the physical interaction between AhR and RXRα has been documented. The interaction between AhR and RXRα may also

contribute to the mutual regulation of transcription activity of AhR and RXRα, and will possibly provide an explanation for the dirrect regulation of the transcriptional activity of AhR and RXRα. However, the role of the interaction between RXRα and AhR in the regulation of PXR transcriptional activity is not clear.

Among AhR and PXR target genes presented in this dissertation, the expression of mouse *Gsta1* and *Cyp1a2* gene expression behaves differently from other target genes. Mouse *Gsta1* gene expression was known to be activated by mouse PXR (Maglich et al., 2002) and AhR (Ramadoss et al., 2005) alone. We first reported that *mGsta1* was coactivated synergistically by co-treatment of PXR agonist PCN and AhR agonist TCDD. The result suggests that there is an activating or coactivating interaction between PXR transcription activity and AhR transcription activity, resulting in the synergistic activation of *Gsta1* gene expression. However, the mechanism of the interaction between PXR and AhR transcriptional activity for the mouse *Gsta1* gene expression remains unclear. It might be different from the mechanism that accounts for the enhanced expression of PXR-regulated reporter gene expression and the repression of AhR-regulated reporter gene expression by the interaction between AhR and PXR or RXRα. For the gene expression profile of other AhR and PXR target genes in mice on the exposure to TCDD and PCN, the survey is going on.

There is no interaction between PXR and ARNT, or between RXRα and ARNT. However, the small interfering RNA targeting ARNT decreased the enhanceability of AhR, suggesting that ARNT is also involved into the regulation, might through its prototypical partner AhR. This is different from the case of ERα and ARNT. Brunnberg et al reported that ARNT (aryl hydrocarbon receptor nuclear translocator) functions as a potent coactivator of ERα- and ERβ- dependent transcription. The coactivating effect of ARNT depends on physical interaction with the ERs and involves the C-terminal domain of ARNT and not the structurally conserved basic helix-loop-helix and PAS (Per-ARNT-Sim) motifs. ARNT/ER interaction requires the E2-activated ligand binding domain of ERα or ERβ. Furthermore ARNT was recruited to a natural ER target gene promoter in a

estrogen-dependent manner, supporting a physiological role for ARNT as an ER coactivator(Brunnberg et al., 2003).

Crosstalk between AhR and nuclear receptors might be a common phenomenon. The extensive studies may provide clues to further study on the indivual crosstalk between AhR and each nuclear receptor, and uncover the underlying mechanism and the association with the physiological effects. In considering RXRα is a partner of many nuclear receptors including PXR, PPARs, RAR, VDR, FXR, LXR, RXR and CAR, the interaction between AhR and RXR may play important roles in regulation of transcription activity of these nuclear receptors and thus impact many important physiological functions in response to AhR and nuclear receptor ligands exposure.

Both AhR and PXR are regulators of many genes of phase I, II drug metabolism enzymes and transporters, understanding more on the crosstalk between AhR and PXR will help in drug development, chemotherapy and risk assessment.

4.3. A/B domain of PXR coactivated ligand binding domain of PXR and hinge domain corepressed PXR transcriptional activity

Nuclear receptors share a common structural organization. The N-terminal region (A/B domain) is highly variable, and contains at least one constitutionally active transactivation region (AF-1) and several autonomous transactivation domains (AD); A/B domains are variable in length, from less than 7 to more than 600 amino acids, and their 3D structures are not known(Robinson-Rechavi et al., 2003).

We have investigated the role of the domain or the combination of the domains of PXR in regulation of PXR transcription activity. The result showed that A/B domain of PXR activated the transcription of the reporter gene and co-activated the ligand-dependent activation (Fig. 28). This observation is consistant with that of other nuclear receptors.

