XENOBIOTIC REGULATION OF THE ATP BINDING CASSETTE TRANSPORTER ABCB6 AND ITS SIGNIFICANCE TO HEPATIC HEME HOMEOSTASIS

BY

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ABSTRACT

Heme is indispensable for mammalian life. It is an essential component of numerous heme proteins, with functions including oxygen transport and storage, energy metabolism, drug and steroid metabolism and signal transduction. Under normal physiological conditions intracellular free heme levels are extremely low because increased levels of free heme are cytotoxic and accordingly, heme biosynthesis is tightly regulated. Although, 5-aminolevulinic acid synthase (ALAS) mediated regulation of heme synthesis is considered the key step in heme biosynthesis, recent reports have identified a second regulatory step in heme biosynthesis mediated by the mitochondrial ATP binding cassette transporter b6 (Abcb6). Abcb6 expression is directly related to enhanced *de novo* porphyrin biosynthesis, and Abcb6 overexpression activates the expression of genes important for heme biosynthesis. Thus, Abcb6 represents a previously unrecognized rate-limiting step in heme biosynthesis.

The dissertation outlines the progress made since its initiation in understanding the mechanism(s) that regulate Abcb6 expression and the significance of Abcb6 expression to cellular heme homeostasis.

Exposure to therapeutic drugs and environmental contaminants leads to an increase in heme demand to compensate for the increased expression of the heme-dependent cytochrome P450s (P450s) detoxifying enzymes. Cells respond to this increasing heme demand by increasing heme synthesis. Thus, exposure to environmental contaminants serves as an optimal *in vivo* and *in vitro* model system to study mechanisms that regulate heme synthesis. In this model, Abcb6 expression was induced in response to exposure to xenobiotics [polyaromatic hydrocarbons (PAHs), 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and pregnenolone 16α-carbonitrile (PCN)] suggesting a co-ordinate induction of Abcb6 to support the increased heme synthesis. Increased Abcb6 expression in response to cellular heme

demands was mediated by the xenobiotic sensing nuclear receptors aryl-hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), and the pregnane-X receptor (PXR).

Exposure to environmental contaminants also leads to the generation of oxidative stress, a primary mechanism by which these compounds cause cellular damage. Cells respond to this increased oxidative stress by activating anti-oxidant defense mechanisms, whose principal components include hemo-proteins (such as catalase, superoxide dismutase, etc). Arsenic, an environmental contaminant and a major hazard following occupational exposure exerts its chronic toxicity through the generation of reactive oxygen species. Of importance, exposure to arsenic also activates the antioxidant defense mechanism. Thus, exposure to arsenic serves as a good model system to evaluate *in vivo* and *in vitro* oxidative stress response. In this model system, sodium arsenite induced Abcb6 expression in a dose-dependent manner both in mice fed sodium arsenite in drinking water and in cells exposed to sodium arsenite *in vitro*. Arsenite-induced Abcb6 expression was transcriptionally regulated but was not mediated by the redox sensitive transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2).

The significance of Abcb6 expression to cellular heme homeostasis under conditions of heme demand was evaluated *in vitro* by both gain of function (cells engineered to overexpress Abcb6) and loss of function (cells where endogenous Abcb6 expression was knocked down using Abcb6 specific ShRNA) analysis. Loss of Abcb6 expression in these *in vitro* model systems significantly compromises the ability of cells to respond to increased heme demand and the ability to protect against oxidative stress following exposure to environmental contaminants.

To understand the significance of Abcb6 function to heme homeostasis *in vivo*, we generated mice carrying homozygous deletion of the Abcb6 allele (Abcb6 null mice). Abcb6 null animals appear phenotypically normal with a trend towards decreased hepatic heme levels,

although, the decreased heme levels did not appear to be statistically significant. Interestingly however, Abcb6 null mice demonstrate genotypic changes that suggest a role for Abcb6 in lipid and cholesterol homeostasis. Abcb6 null mice have increased fasting serum cholesterol and increased accumulation of androstone metabolites. Abcb6 null mice also show decreased expression and activity of a specific set of P450s suggesting a role for Abcb6 in drug metabolism and disposition.

Mitochondrial ABC transporters are difficult to study because of the two-membrane architecture of mitochondria, problems associated with analyzing transport process, and the high abundance of other ATPases and carriers/transporters. Thus, the development of an *in vitro* system with pure and active protein is a prerequisite toward understanding the mechanistic relationships between ATP binding and hydrolysis and coupling of these events to translocation of substrates across the lipid membranes. Towards this end, we developed an *in vitro* liposomal transport system with pure and active Abcb6 protein. Reconstitution of Abcb6 into liposomes allowed biochemical characterization of the ATPase including (i) substrate stimulated ATPase activity (ii) transport kinetics of its proposed endogenous substrate coproporphyrinogen III and (iii) transport kinetics of substrates identified using a HTS assay.

In summary, this dissertation provides insight into the mechanisms that regulate Abcb6 expression in response to increasing heme demand and the *in vitro* significance of Abcb6 to cellular heme homeostasis. Development of the Abcb6-null mice suggests that loss of Abcb6 does not severely affect heme-dependent functions in the liver probably because of the activation of compensatory mechanisms that balance the loss of Abcb6. More interestingly, Abcb6-null mice show a phenotype that is characteristic of the deficiency of a protein that is involved in cholesterol and lipid homeostasis. Development of the Abcb6-null mice and the development of an *in vitro* system with purified Abcb6 should serve as useful tools to understand the transport function of Abcb6 and its role in normal and patho-physiology.

Dedicated to my grandparents:

The late Shankarrao and Kaushlya Chavan

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LIST OF ABBREVIATIONS

Abbreviation	Full name
3-AT	3-Aminotriazole
Abc	ATP-binding cassette
AhR	Aryl hydrocarbon Receptor
ALA	Delta-aminolevulinate
ALAD	Aminolevulinate dehydratase
ALAS1	Delta-aminolevulinate synthase 1
ALAS2	Delta-aminolevulinate synthase 2
CAR	Constitutive Androstane Receptor
CFTR	Cystic fibrosis transmembrane conductance regulator
COPIII	Coproporphyrinogen III
CPO	Coproporphyrinogen oxidase
DCF	2, 7-dichlorodihydrofluorescein diacetate
DUH	Dyschromatosis universalis hereditaria
FECH	Ferrochelatase
FLVCR	Feline leukemia virus subgroup C cellular receptor
FP	Familial Pseudohyperkalemia
HAP1	Heme activator protein 1
HMB	Hydroxymethylbilane
HMT1	Heavy metal tolerance factor 1
HO	Heme oxygenase
HRI	Heme-regulated inhibitor kinase
HRM	Heme Regulatory Motif
IRP2	Iron Regulatory Protein 2
Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
Mdr	Multidrug resistance protein
MDZ	Midazolam
MFS	Major facilitator superfamily
Mrp	Multidrug resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced

NBD	Nucleotide binding domain
Nrf2	Nuclear factor E2-related factor 2
P450	Cytochrome P450
PAH	Polyaromatic hydrocarbon
PBG	Porphobilinogen
PBGD	Porphobilinogen dehydratase
PBR	Peripheral-type benzodiazepine receptor
PCN	5-pregnen-3β-ol-20-one-16α-carbonitrile
POR	Cytochrome P450 reductase
PPIX	Protoporphyrin IX
PPO	Protoporphyrin oxidase
PXR	Pregnane X receptor
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCPOBOP	(1,4-bis(2-(3,5-dichloropyridyloxy))benzene)
TMD	Transmembrane domain
UPLC-TOFMS	Ultra-performance liquid chromatography time-of-flight mass spectrometry
UROIII	Uroporphyrinogen III
UROIIIS	Uroporphyrinogen III synthase

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LIST OF FIGURES

The portions of this dissertation are reproduced from the following publications, with permission from the publishers:

1. **Chavan H**, Oruganti M, & Krishnamurthy P (2011) The ATP-binding cassette transporter Abcb6 is induced by arsenic and protects against arsenic cytotoxicity. *Toxicol Sci* 120(2):519-528.

2. **Chavan H** & Krishnamurthy P (2012) Polycyclic aromatic hydrocarbons (PAHs) mediate transcriptional activation of the ATP binding cassette transporter Abcb6 gene via the aryl hydrocarbon receptor (AhR). *Journal of Biological Chemistry* 287(38):32054-32068.

3. **Chavan H**, Taimur Khan MM, Tegos G, & Krishnamurthy P (2013) Efficient Purification and Reconstitution of ATP Binding Cassette Transporter B6 (Abcb6) for Functional and Structural Studies. *Journal of Biological Chemistry* 288(31):22658-22669.

Chapter 1. **BACKGROUND AND INTRODUCTION**

1.1. Heme as a magnificent molecule with multiple functions

Heme and porphyrins are vital for cell survival. Heme, the iron and protoporphyrin IX complex senses and utilizes oxygen in nearly all living cells. It is an essential component of various hemoproteins, including those involved in oxygen transport and storage (hemoglobin, myoglobin), mitochondrial electron transport (complexes II-IV), drug and steroid metabolism (cytochromes), signal transduction (nitric oxide synthases), and regulation of antioxidant-defense enzymes (1).

Heme is also a regulatory molecule; its intracellular localization (cytosolic vs nuclear) and concentration affects gene transcription and translation (1). Binding of heme to selected transcription factors such as HAP1 and Bach1 markedly alters gene expression patterns, orchestrating cell-wide multi-component responses to different stimuli. In erythroid cells, heme deficiency results in inhibition of protein synthesis, because of the activation of the heme-regulated inhibitor kinase (HRI) (2). Iron concentration in the cell is sensed and regulated by the heme-mediated oxidization and subsequent degradation of iron regulatory protein 2 (IRP2) (3). Heme also binds to certain types of potassium channels, thereby inhibiting transmembrane K+ currents (4).

While heme and porphyrins are considered as the “pigments of life” and are essential for life, excess intracellular heme, porphyrins or both is highly toxic to cells. The damaging effect of excess heme is, at least in part, due to its iron-induced pro-oxidant effect on DNA, proteins, membrane lipids, and the cytoskeleton (5-9). This effect is caused by iron catalyzing the Fenton reaction. An elevated level of non-iron protoporphyrin has been linked to numerous diseases and pathologic conditions (e.g. anemia, lead poisoning and thalassemias) (10-17). The damaging effect of excess protoporphyrins is caused by their ability to absorb energy leading to

photosensitization. Photosensitizers, such as protoporphyrin, accumulate in membranes and upon exposure to light (430–635 nm) cause the release of singlet oxygen, which causes cellular damage. Therefore, the amount of cellular heme and porphyrins need to be tightly regulated. Cellular heme homeostasis is achieved by a fine balance between biosynthesis, utilization (for hemoproteins), transport (into and out of cells and organelles to prevent porphyrin over accumulation) and catabolism (by hemeoxygenase).

1.2. Pathologies associated with disruption of heme homeostasis

Heme synthesis is orchestrated by eight enzymes and few transporters in a regulated manner. Any defect in the heme biosynthesis pathway enzymes (except *ALAS1*) can alter porphyrin homeostasis and lead to accumulation of porphyrins, clinically known as the porphyrias. Table 1.1 provides a list of pathologies associated with defects in enzymes of heme biosynthesis.