The N-terminal A/B domain (AF-1) of estrogen receptor (ER) α and β is required in 17 β-estradiol-induced functional synergism between AF-1 and AF-2. The intact serine residue at position 118 (S (118)) in ER AF-1 can be phosphorylated by MAP kinase

(MAPK)(Tremblay et al., 1999). The physical interaction between COUP-TFI and hER α increases the affinity of hER α for ERK2/p42 (MAPK), resulting in an enhanced phosphorylation state of the hER α Ser118. hER α thus acquires a strengthened AF-1 activity due to its hyperphosphorylation(Metivier et al., 2002). Phosphorylation of AF-1 also leads to the recruitment of steroid receptor coactivator-1 (SRC-1) (Tremblay et al., 1999). AF-1 and AF-2 cooperatively recruit SRC-1 by both the AF-1 α-helical core and AF-2 regions and that it is stabilized by a direct interaction between the B and C-terminal domains. This interaction of SRC-1 with the AF-1 α-helical core is essential for both E2- and OHT-induced ERα activity(Metivier et al., 2001). ERα AF-1 domain interacts with a subfamily of RNA-binding DEAD-box proteins (p72/p68) in AF-2 associated coactivator complexes containing the SRC-1/TIF2 family, CBP/p300 and steroid receptor RNA activator (SRA) (Watanabe et al., 2001). However, neither any of the SRC-1/TIF2 family coactivators nor TRAP220/DRIP205 is potent, ligand-induced functional synergism between AF-1 and AF-2 is mediated through p300 by its direct binding to the A/B regions of ERα and ERβ(Kobayashi et al., 2000). ERα AF-1 domain is required for coactivation of ER by SRA (Deblois and Giguere, 2003). ERα AF-1 domain activity can be specifically coactivated by a putative human homologue of the yeast DNA repair and transcriptional regulator MMS19 (hMMS19) (Wu et al., 2001). Prenylated proteins (at least RhoA, RhoB and/or RhoC) antagonize the ability of ERα and ERβ potentially acting through interfering both AF-1 and AF-2 transcriptional activities(Cestac et al., 2005). ER AF-1 activity is enhanced through modification of AF-1-associated coactivator proteins by the Src/JNK pathway(Feng et al., 2001).

The transcriptional activity of nuclear retinoic acid receptors (RARs), which act as RAR/retinoid X receptor (RXR) heterodimers, depends on two activation functions, AF-1 and AF-2, which are targets for phosphorylations and synergize for the activation of retinoic acid target genes RARs through the cyclin-dependent kinase (cdk)-activating kinase (CAK) subcomplex (cdk7/cyclin H/MAT1) of the general transcription factor TFIIH (Rochette-Egly et al., 1997; Bour et al., 2005a). The RARα AF-1 and AF-2 activation functions, but not their phosphorylation sites, are involved in the induction of

RA-responsive genes in a differential promoter context-dependent manner(Rochette-Egly et al., 2000). p160 coactivator TIF2, not SRC-1, is able to bridge the two activation domains of the retinoic acid (RA) receptor isotype RARα1, resulting in synergistic activation of transcription. Bridging requires the presence of motifs in region A of RARα1 and in the activation domain AD1 of TIF2(Bommer et al., 2002). RARγ phosphorylation of the AF-1 domain might control RARγ-mediated transcription through triggering the dissociation of vinexin β which is a multiple SH3 motif-containing protein associated with the cytoskeleton and also present in the nucleus. (Bour et al., 2005b).

The HNF4 AF-1 interacts with multiple transcriptional targets, including the TATA-binding protein; the TATA-binding protein-associated factors TAFII31 and TAFII80; transcription factor IIB; transcription factor IIH-p62; and the coactivators cAMP-responsive element-binding protein-binding protein, ADA2, and PC4 that regulate distinct steps of transcription may provide a mechanism for synergistic activation of gene expression by AF-1 (Green et al., 1998). The amino-terminal region of TRβ1 contains an activation domain (AF-1) that can modulate the function of the receptor and may allow for the fine-tuning of receptor activity in various target tissues. N-terminal truncated rat TRβ1 was impaired in hormone-dependent activation in both yeast and mammalian cells. The truncated receptor displayed enhanced homodimer binding on several different TREs(Wilkinson and Towle, 1997). GR AF-1 is capable of recruiting both positive and negative regulatory factors that differentially regulate GR transcriptional enhancement. DRIP150 and DRIP205 functionally link GR AF-1 and AF-2, and represent important mediators of GR transcriptional enhancement(Hittelman et al., 1999). MR A/B domain functionally interacts with TIF2 and p300 in the cells(Fuse et al., 2000). NOR-1 transactivates gene expression in a cell- and target-specific manner; moreover, it operates in an activation function AF-1-dependent manner. The N-terminal AF-1 domain preferentially recruits the steroid receptor coactivator (SRC). Furthermore, SRC-2 modulates the activity of the AF-1 domain but not the C-terminal ligand binding domain (LBD). In contrast, the N-terminal AF-1 is necessary for