Effect on heme biosynthesis can alter the hemoprotein function; one such example is P450, a major drug metabolism enzyme family. In 1972 Kappas *et al.* showed that lead and methyl mercury affected heme biosynthesis in rats and as a consequence their P450 enzymes were decreased which affected hexabarbital metabolism and increased animals sleeping time after hexabarbital administration (18). In another case of acquired heme deficiency by lead acetate Meyer *et al.* showed that heme deficiency decreases content and heme saturation of Cyp1a (19). *PBGD*$^{-/-}$ mice also showed resistance to phenobarbital induced change in P450 activity (20). HL-60 cells with succinylacetone were demonstrated to decrease the activity of another hemoprotein, catalase, but the mechanism of this decrease has not been defined.

Table 1.1. Pathologies associated with disruption of heme homeostasis

DISEASE/DISORDERS/PHENOTYPE	GENE
PORPHYRIAS	
5-Aminolevulinic acid dehydratase porphyria	*5-Aminolevulinic acid dehydratase*
Acute intermittent porphyria	*Porphobilinogen deaminase*
Congenital erythropoietic porphyria	*Uroporphyrinogen III cosynthase*
Porphyria cutanea tarda	*Uroporphyrinogen decarboxylase*
Hepatoerythropoietic porphyria	*Uroporphyrinogen decarboxylase*
Hereditary coproporphyria	*Coproporphyrinogen oxidase*
Variegate porphyria	*Protoporphyrinogen oxidase*
Erythropoietic protoporphyria	*Ferrochelatase*
ANEMIA	
X-Linked Sideroblastic Anemia	*5-aminolevulinic acid synthase (ALAS2)*
X-Linked Sideroblastic Anemia with Ataxia	*ABCB7*
OTHERS	
Cyp2a5	*Porphobilinogen deaminase*
Lead Poisoning	*5-Aminolevulinic acid dehydratase*
Hepatorenal tyrosinemia	*5-Aminolevulinic acid dehydratase*
Ocular Coloboma	*Abcb6*

1.3. Cellular heme homeostasis

Cellular heme homeostasis is achieved by a fine balance between biosynthesis, catabolism (by hemeoxygenase), utilization (for hemoproteins) and transport (into and out of cells and organelles to prevent porphyrin over accumulation).

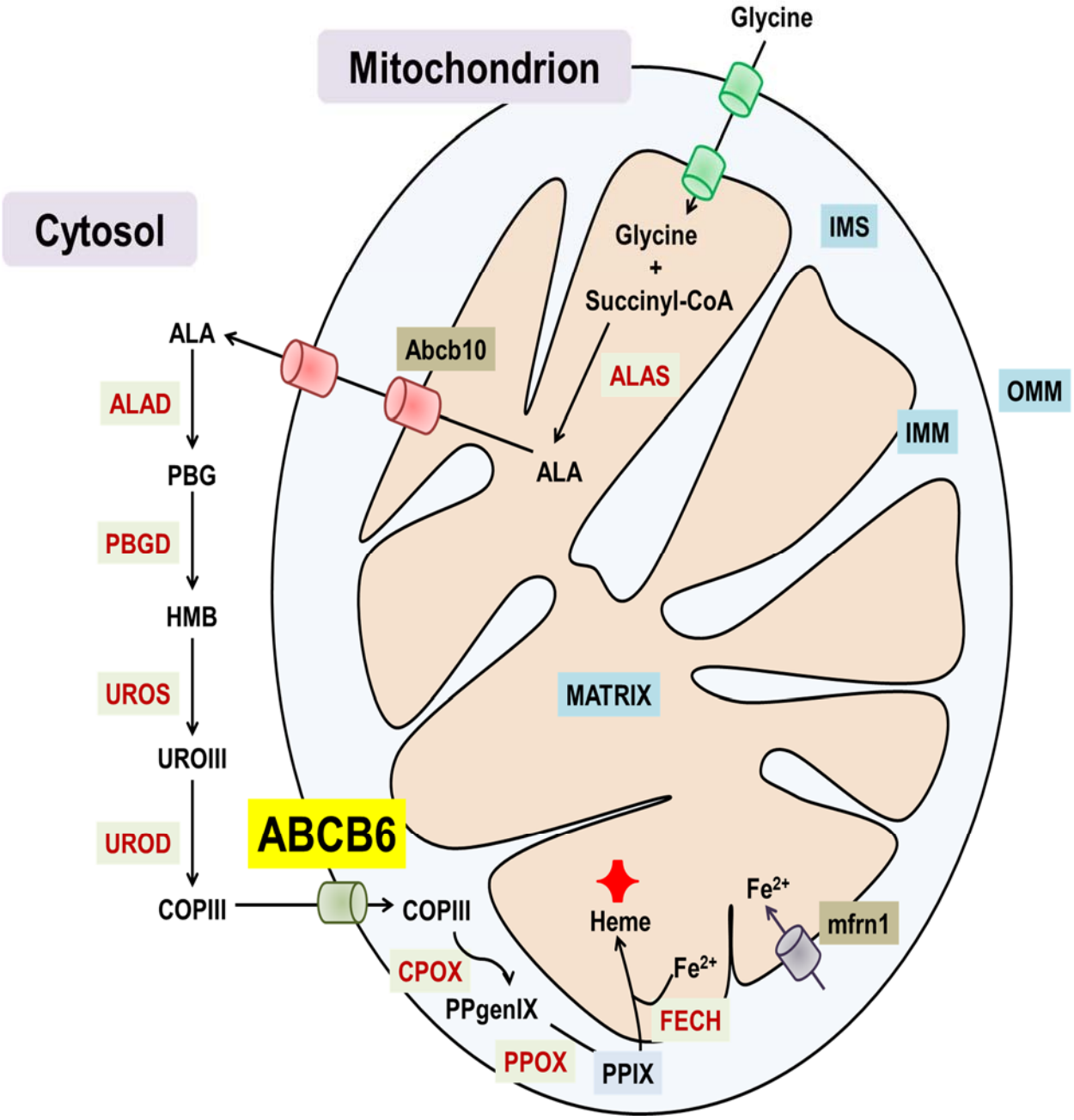
1.3a. Heme biosynthesis

The biosynthesis of heme, identical in all mammalian cells, is initiated from simple molecules and comprises eight enzymatic steps (Figure 1.1). David Shemin and Albert Neuberger first elucidated that heme biosynthesis begins with condensation of glycine and Succinyl CoA to form 5-aminolevulinic acid (ALA) (21,22). This reaction is catalyzed by ALA synthase (ALAS) which is located in the mitochondria and is the rate limiting enzyme of the pathway. ALA synthesized in the mitochondria is thought to be transported by the mitochondrial ABC transporter Abcb10 that localizes to the inner mitochondrial membrane (23).

Once in the cytosol, condensation of two ALA molecules forms the monopyrrole porphobilinogen (PBG). This reaction is catalyzed by aminolevulinate dehydratase (ALAD) (24,25). Four molecules of PBG form tetrapyrrole with the help of cytosolic porphobilinogen deaminase (PBGD) (26,27). The tetrapyrrole polymer formed is unstable and designated as hydroxymethylbilane (HMB). HMB serves as the substrate for the next enzyme in the heme synthesis pathway, uroporphyrinogen III synthase (UROIIIS). UROIIIS catalyzes the conversion of HMB to uroporphyrinogen III (UROIII) which is converted to coproporphyrinogen III (COPIII) by uroporphyrinogen decarboxylase (UROD) (27).

Cytosolic COPIII is an anionic molecule and requires active transport into the mitochondria which is mediated by the outer mitochondrial ABC transporter Abcb6 (28,29). Once inside the mitochondria oxidative decarboxylation of COPIII by coproporphyrinogen oxidase, a mitochondrial intermembrane space enzyme, converts COPIII to protoporphyrinogen IX. Protoporphyrinogen IX is then oxidized to protoporphyrin IX (PPIX) by the enzyme protoporphyrinogen oxidase (PPO) (27). The final step in the heme biosynthesis pathway occurs on the inner surface of the inner mitochondrial membrane where iron is inserted into PPIX by ferrochelatase (FECH) (27).

Figure 1. 1.



Model of heme biosynthesis pathway.

Figure 1. 1. (continued).

Heme biosynthesis occurs in 8 steps through coordination between cytosolic and mitochondrial enzymes. These steps begin in the mitochondria when one of the rate limiting enzymes, delta-amino-levulinate synthase (ALAS), condenses glycine and succinyl-CoA to form amino-levulinic acid (ALA). ALA is then transported into the cytosol by the ABC transporter Abcb10, where successive enzymes catalyze the formation of coproporphyrinogen III (COPIII). COPIII is transported back into the mitochondria for further oxidation by coproporphyrinogen oxidase (CPO) tethered to the inner membrane. Transport of COPIII into the mitochondria is an energy dependent mechanism. Abcb6 is ideally located in the outer membrane where it can move COPIII from the cytoplasm into the mitochondria using ATP hydrolysis as the source of energy. COPIII is further decarboxylated by CPO to form PPIX which combines with iron to form heme. The final step of iron incorporation is catalyzed by enzyme ferrochelatase (FECH). OMM: Outer mitochondrial membrane; IMM: inner mitochondrial membrane; IMS: Inter membrane space.

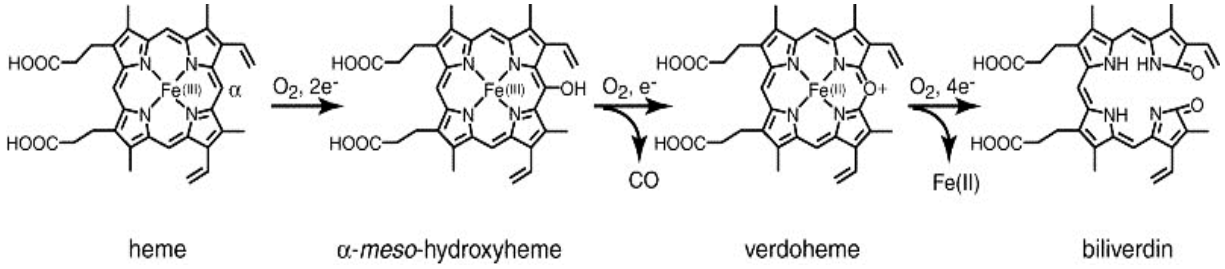
1.3b. Heme catabolism

Heme catabolism is mediated by the enzyme heme oxygenase (HO). Heme degradation by HO involves three sequential oxidation reactions. (Figure 1.2). Overall heme is converted to biliverdin, carbon monoxide (CO), and iron. The released iron is largely recycled for heme production; carbon monoxide functions as a cell-signaling molecule, and biliverdin is immediately converted to bilirubin, a powerful antioxidant (30,31).

Heme oxygenase is represented in 2 isoforms, Heme oxygenase-1 (HO-1) and Heme oxygenase-2 (HO-2), encoded by genes on ch22q12 and ch16p13.3 respectively. HO-1 is an inducible isoform upregulated by heme and oxidative stress signals such as hypoxia, inflammatory cytokines, metals and hydrogen peroxide (31-33). Studies with HO-1 null mice provided insight into the role of HO-1 as an important enzymatic antioxidant system. HO-1 deficient mice demonstrate growth retardation, tissue iron overload, and vulnerability to oxidative stress, phenotypes that are similar to those found in patients with HO-1 deficiency (33-35). The protective effects of HO-1 are related to this enzyme's capacity to provide biliverdin and bilirubin, both of which are powerful antioxidants.

HO-2 was initially thought to be a constitutively-induced enzyme but recent reports suggest that HO-2 expression might also be regulated depending on the cellular microenvironment (36,37). Like HO-1, HO-2 also plays an important role in protecting against oxidative injury. Mice lacking HO-2 had substantially increased mortality following chronic hyperoxia (32). Interestingly, loss of HO-2 in HO-2 knockout mice results in a compensatory increase in HO-1 expression. However this was not sufficient to protect the HO-2 knockout mice from hyperoxia-induced oxidative injury (38,39).

Figure 1.2.



Heme catabolism

Three steps in the degradation of heme catalyzed by hemeoxygenase (HO). First, heme binds to HO as a substrate and activates the first O_2 mediated oxidation. Second, HO regiospecifically hydroxylates α-meso carbon atom of the porphyrin ring which immediately reacts with a second O_2 molecule to yield verdoheme and carbon monoxide (CO). Finally a third oxidation reaction cleaves the heme macrocycle to form biliverdin and Fe(II).

1.3c. Regulation of heme biosynthesis and heme catabolism

Heme biosynthesis is regulated by the rate-limiting enzyme ALAS which catalyzes the first step in heme biosynthesis (40). In animals, two separate genes have been identified for ALAS; one encodes the erythroid specific form (ALAS2 or ALAS-E) and the other, the housekeeping gene (ALAS1 or ALAS-H) which is ubiquitously expressed (32). The evolution of two isoforms for the same enzyme is probably associated with the differential requirements of heme in different cells/tissues. ALAS1 controls the production of heme for basic cellular functions while ALAS2 is mainly involved in the synthesis of heme for the production of hemoglobin in differentiating erythrocytes. Likely to accommodate the different functional requirements, the two genes for ALAS are under separate control mechanisms, and in man, are localized to different chromosomes (32).

Heme synthesis in non-erythroid cells: role of ALAS1. ALAS1 is mainly involved in the production of heme for basic cellular functions and as such, ALAS1 synthesis is negatively regulated by heme, the end product of the heme biosynthetic pathway (Figure 1.3) (41). Conversely, ALAS in the liver can be induced by treatment with chemicals, particularly those that induce the expression P450s and those that decrease free heme concentration, which can be accomplished by decreasing its synthesis or by increasing its catabolism (42,43). The most important effect of heme on hepatic heme synthesis appears to occur at the level of repression of ALAS synthesis. ALAS repression occurs at four different levels which are all influenced by heme; 1) direct inhibition of ALAS activity, 2) repression of the synthesis of mRNA for ALAS, 3) repression of ALAS at the translational level, and 4) inhibition of the translocation of the cytoplasmic form of the enzyme into the mitochondrial matrix (32,44). Importantly, heme mediated repression of ALAS1 is responsible for rendering this enzyme the rate limiting step in non-erythroid heme biosynthesis.

Heme synthesis in erythroid cells, role of iron: ALAS2 is solely involved in synthesizing heme for the production of hemoglobin in differentiating erythrocytes (1). Thus, erythroid differentiation requires a coordinate regulation of expression of globin genes and the genes for the enzymes of the heme biosynthetic pathway (1,32). Regulation of ALAS2 is therefore very complex and occurs at many different levels. In erythroid cells, heme does not inhibit the synthesis of ALAS2; rather heme treatment of erythroid cells significantly increases the incorporation of radiolabeled Glycine into heme, indicating that all enzymes in the pathway including ALAS2 are increased. ALAS2 expression is regulated at the posttranslational level, by iron. ALAS2 mRNA contains an Iron Response Element (IRE) at its 5' UTR, this localization of the IRE dictates that the translation of erythroid ALAS mRNA depends on the availability of iron (Figure 1.3) (45). Another important feature of ALAS2, with possible implications for heme synthesis regulation in erythroid cells, is that this enzyme associates with succinyl CoA synthase. It seems that succinyl CoA serves as an anchor protein for erythroid specific ALAS2 and may provide succinyl CoA more efficiently to ALAS (41). In erythroid cells, heme does not inhibit ALAS2 activity; rather it blocks the uptake of iron from transferrin. Hence, although hemoglobin-synthesizing cells remain faithful to the basic principles whereby heme feedback inhibits heme synthesis, this feedback regulation is executed differently in erythroid cells than non-erythroid cells (32,41).

The mechanisms controlling heme catabolism involves transcriptional regulation of HO-1 gene through the transcriptional repressor Bach 1. Bach 1 is a heme regulated repressor, and is able to bind to Maf (musculoaponeurotic fibrosarcoma oncogene homolog) recognition element (MARE) as a hetero-dimer with a small Maf family member (46). When a small Maf transcription factor binds to MARE site with NF-E2 (p45) or Nrf2, they can stimulate transcription of genes, while heterodimers consisting of small Maf and Bach 1, on MARE site suppresses transcription of the target genes (46,47). Heme modulates the Heme Regulatory Motif (HRM) on Bach 1

which then reverses the repressor function of Bach 1 at the MARE of HO-1 to induce its expression (41,48,49).

Figure 1.3.

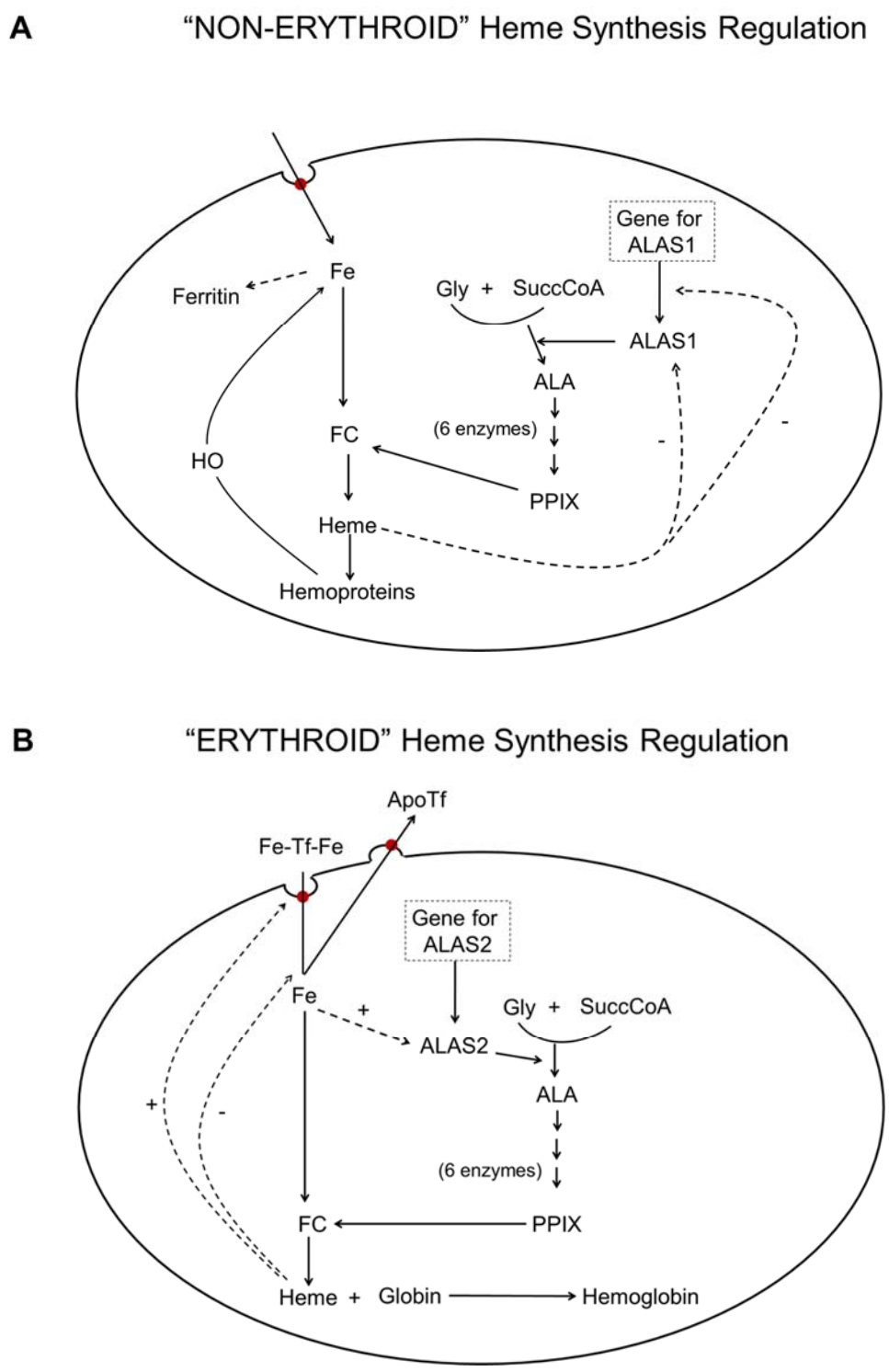


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Figure 1.3. (continued)

Regulation of heme synthesis in A) non-erythroid cells B) erythroid cells.

Heme synthesis involves eight distinct enzymes (see Figure 1.1). Differences in Fe metabolism and in genes for delta-amino levulinate synthase (ALAS) probably account for the differences in the rate of heme synthesis and its regulation in erythroid cells (B) compared with that in nonerythroid cells (A). In nonerythroid cells, the rate-limiting step of heme production is that catalyzed by the ALAS1. The synthesis and possibly the activity of ALAS1 is subject to feedback inhibition by heme, and this is probably one of the factors making ALAS1 the rate-limiting enzyme of the heme pathway. On the other hand, in erythroid cells, heme does not inhibit either the activity or the synthesis of ALAS2, but does inhibit cellular iron acquisition from transferrin without affecting its utilization for heme synthesis. This negative feedback is likely to explain the mechanism by which the availability of transferrin iron limits heme synthesis rate. Since the 5′ untranslated region of ALAS2 mRNA contains iron response element (IRE), the availability of iron controls ALAS2 translation, the rate of ALA formation, and consequently the overall rate of heme synthesis. In addition, iron may play a role in the synthesis and/or stability of ALAS2 mRNA. Heme is also essential for globin translation and may also be involved in globin gene transcription. In contrast, in nonerythroid cells the removal of iron from transferrin is not regulated by intracellular heme and the ubiquitous ALAS1 mRNA does not contain the IRE so that Fe availability does not control the overall heme synthesis rate. (Tf, transferrin; Gly, glycine; PPIX, protoporphyrin IX; HO, heme oxygenase; FC, ferrochelatase.)

1.3d. Heme and porphyrin transport

Heme is readily associated with membranes because of its lipophilic nature but, contrary to earlier beliefs, these compounds (heme and porphyrins) are incapable of readily traversing the biological membranes. Heme and porphyrins carry anionic carboxylate side chains which introduce negative charges to the molecule, limiting their ability to diffuse across membranes. So an energy-dependent mechanism is required to move the carboxylate side chains through the lipid bilayer. Furthermore, kinetic studies in model membrane lipid bilayers and isolated membranes have indicated that transmembrane diffusion of heme is inadequate to support the synthesis of hemoproteins. In addition, the reduction in cellular energy redistributes intracellular heme, suggesting that intracellular heme gradients are maintained actively. In fact, the lipophilic nature of heme and porphyrins causes these molecules to accumulate in the lipid bilayer of membranes rather than be transported across the membrane.