cofactor recruitment and can independently conscript coactivators. The purine anti-metabolite 6-mercaptopurine, a widely used antineoplastic and anti-inflammatory drug, activates NOR-1 in an AF-1-dependent manner(Wansa et al., 2003). Nur factors behave as endpoint effectors of the PKA signaling pathway acting through dimers and AF-1-dependent recruitment of coactivators(Maira et al., 2003).

The physiological effect of A/B domain of PXR is not known. The evidence on the physiological effects of the A/B domain of nuclear receptors remains to be accumulated. Analysis of deleted mutants of ERα indicates that the transcriptional activation function (AF)-1 is required for ERα-mediated transcription as well as for the inhibition of cell migration induced by cell adhesion on extracellular matrix (ECM) proteins. In addition, the nuclear localization signal region and some serine residues in the AF-1 of the ERα are both required for the regulation of cell invasiveness as observed in HeLa cells(Sisci et al., 2004). The physiological role of A/B domain (AF-1) of HNF4α has been first investigated in vivo using knock-in mice of HNF4α1 with AF-1 transactivation domain and HNF4α7 without AF-1 transactivation domain. Hnf4α gene disruption causes embryonic lethality. The 'α7-only' and 'α1-only' mice are viable, indicating functional redundancy of the isoforms. However, the former show dyslipidemia and preliminary results indicate impaired glucose tolerance for the latter, revealing functional specificities of the isoforms(Briancon and Weiss, 2006).

Between the DNA-binding and ligand-binding domains is a less conserved region (D domain) that behaves as a flexible hinge between the C and E domains, and contains the nuclear localization signal (NLS), which may overlap on the C domain. The hinge (D) domain which is located between the DNA binding (C) domain and the ligand binding (EF) domain, is less conserved among the nuclear receptors. The largest domain is the moderately conserved ligand-binding domain (LBD, E domain), whose secondary structure of 12 α-helixes is better conserved than the primary sequence. The E domain is responsible for many functions, mostly ligand induced, notably the AF-2 transactivation function, a strong dimerization interface, another NLS, and often a repression function. Nuclear receptors may or may not contain a final domain in the C-terminus of the E

domain, the F domain, whose sequence is extremely variable and whose structure and function are unknown(Robinson-Rechavi et al., 2003).

We also detected that D domain of PXR repressed the activation of A/B domain and/or ligand-binding domain. In the presence of ligand, the constructs containing D/E/F domains were activated. It is not clear whether the activation was due to the removal of the suppressive property of the D domain or the suppressive property was overcomed by the activation of ligand-binding domain. The physiological role of the D domains of PXR is not known. The role of D domain of other nuclear receptors has been reported.

The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT(Jackson et al., 1997). TR EF domain lacked T3 binding activity and additional D domain was required for its ligand binding. The D domain of TR is required but that of RXR is not necessary for the heterodimerization. The D domain was required for receptor specific DNA recognition. The D domain of TR cannot substitute for that of VDR in context of specific DNA recognition. These data suggest that the D domain is important to maintain the integrity of the functional structure of the nuclear receptors with regard to ligand binding, protein-protein interaction and DNA recognition. (Miyamoto et al., 2001). The TR D-domain has the potential to form functionally important extensions of the DBD and LBD or unfold to permit TRs to adapt to different DNA response elements. Mutations of the D domain LXXLL-like motif indeed selectively inhibit TR interactions with an inverted palindromic response element (F2) in vitro and TR activity at this response element in cell-based transfection experiments(Nascimento et al., 2006).