1.3d.i. Heme transport across the plasma membrane

Heme carrier protein 1 (HCP-1) a heme uptake carrier

HCP1 was originally isolated from mouse duodenum using subtractive hybridization (50). HCP1 gene codes for a 50-kDa protein with 9 predicted transmembrane domains (TM) that is highly conserved across species and has a striking homology to bacterial metal-tetracycline transporters, which belong to a large family of transporters known as the major facilitator superfamily (MFS) (50,51). MFS proteins transport small molecules (e.g., sugars, amino acids) across membranes by using either ion or proton gradients as an energy source.

HCP1 protein localizes to the apical membrane of the duodenum, and plays a role in the uptake of dietary heme (51). *Xenopus* oocytes and HeLa cells expressing HCP1 demonstrate a

2- to 3-fold increase in heme uptake that is both saturable and temperature dependent (52-54). Furthermore, HCP1-specific siRNA reduces HCP1-induced increase in ^{55}Fe-heme uptake (51). The strongest *in vivo* evidence supporting the role of HCP1 in intestinal heme transport comes from blocking studies using HCP1-specific antisera. The uptake of ^{55}Fe into everted duodenal sacs was blocked by HCP1 antisera but not by preimmune sera (51). The source of energy for HCP1-mediated heme transport is yet to be defined, though it most likely involves co-transport of a proton or ion along a favorable concentration gradient similar to the mechanism used by other MFS transporters. Moreover, effective competition of HCP1-mediated transport revealed that while the porphyrin ring is important, the presence of a metal ion (Zn^{+2} or Fe^{+2}) is not.

Feline leukemia virus receptor C (FLVCR1a) a heme efflux protein

FLVCR was originally cloned as a cell surface protein that served as a receptor for feline leukemia virus, subgroup C. FLVCR is highly expressed in tissues that either transport heme (i.e., intestinal or hepatic cells) or synthesize high levels of heme (erythroid cells). Like HCP1, FLVCR is a MFS protein that transports small solutes across membranes by using the energy of ion-proton gradients. However, unlike cells that express *HCP1*, cells engineered to express *FLVCR* show significant reduction in cellular heme content, which was assessed by measuring the retention of radioactive hemin (52,53). The level of ^{55}Fe-hemin was substantially reduced only in FLVCR-expressing cells and not in cells engineered to express a nonfunctional FLVCR (53,55). Moreover, FLVCR-mediated heme export was time and temperature dependent. Finally, FLVCR mediates unidirectional heme transport and does not appear to import heme (53,55). Even under conditions of low intracellular heme concentration, FLVCR does not reverse function to increase heme uptake.

Abcg2/Bcrp in the efflux of heme and porphyrins

Abcg2 (Bcrp) is a "half" transporter and a member of the ATP-binding cassette (ABC) family that is capable of moving substrates against a concentration gradient. Abcg2 localizes to the plasma membrane and was initially identified in a cell line that had a specific resistance phenotype characteristic of an ABC transporter (i.e., an ATP-dependent reduction in the intracellular accumulation of anticancer drugs) (56-58). Subsequent studies showed that Abcg2 is expressed in normal tissues, with the highest expression in placental syncytiotrophoblasts. These cells are in contact with the maternal blood; and therefore Abcg2 is thought to protect the human fetus by transporting drugs and toxins from the placenta back into maternal circulation (59-61).

Role of ABCG2 in porphyrin transport was noted when *Bcrp*-null mice were inadvertently exposed to a dietary modification that caused phototoxicity. The underlying cause of skin phototoxicity was the increased accumulation of pheophorbide, a phototoxic chlorophyll-degradation product (62). Subsequent studies confirmed that pheophorbide was indeed an Abcg2 substrate (62,63). It is notable that the structure of pheophorbide is very closely related to that of porphyrins, which are strong photosensitizing agents. The possibility that Abcg2 interacts with heme and protoporphyrin molecules was assessed by affinity-precipitation studies using hemin-agarose. Abcg2 was precipitated by hemin-agarose, and its binding was specific as hemin decreased the amount of precipitated heme in a dose-dependent manner (64). The tetrapyrrole structure is an important molecular feature in the interaction with Abcg2, because porphobilinogen, a monopyrrole precursor of tetrapyrroles, was unable to compete with the Abcg2‑hemin-agarose complex (64).

1.3d.ii. Intracellular heme/porphyrin transport

In mammalian cells, transport of heme and porphyrins takes place not only across the plasma membrane but also across the membranes of intracellular organelles. For example, intracellular transport of heme takes place across mitochondrial membranes during heme synthesis, across the lysosomal membrane during hemoglobin catabolism, across the nucleus where it can activate or repress transcription, and across the endoplasmic reticulum for synthesis of P450s and for heme oxygenase. Further, the biological effects of heme are highly compartment-specific.

A) Mitochondrial heme transporters

One of the most intriguing aspects of heme metabolism is the subcellular compartmentalization of the enzymes of heme synthesis and catabolism. Heme synthesis is initiated in the mitochondrial by ALAS, and continues via intermediates synthesized in the cytosol, which is followed by the return of corporporphyrinogen III to the mitochondrion for final steps in heme formation (Figure 1.1). Thus, transfer of tetrapyrole intermediates across mitochondrial membranes must occur in the later stages of heme synthesis and presents an interesting problem in terms of the intracellular movement of pyrrolic compounds.

Peripheral benzodiazepine receptor

The peripheral-type benzodiazepine receptor (PBR) was initially identified as a benzodiazepine (diazepam)-binding receptor located in the outer mitochondrial membrane (65-67). To determine if an endogenous ligand existed, tissue extracts were prepared and assayed for their ability to displace radiolabeled benzodiazepines. Analysis of the activity that displaced radiolabeled benzodiazepines revealed that porphyrins were potent disrupters of the interaction

between radiolabeled benzodiazepine and PBR (66). Further analysis indicated that among the porphyrins, PPIX was most potent, whereas hemin was less effective (66). These studies supported the idea that porphyrins are endogenous ligands. While these studies were interpreted as demonstrating PBR transports PPIX, a closer inspection reveals that these studies do not effectively discriminate between PPIX transport and binding. Furthermore, given the relatively low affinity of the PBR for a porphyrin precursor such as COPIII and the lack of energy requirement, it seems unlikely that PBR is a porphyrin transporter.

Mitochondrial ATP-binding cassette transporter Abcb6

Abcb6 is highly expressed in fetal liver, erythroid cells and in adult tissues that have substantial heme requirements because of their high metabolic activity (e.g., heart and skeletal muscle). Abcb6 is a specific heme-binding protein by virtue of its ability to be "pulled down" by the affinity resin hemin-agarose and the requirement of a specific domain (28). The interaction between Abcb6 and hemin-agarose is specifically disrupted only by tetrapyrole-containing molecules (hemin and other protoporphyrins such as COPIII) and not by monopyrole-containing molecules such as porphobilinogen. Moreover, COPIII, an oxidized form of the ultimate cytosolic protoporphyrin heme biosynthetic precursor, was highly effective in disrupting Abcb6 interaction with hemin-agarose (28,68,69). Furthermore, in transport assays, COPIII (the oxidized form of coproporphyrin) functioned as a better competitor for ^{55}Fe-Heme uptake than other porphyrins (28). This is interesting, since the reduced form COPIII is the final byproduct of heme biosynthesis in the cytoplasm and has to be transported into the mitochondria for further catalysis, a process which has been shown to require an energy dependent mechanism. In this context, the ideal localization of Abcb6 to the outer mitochondrial membrane and its ability to interact and transport coproporphyrinogen suggests that COPIII is the endogenous substrate for Abcb6 *in vivo*.

Feline leukemia virus 1b

FLVCR1b is an isoform which contains a shortened N terminus of the previously described FLVCR1a mRNA (described above). The protein encoded by the FLVCR1b transcript has a mitochondrial targeting signal at the N terminus (70,71). Flvcr1b is ubiquitously expressed in mouse tissues with highest transcript levels in the brain, followed by tissues with high heme synthesis rates, such as the heart, skeletal muscle, bone marrow, and spleen (70,71). Knockdown of the FLVCR1b isoform impaired erythroid differentiation *in vitro* (70,72). Overexpression of the protein increases cytosolic heme and that knockdown of FLVCR1b results in mitochondrial heme retention, suggest that the FLVCR1b could serve as a heme exporter at mitochondria (70,71). However, FLVCR1b mediated direct transport of heme out of mitochondria or not needs to be demonstrated.

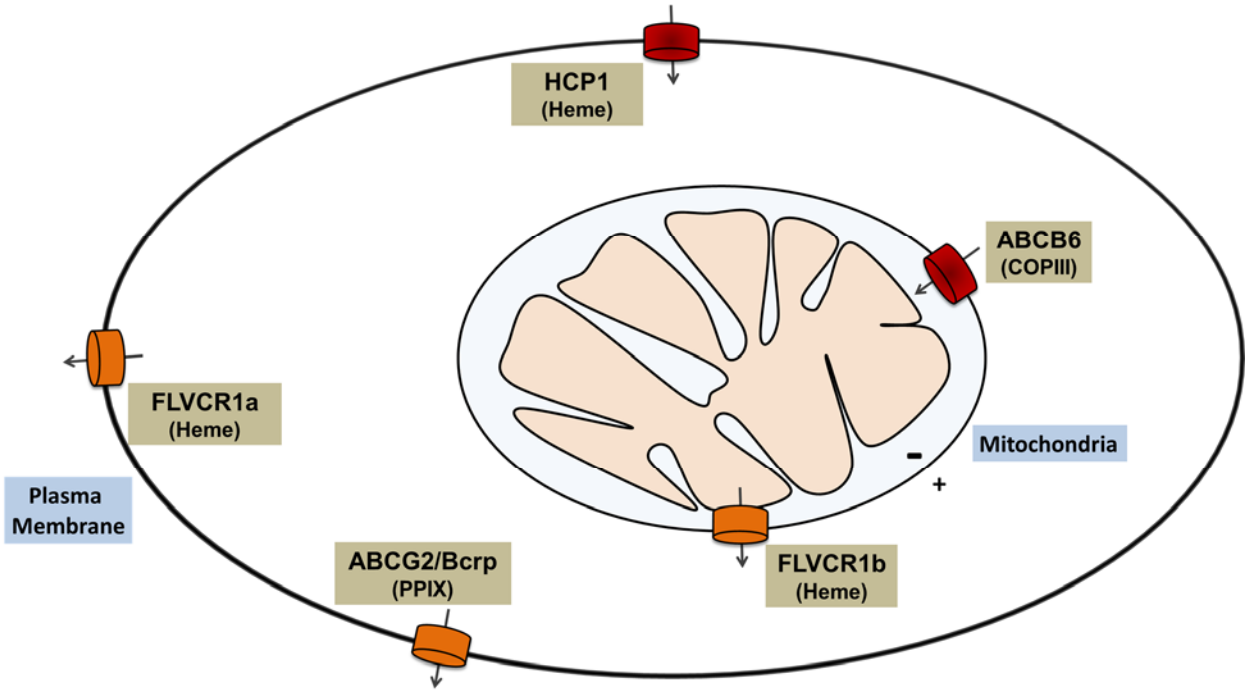
B) Endoplasmic reticulum heme transporters (Ligandin)

Transport of newly synthesized heme from mitochondria to the apocytochrome in vesicles has been reported to be mediated by ligandin (73). However, ligandin mediated transport process in intact cells is not demonstrated yet.

C) Peroxisomal heme transporters

Peroxisomes which, like mitochondria, are a major site of oxygen utilization, contain most of the cell's catalase the antioxidant hemoprotein that neutralizes the potentially lethal oxidant hydrogen peroxide. Catalase is not synthesized as a precursor, but is released into the cytosol as apocatalase. Concomitant with transport across the peroxisomal membranes, the chains are assembled into tetramers, and heme transported from the mitochondria is added. However, the details of this process are not defined (74).

Figure 1.4.



Model for heme/porphyrin transporters

Heme/porphyrin transporters are located at the plasma membrane and mitochondria. FLVCR1a and Abcg2 localize to the plasma membrane and efflux intracellular porphyrin/heme whereas HCP1 is located on the plasma membrane and imports extracellular heme. The mitochondrial transporter Abcb6 localizes to the outer mitochondrial membrane and transports heme/porphyrins from the cytoplasm into the mitochondria. FLVCR1b is the mitochondrial heme exporter.

1.4. The human ATP binding cassette transporter subfamily

1.4a. Evolution of ABC transporter superfamily

All cells are separated from the external milieu by lipid membranes, which are crucial to the life of the cell. This lipid bilayer functions as a barrier that defines the cellular boundary and maintains the essential difference between the cytosol and the extracellular environment. In addition, inside a eukaryotic cell, the membranes of the endoplasmic reticulum, golgi apparatus, mitochondria, and other membrane-enclosed organelles maintain the characteristic differences between the content of each organelle and the cytosol. However, in order to benefit from this barrier, cells have had to evolve ways of transferring specific water-soluble molecules and ions across their membranes. This necessitates the development of specialized transmembrane proteins that regulate the selective passage of specific molecules across these membranes to not only acquire nutrients and excrete waste products, but also perform a multitude of regulatory and other functions. The importance of membrane transport to the cell is reflected in the large number of genes in all organisms that code for transport proteins, which make up almost 20% of membrane proteins in all cells, with some specialized mammalian cells devoting up to two-thirds of their total metabolic energy consumption to membrane transport processes.

The evolution of transport proteins in cells can be grouped under two broad classes, transporters and channels, both of which form continuous protein pathways across the lipid bilayer. Whereas transmembrane movement of solutes mediated by channel proteins is always passive, solute movement mediated by transporters can be either active or passive. Transporters belong to a small number of protein families that bind specific solutes and transfer them across the lipid bilayer by undergoing conformational changes that expose the solute-binding site sequentially on one side of the membrane and then on the other. Each transporter

family contains proteins of similar amino acid sequences that are thought to have evolved from a common ancestral protein and to operate by a similar mechanism. The superfamily of ABC transporters is the largest family of membrane proteins and is especially important clinically.

1.4b. General features of ABC transporters

Transport proteins are designated as ABC transporters based on the highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily. The ATP-binding cassette (ABC) consists of two conserved sequence motifs known as the Walker A and Walker B motifs that bind and hydrolyze ATP. In addition all ABC transporters also share a third sequence motif known as the Walker C or the signature motif, located just upstream of the Walker B site (Figure 1.5) (4). The Walker C motif distinguishes ABC transporters from other ATP-binding proteins and is thought to be essential for ATP hydrolysis. The ABC transporters use the energy of ATP hydrolysis to drive the transport of various molecules across the cell membranes (1–3) (Figure 1.5).

ABC transporters require the function of multiple polypeptides/protein domains, organized in a characteristic fashion (Figure 1.5). The typical transporter consists of four membrane-associated domains. Two of these domains are highly hydrophobic and each consists (normally) of six membrane-spanning segments. These domains form the pathway through which substrate crosses the membrane and, in large part, are believed to determine the substrate specificity of the transporter. The other two domains are the nucleotide binding domains, described above, that are peripherally located at the cytoplasmic face of the membrane, bind ATP and couple ATP hydrolysis to the transport process. In addition, certain ABC transporters have additional domains that serve regulatory or other peripheral functions. For example, the cystic fibrosis gene product, CFTR, has a fifth domain, the R-domain, which has no equivalent in any other ABC transporter and serves a regulatory function (75,76). The

ATP-binding domain of the maltose transporter (MalK) has a C-terminal extension that has an enzymatic function apparently independent of the transport process (77).

The eukaryotic ABC genes are organized either as full transporters containing two TMDs and two NBDs within a single polypeptide chain, or as half transporters with one TMD and one NBD. The latter must form either homodimers or heterodimers to form a functional transporter. In eukaryotes, most functional ABC proteins move substrates from the cytoplasm to the outside of the cell or into an intracellular compartment such as endoplasmic reticulum (ER), mitochondria, peroxisome and lysosome.

Figure 1.5.

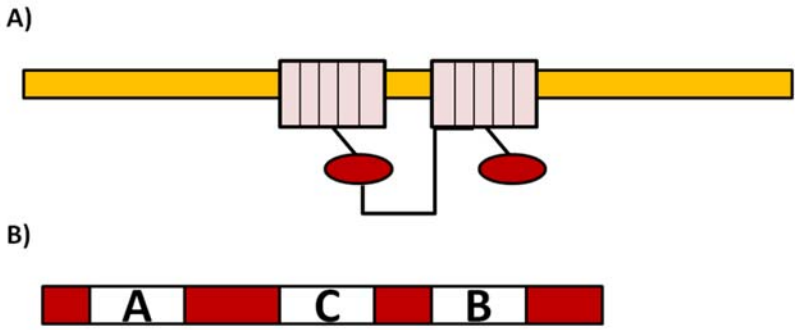


Diagram of a typical ABC transporter protein

A) A diagram of the structure of a representative ABC protein is shown with the lipid bilayer in *yellow*, the transmembrane (TM) domains in *crimson*, and the nucleotide binding domains (NBD) in *magenta*. Although the most common arrangement is a full transporter with motifs arranged N-TM-NBD-TM-NBD-C, as shown, NBD-TM-NBD-TM, TM-NBD, and NBD-TM arrangements are also found. B) The NBD of an ABC gene contains the walker A and B motifs found in all ATP-binding proteins. In addition, a signature or C motif is also present.

1.4c. Classification and nomenclature of ABC gene subfamilies

ABC genes are broadly dispersed in eukaryotic genomes and are highly preserved between species, demonstrating evolutionary conservation of these genes. The genes can be divided into subfamilies based on similarity in gene structure (half *versus* full transporters), and on sequence homology in the NBD and TMDs. Human and mouse ABC genes have standard nomenclature, developed by the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC). Details of the nomenclature scheme can be found at: http://www.genenames.org/genefamilies/ABC.

Several sequences in the genome with homology to ABC genes are positioned in incompletely sequenced regions and may represent pseudogenes. The existing eukaryotic genes are grouped into seven major subfamilies, A to G, based on sequence alignment of the NBDs and phylogenetic analysis. A list of all known human ABC genes and pseudogenes is presented in Table 1.2 with their chromosomal location and known function, when defined.

Table 1.2. List of human ABC genes, chromosomal location, and function.

Approved Symbol	Alias	Chromosome	Function
ABCA1	ABC1, HDLDT1	9q31	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34	Drug resistance
ABCA3	ABC3	16p13.3	Surfactant secretion?
ABCA4	STGD1, ABCR, RP19, STGD	1p22	*N*-Retinylidiene-PE efflux
ABCA5		17q24.3	
ABCA6		17q21	
ABCA7		19p13.3	
ABCA8		17q24	
ABCA9		17q24	
ABCA10		17q24	
ABCA11P#	ABCA11	4p16.3	
ABCA12	ICR2B	2q34	
ABCA13		7p12.3	
ABCA17P#		16p13.3	
ABCB1	PGY1, MDR1, CLCS	7q21.12	Multidrug resistance
TAP1	ABCB2	6p21.3	Peptide transport
TAP2	ABCB3	6p21.3	Peptide transport
ABCB4	PGY3, MDR3	7q21	PC transport
ABCB5		7p14	
Abcb6		2q36	Iron transport
ABCB7	ABC7	Xq13.3	Fe/S cluster transport
ABCB8		7q36.1	
ABCB9		12q24	
ABCB10		1q32	
ABCB11	BSEP, PFIC2	2q24	Bile salt transport
ABCC1	MRP, MRP1	16p13.1	Drug resistance
ABCC2	CMOAT	10q24	Organic anion efflux
ABCC3		17q21	Drug resistance
ABCC4		13q31	Nucleoside transport
ABCC5		3q27	Nucleoside transport
ABCC6	ARA, PXE	16p13.11	
CFTR	CF, ABCC7	7q31-q32	Chloride ion channel
ABCC8	SUR, HRINS	11p15.1	Sulfonylurea receptor

Table 1.2. (continued)

Approved Symbol	Alias	Chromosome	Function
ABCC9		12p12.1	K(ATP) channel regulation
ABCC10		6p12.3	
ABCC11		16q12	
ABCC12		16q12.1	
ABCC13#		21q11.2	
ABCD1	ALD	Xq28	VLCFA transport
ABCD2	ALDL1	12q12	
ABCD3	PXMP1	1p21.3	
ABCD4	PXMP1L	14q24	
ABCE1	RNASEL1, RNASELI, RNS4I	4q31	Oligoadenylate binding protein
ABCF1	ABC50	6p21.33	
ABCF2		7q36.1	
ABCF3		3q27.1	
ABCG1		21q22.3	Cholesterol transport?
ABCG2		4q22.1	Toxin and drug efflux
ABCG4		11q23	
ABCG5		2p21	Sterol transport
ABCG8		2p21	Sterol transport

ABCA

The human ABCA subfamily comprises 12 full transporters (Table 1.2). Two members of this subfamily, the ABCA1 and ABCA4 proteins, have been studied extensively. The mammalian ABCA1 is required for cholesterol transport from peripheral cells such as macrophages into high-density lipoprotein (HDL) particles (78). Thus, mutation in huABCA1 is associated with disorders of cholesterol transport and HDL biosynthesis like Tangiers disease and familial HDL deficiency (79-82). The murine *Abca1*$^{-/-}$ phenotype corroborate the human Tangiers disease and also show placental malformation and altered steroidogenesis (83,84). The huABCA4 protein is restricted to the photoreceptor cells and facilitates transport of vitamin A derivatives in the outer segments of rod photoreceptor cells and performs a crucial step in the vision cycle. The murine *Abca4*$^{-/-}$ displays delayed adaptation to dark and is a model for juvenile macular degeneration (85,86).

ABCB

The ABCB subfamily is unique among the ABC subfamilies in two ways. First, the complement of ABCB genes is identical in all mammals characterized to date, except that the gene encoding P-glycoprotein (Abcb1) is duplicated in the rodent genomes. This suggests that the ABCB genes have important, conserved functions that are similar in all mammals. Second ABCB subfamily is unique as it contains both full and half transporters.