The AR hinge region exerts its repressor effect on ligand-activated and coactivator-mediated AF2 activity of the AR LBD(Wang et al., 2001). AR gene mutations identified in human prostate cancer and the autochthonous transgenic adenocarcinoma of the mouse prostate model that colocate to residues (668)QPIF (671) at the boundary of the hinge and ligand-binding domain, resulting in receptors that exhibit 2- to 4-fold increased activity compared with wild-type AR in response to dihydrotestosterone,

estradiol, progesterone, adrenal androgens, and the AR antagonist, hydroxyflutamide, without an apparent effect on receptor levels, ligand binding kinetics, or DNA binding. The expression of these or similar variants could explain the emergence of hormone refractory disease in a subset of patients. Homology modeling indicates that amino acid residues (668) QPIF (671) form a ridge bordering a potential protein-protein interaction surface. The naturally occurring AR gene mutations reported in this study result in decreased hydrophobicity of this surface, suggesting that altered receptor-protein interaction mediates the precocious activity of the AR variants(Buchanan et al., 2001).

Sepecific residues in the D domains of LRH-1 are downstream targets of mitogenic stimuli which may contribute to proliferative functions of LRH-1(Xu et al., 2003). A domain in hinge region imposes a strong repression on the transcriptional activity of hB1F, which is important for the function of hB1F on regulating the activity of HBV enhancer II/core promoter. Mutations of the core residues in this domain abrogate the repression. Moreover, the repression is not affected by the silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) and steroid receptor coactivator 1 (SRC-1)(Xu et al., 2003).

We have investigated the co-activational effect of A/B domain and the co-repressive effect of D domain of PXR on PXR transcriptional activity. Like in the other nuclear receptors, such as ER, These domains may interact with coactivators or corepressors to exert their effects. What co-factors are included and how they interact with these domains remain to be investigated.

4.4 Green tea polyphenols enhanced PXR-activated CYP3A4-Luc gene expression

Tea is a natural and historic beverage, consumed worldwide although at greatly varying levels. Tea is now grown in ~30 countries, but geologic and botanic evidence suggest that the tea plant originated from China. As one of the most ancient and commonest beverages, tea has been consumed for thousand years in the orient and plays a central part in Chinese culture, with tea-drinking practices specific to different areas. Among three types of tea green tea, oolong tea and black tea with different types and

concentration of polyphenols, green tea is prepared in such a way as to preclude the oxidation of green leaf polyphenols. Green tea, which is the main tea beverage in Japan and many parts of China, accounts for ~20% of worldwide tea production, whereas <2% of tea production is oolong tea, consumed mainly in southern China and Taiwan. including the southeastern region of China(Graham, 1992; Phipps, 1999; Zhang et al., 2006). Tea is one of the most consumed beverages worldwide, and its beneficial effects on health have been documented(Liao et al., 2001; Cooper et al., 2005a; Cooper et al., 2005b; Fujiki, 2005; Shimbo et al., 2005; Pastore and Fratellone, 2006).

In China, there is a common sense that no drinking tea while taking medicine mainly due to that the tannins in the drinking tea react with components of the medicine. Still, there id drug metabolism enzyme system in the body, they may deactivate the medicine and cause undesired drug-drug interactions. The effect of green tea polyphenols on the activity and regulation of drug metabolism enzymes has been studied and reporterd. Decaffeinated green tea (Camellia sinensis) is unlikely to alter the disposition of medications primarily dependent on the CYP2D6 or CYP3A4 pathways of metabolism(Donovan et al., 2004). Green tea drinking inhibited CYP1A activity by green tea polyphenols. Green tea may work to biotransform CYP1A inducing carcinogens into non-carcinogenic metabolites by modulation of other microsomal enzymes rather than CYP1As(Yang et al., 2003). In LS-180 cells green tea extract (GTE), but not epigallocatechin gallate (EGCG), significantly induced CYP1A2 mRNA expression, whereas neither CYP1A1 nor CYP3A4 mRNA expression was modulated by GTE or EGCG. In Caco-2 cells CYP1A1 as well as CYP1A2 mRNA expression was significantly increased in a dose-dependent manner by GTE and EGCG. GTE or EGCG significantly inhibited CYP1A2 and CYP3A4 function in a dose-dependent manner. Therefore, it appears that green tea moderately modulates the expression of drug-metabolizing enzymes but non-specifically inhibits the function of human CYPs(Netsch et al., 2006). Individual polyphenols as well as polyphenol-rich plant extracts may affect phase I and II enzyme expression by distinct mechanisms that must be elucidated(Kluth et al., 2007). Catechins do not induce CYP3A4 activity.The induction effect of catechins