The human ABCB subfamily comprises 11 transporters. ABCB1 is the first human ABC transporter cloned and characterized for its ability to confer multi drug resistance (MDR) phenotype to cancer cells. The ABCB2 and ABCB3 (TAP) genes are half transporters which heterodimerize to transport peptides into the endoplasmic reticulum, which then complex with HLA class I molecules for presentation on the cell surface (87-89). The ABCB4 and ABCB11

proteins are both located in the liver and are involved in the secretion of bile acids (90-92). Mutation in ABCB4 leads to cholethiasis while mutation in ABCB11 leads to cholestasis (90,91). The ABCB9 half transporter, homolog of the TAPs, has been localized to lysosomes (93). The remaining four half transporters, Abcb6, ABCB7, ABCB8, and ABCB10, localize to the mitochondria, where they function in iron metabolism, heme biosynthesis and transport of Fe/S protein precursors (28,94-97).

ABCC

The ABCC subfamily contains 13 full transporters of diverse functions that include ion transport, cell-surface receptor, and metabolite secretion activities. The ABCC7/CFTR gene encodes for a chloride channel that is mutated in patients with cystic fibrosis (11). ABCC8 and ABCC9 proteins regulate potassium channels involved in modulating insulin secretion. The rest of the subfamily (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10 and ABCC13) is composed of nine multidrug resistance associated proteins (MRPs) which are involved in the cellular export of toxic compounds and therapy drugs either complexed with glutathione or co-transported with glutathione

ABCD

The ABCD subfamily contains four genes encoding half transporters. All ABCD members are localized in the peroxisome. ABCD1 and ABCD2 function as homo- and/or heterodimers and regulate the transport of very long chain fatty acids (97). Homozygous deletion of ABCD1 and ABCD2 gene in mice lead to accumulation of very long chain fatty acid resulting in the development of late onset neurodegenerative disease (98). Physiological function of ABCD3 and ABCD4 is not known yet.

ABCE and ABCF

The ABCE and ABCF subfamilies are unique because they have no TMD and are not known to be involved in any membrane transport functions. The ABCE subfamily is solely composed of the oligo-adenylate-binding protein. Mice homozygous for the *Abce* gene disruption exhibit embryonic lethality at embryonic day E7. ABCF1 is associated with the ribosome and appears to play a role in the activation of elf2α kinase similar to its yeast homolog GCN20 (99,100).

ABCG

The human ABCG subfamily is composed of six “reverse” half transporters that have an NBD at the N terminus and a TMD at the C-terminus. The mammalian ABCG1 protein is involved in cholesterol transport (18). ABCG2, one of the well characterized members of this family, was originally identified as a gene overexpressed in several drug-resistant cell lines. The protein is found in the intestine, placenta, and liver and regulates the transport of drugs and toxins into and out of these tissues. Recent studies suggest that ABCG2 may also play a role in heme and porphyrin homeostasis (64,101).

The remaining ABCG family genes are involved in transporting cholesterol and other sterols. The ABCG5 and ABCG8 are both required for transporting sterols, such as cholesterol and plant and shellfish sterols, out of intestinal cells and from the liver in to the bile (102-104). ABCG1 and ABCG4 are closely related genes that are both induced by cholesterol. ABCG1 is predominantly found in macrophages and ABCG4 in the brain (105,106). However, both genes have several alternatively spliced transcripts some of which are exclusively expressed in other tissues such as the lung and thymus (107).

1.4d. ABC genes and human genetic disease

Many ABC genes were originally discovered during the positional cloning of human genetic disease genes. To date, 17 ABC genes have been linked to disorders displaying Mendelian inheritance (19) (Table 1.3). As expected from the diverse functional roles of ABC genes, the genetic deficiencies that they cause also vary widely. Because ABC genes typically encode structural proteins, all of the disorders are recessive or X-linked recessive and are attributable to a severe reduction or lack of function of the protein. However, heterozygous variants in ABC gene mutations are being implicated in the susceptibility to specific complex disorders.

Few ABC gene mutations are lethal. Untreated cystic fibrosis (ABCC7 /CFTR) is typically lethal in the first decade, and adrenoleukodystrophy (ABCD1 /ALD) can also be fatal in the first 10 years of life (108-110). The only mutations described in ABCB7 are missense alleles, and the yeast homolog is essential to mitochondria, suggesting that this gene is essential. The only developmental defect ascribed to an ABC gene is the congenital absence of the vas deferens that occurs in both cystic fibrosis patients and patients with less severe alleles that present male sterility as their only phenotype (111,112). Thus, most ABC genes do not play an essential role in development.

Table 1.3. ABC genes: human ABC genes and mendelian disorders

Gene	Mendelian disorder	Complex disease	Animal model
ABCA1	Tangier disease, FHD	HDL levels	Mouse, chicken
ABCA3	Surfactant deficiency		
ABCA4	Stargardt/FFM, RP, CRD	AMD	Mouse
ABCA12	Lamellar ichthyosis		
ABCB1	Ivermectin susceptibility	Digoxin uptake	Mouse, dog
ABCB2	Immune deficiency		Mouse
ABCB3	Immune deficiency		Mouse
ABCB4	PFIC3	ICP	
ABCB7	XLSA/A		
ABCB11	PFIC2		
ABCC2	Dubin-Johnson Syndrome		Rat, sheep, monkey
ABCC6	Pseudoxanthoma elasticum		Mouse
ABCC7	Cystic Fibrosis, CBAVD	Pancreatitis, bronchiectasis	Mouse
ABCC8	FPHHI		Mouse
ABCC9	DCVT		
ABCD1	ALD		Mouse
ABCG5	Sitosterolemia		Mouse
ABCG8	Sitosterolemia		Mouse

FHD, familial HDL deficiency; FFM, fundus flavimaculatis; RP, retinitis pigmentosum 19; CRD, cone-rod dystrophy; AMD, age-related macular degeneration; PFIC, progressive familial intrahepatic cholestasis; ICP, intrahepatic cholestasis of pregnancy; XLSA/A, X-linked sideroblastosis and anemia; CBAVD, congential bilateral absence of the vas deferens; FPHHI, Familial persistent hyperinsulinemic hypoglycemia of infancy; ALD, adrenoleukodystrophy, DCVT, dilated cardiomyopathy with ventricular tachycardia

1.4e. Mouse ABC genes and knockouts

Mice have 52 ABC genes and most of the human genes have a clear mouse ortholog, indicating that the functions of the mouse genes should be highly similar to human genes; however, there are several exceptions (Table 1.4). Biological functions of some of these transporters has been facilitated by both genetic defects, so called 'accidents of nature' and the adventitious discovery of their role in causing chemotherapeutic drug resistance.

A number of ABC genes have been disrupted in the mouse including those mutated in human diseases (Table 1.4). The *Abca1*$^{-/-}$, *Cftr*$^{-/-}$, *Abce1*$^{-/-}$ *and Abcc4*$^{-/-}$ mice show compromised viability; however, the remaining knockouts are viable and fertile, and many show either no phenotype or a phenotype only under stressed conditions.

Table 1.4. ABC genes: mouse ortholog for human gene

Human gene	Mouse gene	Location	Knockout
ABCA1	Abca1	4, 23.1 cM	Y
ABCA2	Abca2	2, 12.6	Y
ABCA3	Abca3	Unknown	Y
ABCA4	Abca4	3, 61.8	Y
ABCA5	Abca5	Unknown	Y
ABCA6	Abca6	Unknown	N
ABCA7	Abca7	10, 44	Y
ABCA8	Abca8a	Unknown	N
	Abca8b	11, 69	N
ABCA9	Abca9	Unknown	N
ABCA10	Abca10		
ABCA12	Abca12	1C1	Y
ABCA13	Abca13	11A1	
ABCB1	Abcb1a	5, 1	Y
	Abcb1b	5, 1	Y
ABCB2	Abcb2 (Tap1)	17	Y
ABCB3	Abcb3 (Tap2)	17	N
ABCB4	Abcb4	5, 1	Y
ABCB5	Abcb5	12, 60	
ABCB6	Abcb6	1, C3	Y
ABCB7	Abcb7	X, 39	N
ABCB8	Abcb8	Unknown	N
ABCB9	Abcb9	5, F	N
ABCB10	Abcb10	8, 67	Y
ABCB11	Abcb11	2, 39	Y
ABCC1	Abcc1	16	Y
ABCC2	Abcc2	19	Y
ABCC3	Abcc3	Unknown	Y
ABCC4	Abcc4	13, E4	Y
ABCC5	Abcc5	16, 14	Y
ABCC6	Abcc6	7, B3	Y
ABCC7	Abcc7 (Cftr)	6, 3.1	Y
ABCC8	Abcc8	7, 41	Y
ABCC9	Abcc9	6, 70	Y
ABCC10	Abcc10	Unknown	N

Table 1.4. (continued)

Human gene	Mouse gene	Location	Knockout
ABCC11	Abcc11	8, 44-45	
ABCC12			
ABCD1	Abcd1	X, 29.5	Y
ABCD2	Abcd2	15, E-F	N
ABCD3	Abcd3	3, 56.6	N
ABCD4	Abcd4	12, 39	N
ABCE1	Abce1	8, 36	N
ABCF1	Abcf1	17, 20.5	N
ABCF2	Abcf2	13, 40	N
ABCF3	Abcf3	16, 22	N
ABCG1	Abcg1	17, A2-B	N
ABCG2	Abcg2	6, 28.5	Y
	Abcg3	5, 59	N
ABCG4	Abcg4	9, syntenic	N
ABCG5	Abcg5	17, syntenic	N
ABCG8	Abcg8	17, syntenic	N

Y= Yes; N= No

1.5. Mitochondrial ATP binding cassette transporter Abcb6

Mitochondria are dynamic organelles that play a pivotal role in energy metabolism. Mitochondria are also involved in regulation of heme biosynthesis, iron homeostasis and biogenesis of iron–sulfur clusters. In addition, mitochondria play a major role in the regulation of apoptosis and are the major source of reactive oxygen species (ROS). Because of its central role in several vital processes, mitochondrial dysfunction is linked to human diseases. Reduced ATP level is associated with neurological disorders and mutations in mitochondrial DNA can cause MELAS (Mitochondrial Encephalopathy and Lactic Acidosis with Stroke like episodes) or Leigh's syndrome (113). Dysfunctional mitochondrial ATP binding cassette (ABC) proteins are also linked to human diseases.

There are four mitochondrial ABC transporters known to date, Abcb6, Abcb7, Abcb8, and Abcb10. All of them play a role in some aspect of heme and iron homeostasis (28,94,95,97). Abcb7 localizes to the mitochondrial inner membrane and regulates mitochondrial iron homeostasis by forming iron–sulfur clusters (95). Defect in ABCB7 causes a rare type of X-linked sideroblastic anemia with cerebella ataxia (XLSA/A) (95,114). ABCB8 is involved in mitochondrial iron export, is essential for baseline cardiac function and appears to protect cells against ischemia and oxidative stress (94, 97). Genetic deletion of ABCB8 in mouse hearts results in mitochondrial iron accumulation and cardiomyopathy. Abcb10 localizes to mitochondrial inner membrane and exports ALA from the mitochondria into the cytosol. Abcb10 also plays important role in oxidative stress response and improves cardiac recovery after ischemia–reperfusion (94). Abcb6 localizes to the outer mitochondrial membrane and is shown to transport COPIII into mitochondria to complete heme biosynthesis (28). Several mutations in Abcb6 gene has been reported and linked to diseases like ocular coloboma, Dyschromatosis Universalis Hereditaria and dominant familial pseudohyperkalamia (115-117).

1.5a. History and cloning of Abcb6

Abcb6 was initially cloned while screening for novel drug resistance-related genes in the liver using the nucleotide-binding domain of P-glycoprotein as a probe. The partial cDNA sequence isolated from the rat liver was referred to as PRP for Pgp-related protein. The transcript for this gene is ubiquitously expressed with specific induction during hepatocarcinogenesis (118). Subsequent to the initial cloning, *Hirsch-Ernst et al.* and *Mitsuhashi et al.* independently cloned the full length rat cDNA, UMAT (ubiquitously expressed mammalian ABC half-transporter) and the human cDNA, MTABC3 (mammalian mitochondrial ABC protein 3) respectively (119,120). However, to conform with the ABC transporter nomenclature, all forms of the protein are now referred to as Abcb6. The human *Abcb6* is localized to chromosome 2q36, a locus initially thought to encode a defective gene causing GRACILE syndrome, a defect associated with abnormal mitochondrial iron metabolism. However, sequence and quantitative expression analysis subsequently excluded Abcb6 as the primary cause of this syndrome (121).

1.5b. Structure of Abcb6

Abcb6 is a transmembrane protein with 11 predicted transmembrane helices arranged in two TMDs and a NBD. Abcb6s' NBD is located on the cytosolic side of the membrane (122). Abcb6 is a 'half-transporter' and needs to either homodimerize or heterodimerize to form a functional unit of four Membrane spanning domains and two NDBs. Recently, the crystal structure of Abcb6's NBD has been determined in the apo form and in complexes with ATP, ADP and Mg2+. Nucleotide binding appears to induce notable structural changes through the highly conserved Tyr599 and the Walker A motif (122). The overall structure of the NBD of human Abcb6 is similar to the NBD structures of other ABC transporters.

1.5c. Localization of Abcb6

Multiple laboratories have confirmed that in many cell types, Abcb6 is primarily a protein localizing on the outer mitochondrial membrane and the plasma membrane (28,29,123). Interestingly the rat Abcb6 was demonstrated to localize to vesicular membrane, specifically that of the lysosome, suggesting either a species-specific difference in Abcb6 localization or a mis-localization due to overexpression (124,125). However, unlike other mitochondrially targeted ABC transporters, which possess a signal sequence to target them to the mitochondria, Abcb6 lacks a *bona fide* inner mitochondrial matrix-targeting sequence. This finding may explain why it has been reported to localize to both the plasma and mitochondrial membranes. Alternatively, Abcb6 may be subject to species- and/or tissue-specific splice variants (multiple Abcb6 splice variants have been described and are found at http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cig?exdb=AceView&db=36a&term=abcb6) that produce this unique pattern of localization.

1.5d. Physiological function(s) of Abcb6

Abcb6 shares sequence similarity to the yeast mitochondrial transporters HMT1 (~39% identity) and ATM1p (~46% identity). Because of this similarity, Abcb6 was thought to play a role in iron transport and cellular iron homeostasis. In complementation studies, Abcb6 rescued yeast harboring a defective ATM1p from the respiratory effects of mitochondrial iron accumulation (120). While these studies suggested that Abcb6 is a functional orthologue of ATM1p, additional experiments revealed that, paradoxically, a non-functional mutant of Abcb6 provided similar protection. Further, identification of ABCB7, an inner mitochondrial ABC transporter, as the true functional orthologue of ATM1p confirmed the distinction between Abcb6 and ATM1p (114).

Orthologues of Abcb6 span multiple phyla from humans to *C. elegans.* Among the four mitochondrial ABC transporters, only Abcb6 and ABCB8 are found in *C. elegans,* suggesting that they have an evolutionarily conserved function. *C. elegans* requires dietary heme for survival because of incapability to synthesize heme. Abcb6 is highly expressed in the gut of *C. elegans*, and this location is important for the uptake of dietary heme (101). Based on this observation Abcb6 was thought to play a role in heme or porphyrin homeostasis.

Abcb6 in porphyrin biosynthesis

Recently, *Krishnamurthy et al.* showed that Abcb6 plays a pivotal role in porphyrin biosynthesis. The concentration of intracellular PPIX increased in multiple cell lines expressing Abcb6, but not in those expressing the nonfunctional mutant (28). Moreover, silencing of the *Abcb6* gene expression prevented the increase in porphyrin biosynthesis (28). Abcb6 expression not only increased PPIX biosynthesis but also activated the expression of genes important for heme biosynthesis (28). Thus, Abcb6 appeared to represent a previously unrecognized rate-limiting step in porphyrin biosynthesis.

Initial studies suggested that heme is a transport substrate of Abcb6. However, it appears that Abcb6 transports other tetrapyrole-containing molecules as well, as the binding of Abcb6 to a heme-affinity resin could be disrupted by specific tetrapyrole-containing molecules such as hemin, but not by monopyroles such as porphobilinogen (28,68,101). Moreover, COPIII (the oxidized form of COPIII, the final cytosolic protoporphyrin precursor) was highly effective in disrupting Abcb6 interaction with hemin- agarose [12]. These studies suggested that COPIII is a good candidate for an endogenous Abcb6 substrate. COPIII, the last cytoplasmic heme biosynthesis intermediate, must be transported into the mitochondria, by an energy dependent mechanism, to complete heme biosynthesis [71]. Abcb6 appears to regulate heme biosynthesis

in cells by increasing the uptake of heme precursors (e.g., COPIII). This level of regulation affects the physiology of the cell substantially, as heme is involved in many different cell processes, and the activity and expression of a number of hemoproteins is altered both inside and outside the mitochondria in response to changes in the amount of heme.

Abcb6 in cell growth and proliferation

Abcb6 was first identified as a gene that was upregulated in a rodent model of hepatocarcinogenesis. Abcb6 mRNA increased during liver regeneration in a pattern that paralleled the wave of DNA synthesis [45]. Expression of the transcription factor c-myc shows a similar pattern of increase during liver regeneration, suggesting that c-myc regulates Abcb6. A genome-wide chromatin immunoprecipitation (ChIP) analysis of a Burkitt's lymphoma cell line supported this possibility by showing direct interaction between c-myc and the human Abcb6 promoter [60]. Although c-myc is an oncogenic transcription factor, it also plays a prominent role in regulating proliferative and metabolic genes. Many genes harboring c-myc binding sites have roles in DNA replication, protein synthesis, cell respiration, and energy metabolism [61-63]. Therefore, given the role of Abcb6 in regulating heme synthesis, regulation of Abcb6 by a transcription factor such as c-myc that controls multiple constitutive cell process is reasonable. A ChIP-on-ChIP analysis found that the Abcb6 promoter is also bound by the transcriptional repressor complex p130-E2F4 under conditions of cell cycle arrest [64], a finding suggesting that Abcb6 is switched off by p130-E2F4 when cells stop replicating. The authors of this study developed a motif-scanning algorithm to identify conserved regions in the promoters bound by p130-E2F4 and discovered that some of these promoters, including the *Abcb6* promoter, also harbored consensus nuclear respiratory factor-1 (NRF1) binding sites. NRF1 both contributes to mitochondrial biogenesis and transcriptionally activates nuclear-encoded mitochondrial genes

with respiratory functions (e.g., cytochrome c oxidase) [65]. These findings fit well with Abcb6's ability to regulate biosynthesis of the oxygen-sensing molecule heme.

1.5e. Pathologies associated with Abcb6

An increasing number of human inborn diseases are found to be caused by defects in ABC transporter genes. Abcb6 appears to be a part of this group with several studies reporting an association between non-functional mutations in the Abcb6 gene and human inborn diseases.

Ocular Coloboma

Ocular coloboma is a developmental defect of the eye and results from an abnormal or incomplete closure of the optic fissure. This disorder displays genetic and clinical heterogeneity and its prevalence ranges from 0.5 – 0.75 per 10,000 births. Most interestingly, genes associated with coloboma play important roles in the early development of the human body, especially in the development of the CNS. To date approximately 50% of coloboma cases are associated with genetic mutations (117).

Using a positional cloning approach, *Wang et al.*, identified Abcb6 as the causative gene in a Chinese family affected by autosomal-dominant coloboma (117). A non-functional mutation in the Abcb6 gene was identified in seven affected members of the family and was absent in six unaffected members from three generations. Abcb6s' pathology in coloboma was confirmed in zebra fish where morpholino knockdown of Abcb6 produced a phenotype characteristic of coloboma that replicated the clinical phenotype observed in humans (117). More importantly, the knockdown phenotype in zebra fish, could be corrected with co-injection of the wild-type, but

not mutant Abcb6 mRNA, suggesting that the phenotype observed in zebra fish are due to insufficient abcbc6 function (117).

Abcb6 in Langereis blood group

The International Society of Blood Transfusion has recognized 30 blood group systems as a major concern in transfusion and obstetrics. Among these blood group systems, the high-prevalence blood group antigen Lan, first reported in 1962, can cause acute transfusion reactions and fatal hemolytic disease of the fetus and the newborn (HDFN) (126,127). Until recently, the biochemical and genetic basis of Lan had not been elucidated. However, using a human monoclonal antibody specific to Lan, *Arnaud et al.*, identified Abcb6 as a novel genetic locus encoding for the Lan phenotype (128). Abcb6 was present in the membrane of Lan+ erythrocytes while no Abcb6 was detected in Lan- erythrocytes, suggesting that null alleles of Abcb6 may be responsible for the Lan-phenotype (128). Further, targeted sequencing of Abcb6 in 12 unrelated Lan- individuals identified 10 different null mutations, confirming that null alleles of Abcb6 are responsible for the Lan-phenotype (128).

Familial Pseudohyperkalemia

Familial Pseudohyperkalemia (FP) is a dominant red cell trait characterized by increased serum [K^+] in whole blood stored at or below room temperature (129). This dominantly inherited trait is not accompanied by clinical symptoms or biological signs except for borderline abnormalities of red cell shape. Functional gene mapping and sequencing analysis of the candidate genes by *Andolfo et al*. identified Abcb6 as the causative gene for the FP phenotype (130). Two genomic substitutions altered two adjacent nucleotides within codon 375 of Abcb6 that are predicted to alter protein structure (130). This alteration in Abcb6 structure could potentially lead to red cell K^+ leak that is characteristic of the FP phenotype.

Dyschromatosis Universalis Hereditaria

Dyschromatosis universalis hereditaria (DUH) is a rare autosomal dominant pigmentary genodermatosis characterized by a mixture of hyper-and hypo-pigmented macules distributed randomly over the body. Until recently the causative gene for DUH had not been identified. Using genome-wide linkage analysis coupled with exome sequencing *Zhang et al.* identified mutation in exon 3 of Abcb6 as a contributing factor for DUH (115). Immunohistological examination of biopsy specimens showed that Abcb6 is expressed in the epidermis and had a diffuse cytoplasmic distribution. Subcellular localization of Abcb6 in B16 mouse melanoma cell line revealed that wild type Abcb6 localized to endosome-like compartment and dendrite tips, whereas DUH-causing mutations of Abcb6 resulted in its retention in the Golgi apparatus (115).