on UGT1A1 seems to be modest and highly variable. The effect of acute and prolonged use of green tea on the pharmacokinetics of irinotecan in patients remains to be evaluated(Mirkov et al., 2007).

We detected that green tea polyphenols (GTPs) extract alone didn't induce PXR-mediated CYP3A4-Luc gene expression. They enhanced RIF-induced CYP3A4-Luc gene expression in a dose dependent manner. The result suggests that the components polyphenols of the green tea may enhance the expression of CYP3A4 gene expression induced by PXR-agonists in the medicine. This mechanism probably adds a new explanation into the common sense.

4.5 Pharmacological doses of dietary supplements probably impose little drug-drug interaction

Among extracts surveyed, extracts of Ginger Root, Cats Claw, Dong Quai, Valerian Root, Olive Leaf and Damiana obviously induced PXR-mediated CYP3A4-Luc gene expression. Extracts of Cranberry, Fever Few, Cascara Sagrada and Ginkgo Biloba slightly induced PXR-mediated CYP3A4-Luc gene expression. The Valerian Root extract (5000μg/ml) had the most potency of the induction. The doses of the Valerian Root extract at 5000μg/ml and 2000μg/ml which induced higher expression of lucuferase than rifampicin are much higher than those possible concentrations the target organs can gain. The dose of the Valerian Root extract at 670μg/ml has some inducibility which is less that rifampicin, and the dose of 200μg/ml has marginal inducibility, compared with DMSO.

However, the recommended dose of the Valerian Root extract is one capsule containing 500mg extract daily at bed time. The concentration of the plasma can be estimated by assuming the distribution volumes (Table 14).

The highest plasma concentration of 500mg extract is 167μg/ml according to the estimation, at which marginal induction of PXR-mediated CYP3A4-Luc gene expression was extrapolated. Thus, the recommended dosage for the consumers will not cause the unexpected PXR-mediated gene expression. However, there is uncertainty in this

estimation which doesn't include the value of tissue binding. If the components of high potency for induction have high affinity to the liver, the concentration of the components of high potency will be high to induce the PXR-mediated gene expression. Therefore, the data on the pharmacokinetics of the Valerian Root extract is desirable.

Table 14 Estimation of the plasma concentration of 500mg Valerian Root extract distributing in 70-kg human body*

Compartment	Volume (L)	Concentration (μg/ml)
Plasma water	3	167
Total extracellular water	14	36
Total body water	38	13
Total body	69	7
Tissue binding	?	?

* The reference values come or are derived from (Casarett et al., 2001).

4.6 Aflatoxin B1 enhanced activated-PXR-induced CYP3A4-Luc gene expression

Fungal toxin aflatoxin B_1 (AFB1) has been found mainly in the dietary and several epidemiological studies have established a strong association between dietary aflatoxin B_1 exposure and the development of primary hepatocellular carcinoma(Van Rensburg et al., 1985; Peers et al., 1987; Kolars, 1992; Wogan, 1992) . Although AFB1 is best known as a hepatocarcinogen, epidemiological studies have shown a positive association between human lung cancer occurrence and inhalation exposure to AFB1(He et al., 2006). The worldwide human exposure to aflatoxin B1 (AFB1), particularly in developing countries, remains to be a serious public health concern. The balance between bioactivation to and detoxification of the epoxide determined its effects on human hepatic and extrahepatic carcinogenesis, thus the metabolism of AFB1 plays a key role in mediating the carcinogenicity of AFB1. The induced expression of drug metabolism enzyme genes has quantatively determinant effect on the metabolism activity. The effect of AFB1 on the activity and cytochrome P450s has been studied.

AFB_1 is activated by hepatic cytochrome P450-dependent monooxygenases leading to the formation of several forms of AFB_1 metabolites (Massey et al., 1995). CYP3A4

appears to have a relatively low affinity for AFB1 epoxidation and is primarily involved in AFB1 detoxification through AFQ1 formation in human liver microsomes (Gallagher et al., 1994). CYP3A4 and CYP3A7 have essentially similar capacities to activate AFB1 to produce toxic metabolites(Hashimoto et al., 1995). The CYP3A5 polymorphism is associated with increased levels of the mutagenic AFB1-exo-8, 9-epoxide, particularly in individuals with low CYP3A4, and this may modulate individual risk of HCC(Wojnowski et al., 2004). Cytochrome P450 (CYP)-catalyzed metabolic activation is required for AFB1 to exert its carcinogenicity. The hepatic carcinogen aflatoxin B1 (AFB1) is metabolized in the liver by at least four different P450s, all of which exhibit large interindividual differences in the expression levels. These differences could affect the individual risk of hepatocellular carcinoma (HCC). P450 3A4 contributed a majority of AFBO and AFQ1, and its expression level was the most important determinant of the AFB1 disposition toward these primary metabolites. P450 3A5, which exclusively produced AFBO, was the second-most important enzyme activating AFB1 to AFBO, followed by P450 3A7 and P450 1A2. The relative contribution of AFBO by P450 3A5 strongly depended on the concomitant expression of P450 3A4, and it was as high as 15% in a P450 3A5 high expressor with the lowest P450 3A4 expression of all livers. The P450 1A2-specific AFB1 detoxification product AFM1 was not detected. P450 3A4 expression is the most important determinant of AFB1 activation to AFBO. The contribution of P450 1A2 to AFB1 metabolism appears to be negligible and may have been overestimated. Targeted chemoprevention of AFB1-associated HCC should consider P450 3A4 inhibitors and avoidance of P450 3A4 inducers(Kamdem et al., 2006). CYP2A13-catalyzed metabolic activation in situ may play a critical role in human lung carcinogenesis related to inhalation exposure to AFB1(He et al., 2006).

In a cell-based system, we detected that Aflatoxin B1 alone has marginal effect on the PXR-mediated CYP3A4-Luc gene expression, and enhanced PXR-induced CYP3A4-Luc gene expression by rifampicin. The data suggests that co-exposure of AFB1 and PXR ligand will result in the enhanced CYP3A4 or other PXR-induced gene expression,

and has more complicated effects on the AFB1 metabolism. The carcinogenecity of the AFB1 under this circumstance should be reassessed.

4.7 Effects of metal ions on PXR-induced CYP3A4 gene expression

We detected that $CuSO_4$ and $MnCl_2$ enhanced rifampicin induced luciferase expression (Fig. 34A, B) while $CuSO_4$ and $MnCl_2$ alone had little effects on the PXR-mediated CYP3A4-Luc gene expression (Fig. 34B). This is not the first report on the regulation of drug metabolism gene expression by metal ions. Metal activation of gene expression through several signal transduction pathways, including As (V) induction of GST Ya, FOS, XRE, NFκBRE, GADD153, p53RE, and CRE; Pb (II) induction of GST Ya, XRE, Cyp1A1, and GADD153; Cd (II) induction of NFκBRE, Cyp1A1, XRE, and GST Ya; and Cr (VI) induction of p53RE, XRE, GADD45, HSP70, and CRE promoters (Tully et al., 2000). The effect of copper on the levels of MT2A, HSPA1A, CYP1A1 and HMOX1 expression(Song and Freedman, 2005). The trace metals could influence the carcinogenicity of the PAHs by altering their extent of induction of cytochromes P4501A1, 1A2, and 1B1 (CYP). Both transcriptional and post-translational mechanisms are involved in the trace metal-mediated down regulation of the CYP1 forms. The latter mechanism incorporates induction of heme oxygenase-1 by the metals, with resultant heme catabolism. Thus, trace metals could diminish the carcinogenicity of PAHs(Kaminsky, 2006). However, the mechanism of how metal ions regulated drug metabolism gene expression remains to be investigated.

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