Multi Drug resistance

Drug resistance remains one of the primary causes of suboptimal outcomes in cancer therapy. A growing body of evidence has implicated ABC transporters as potential mediators of drug resistance. Gene amplification and consequent overexpression of Abcb6 has been observed in a considerable number of drug resistant cell lines, suggesting that it has a dominant effect in tumor cells. In addition, treatment of cancer cells with artesunate resulted in increased Abcb6 expression and cross-resistance to cisplatin (41,131,132). These results suggest that Abcb6 could function as a protein capable of conferring multidrug resistance phenotype to cells. However, a role for Abcb6 in substrate efflux across the plasma membrane has not yet been unequivocally demonstrated.

Chapter 2. **STATEMENT OF PURPOSE**

2.1. Objectives of the study

There is a growing awareness of the functional significance of ATP-binding cassette (ABC) transporters in pathophysiological conditions. Most of these ABC transporters are expressed in the plasma membrane and are well characterized. However, the ABC transporters in the intracellular organelles are incompletely understood with respect to their cellular roles. Because of the mitochondria's important role in cellular energy and apoptosis, we have focused on a mitochondrial ABC transporter Abcb6 that is involved in porphyrin transport for two reasons. First, Abcb6 expression regulates cellular heme synthesis. Heme, a complex of iron and PPIX, senses and utilizes oxygen in nearly all living cells and is an essential component of various hemoproteins, including those involved in oxygen transport and storage, electron transfer, drug and steroid metabolism and signal transduction. Second, loss of function mutations in the Abcb6 gene is associated with developmental disorders including improper development of the eye and stunted growth. In contrast, overexpression of functional Abcb6 leads to increased cell growth and proliferation, tumorigenesis and therapy related drug resistance. Thus, the physiological and pathological significance of Abcb6 represents a substantial role in public health-focused research. However, our understanding of Abcb6 appears to be rudimentary at best and several questions remain unanswered, which require further evaluation. The major questions that remain undefined are a) the mechanisms that regulate Abcb6 expression and the significance of such expression to cellular function, b) transport substrates of Abcb6 and their relationship to pathologies associated with Abcb6 expression or loss thereof, and c) the existence and the precise localization of differentially targeted Abcb6 and its functional significance. Answering these questions and understanding their significance is crucial to our ability to evaluate the physiological functions and pathophysiological significance of Abcb6.

As a first step in unraveling the complexity of Abcb6s' biology in its mechanistic detail the dissertation explores the functional consequences of Abcb6 as a regulator of heme synthesis to cellular heme homeostasis.

Specific Aim 1 tests the hypothesis that Abcb6 expression is regulated by signals that promote heme synthesis. This hypothesis was tested in both tissue culture (*in vitro*) and mouse models (*in vivo*) of heme synthesis. In the proposed studies, exposure to xenobiotics, which promotes cellular heme synthesis, essential to support the function and activity of hemoproteins, was used as a model of heme synthesis.

Specific Aim 2 tests the hypothesis that Abcb6 is a determinant of hemoprotein expression and activity *in vitro* and evaluates the consequences of Abcb6 deficiency to cellular heme homeostasis. Studies were conducted *in vitro* in Abcb6 expressing and non-expressing cells to i) determine the significance of Abcb6 expression to xenobiotic mediated heme synthesis; and ii) determine the significance of Abcb6 expression to heme dependent cellular functions, with special emphasis on drug induced porphyria and oxidative stress response.

Specific Aim 3 tests the *in vivo* significance of loss of Abcb6 expression to heme dependent cellular functions. Towards this end, experiments were designed to develop an Abcb6 gene deleted mouse model (*Abcb6*$^{-/-}$ mouse) to evaluate, a) loss of Abcb6 expression to hepatic heme homeostasis, and b) loss of Abcb6 expression to heme dependent hepatic functions, with special emphasis on metabolism and disposition of endo and xenobiotics.

Studies proposed in *specific Aim 4* were designed to define the transport kinetics and substrate specificity of Abcb6 in the absence of contaminating ATPases and carriers/transporters. The rationale for these studies was based on the problems associated with analyzing transport process of mitochondrial proteins because of difficulties associated with the two-membrane

architecture of the mitochondria and the high abundance of other ATPases and carriers/transporters. Thus, the development of an *in vitro* system with pure and active protein, was thought to be a prerequisite toward understanding the mechanistic relationships between ATP binding and hydrolysis and coupling of these events to translocation of substrates across the lipid membranes. Towards this end, *in vitro* liposomal transport system with pure and active Abcb6 protein was developed which allowed biochemical characterization of the ATPase including (i) substrate stimulated ATPase activity (ii) transport kinetics of its proposed endogenous substrate COPIII and (iii) transport kinetics of substrates identified using a HTS assay.

2.2. Significance of the proposed study

Studies assessing the molecular expression and function of transport proteins in the liver have helped to define the adaptive responses of hepatocytes to certain physiological states and to liver injury (133). A growing number of specific transport proteins present at the sinusoidal and canalicular membrane domains of hepatocytes and cholangiocytes have been cloned and functionally characterized (134). These studies have led to our understanding of the molecular basis of several forms of inherited cholestatic liver diseases (135,136). Results from such studies have helped in the development of better diagnostic tools and have resulted in the design of new therapeutic strategies (137-139). However, despite the fact that mitochondrial dysfunction is a major mechanism of liver injury, very little is known about the role of mitochondrial transport proteins in liver function. In particular, the physiological significance of mitochondrial ATP binding cassette transporters to liver function is poorly understood. My contribution will be to demonstrate that the mitochondrial transporter Abcb6, which regulates heme synthesis, plays an important role in hepatic drug metabolism and oxidative stress response. This contribution will be significant because it bridges the gap in our knowledge about

the relationship between mitochondrial ABC transporter Abcb6 and liver function. As this information becomes available, translational clinical trials, specifically targeting Abcb6, can be developed to establish therapeutic approaches to minimize hepatic injury associated with oxidative stress. There is also the potential of specifically targeting Abcb6 to predict adverse drug reactions that are precipitated by alteration to P450 activity mediated by Abcb6. In this context, it is significant to note that variation in P450 expression is believed to influence the therapeutic index of up to one-third of all drugs, and may contribute to inter-individual differences in the generation and elimination of environmental toxins and carcinogens. However, the factors responsible for variation in P450 expression between individuals are unclear. I expect that the studies described in the dissertation will help explain some of these inter-individual differences associated with drug metabolism and disposition. In addition, the research described in the dissertation will be significant because what is learned will contribute not only to our fundamental understanding of the mechanisms that control porphyrin biosynthesis, but also into the manner in which the mitochondrial transporter Abcb6 is integrated into hepatic drug metabolism and disposition.

2.3. Innovation

The research proposed in this dissertation is innovative, because it is the first study to evaluate the role of mitochondrial ABC transporters in liver function. Further, the studies provide a unique opportunity to use the Abcb6 deficient and Abcb6 over-expressing cell culture and mouse models that were developed as a part of the dissertation, to predict adaptive responses of liver, to injury precipitated by pharmacological drugs and environmental chemicals. Further, the development of an *in vitro* cell free system (liposome reconstituted Abcb6) carrying purified Abcb6 provides an opportunity to evaluate Abcb6 transport functions in the absence of contaminating ATPases and carriers/transporters.

Chapter 3. **EXPERIMENTAL MATERIALS and METHODS**

MATERIALS AND METHODS

Materials

8-Azido-[α-32P] ATP was purchased from MP Biomedicals (Solon, OH). Coproporphyrinogen III, Protoporphyrin IX, Hemin and Porphobilinogen were purchased from Frontier Scientific (Logan, UT). N-octyl-β-D-glucopyranoside, Phosphatidylcholine, Phosphatidylethanolamine, Phosphatidylserine and Ergosterol were from Avanti Polar Lipids (Alabaster, AL). Handee spin cups were from Pierce (Rockford, IL). All other chemicals and reagents were obtained from either Sigma-Aldrich or Fluka unless otherwise indicated.

Cell culture

Human liver-derived cell lines Hep3B, Huh7 and HepG2 were from the American Type Culture Collection (Manassas, VA). Hep3B and HepG2 cells were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin. HepG2 Huh7 and Hep3B cells were engineered to overexpress human Abcb6 as described (28). Abcb6-overexpressing cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, 2 mM Lglutamine, and 0.6 μg/ml puromycin.

Overexpression in HEK293 cells for purification

To construct Abcb6-FLAG expressing lentiviral plasmid, the human Abcb6/pcDNA3.1 Topo vector carrying FLAG-tagged Abcb6 was cut with restriction enzymes XbaI and EcoRI, and the Abcb6-FLAG containing fragment was ligated into corresponding restriction sites of Lentiviral vector; pLenti PGK Puro. HEK 293 cells overexpressing the Abcb6-FLAG lentiviral plasmid was generated by infecting cells with filtered viral supernatants followed by selection

with 0.5 μg/mL puromycin. 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. The non-functional mutant of Abcb6 was generated as previously described (28). Briefly, the critical lysine residue in the Walker A domain of Abcb6, which is the nucleotide binding domain in ATP binding cassette transporters that is essential for transport function was changed to alanine by site directed mutagenesis as described (28).

RNA Interference

The small interfering RNA (siRNA) oligonucleotide and negative control scrambled oligonucleotide were custom synthesized by Dharmacon (Lafeyette, CO). Both siRNA and control oligonucleotides were used at a final concentration of 150nM and were added to cells using lipofectamine following the manufacturer's protocol.

siRNA 1: 5' GCGCAUACUUUGUCACUGACA 3'

3' UUCGCGUAUGAAACAGUGACUP 5'

siRNA 2: 5' CCGAAUAGAUGGGCAGGACAU 3'

3' UUGGCUUAUCUACCCGUCCUGP 5'

Scrambled oligo: 5' UAGCGACUAAACACAUCAA 3'

Abcb6-shRNA and scrambled shRNA viral particles were obtained from Sigma. Stable cell lines harboring either Abcb6-shRNA or the scrambled shRNA were generated by transduction of viral particles following the manufacturer's protocol, followed by selection in puromycin (0.6 μg/ml for HepG2, Huh7 and Hep3B cells) as described . Loss of endogenous Abcb6 expression was confirmed by RT-PCR and immunoblot using gene-specific primers and protein-specific antibody.

The siRNA specific for AhR and a scrambled siRNA were a kind gift from Dr. Hongbing Wang (University of Maryland, Baltimore, MD). HepG2 cells at ~60% confluence were

transfected with AhR siRNA or scrambled siRNA (40 pmol/well) using Lipofectamine 2000 reagent (Invitrogen, CA) following the manufacturer's instructions. Twenty-four hours after siRNA transfection, cells were treated with either DMSO or 5 μM B[a]P and incubated for an additional 16 h before harvesting. Total RNA isolated from transfected cells as described below.

Primary mouse hepatocyte culture

Hepatocytes from the liver were isolated using the collagenase perfusion method as described previously (140). Briefly, under pentobarbital anesthesia (50 mg/kg intraperitoneally), liver was perfused with 50 ml of calcium- and magnesium-free Hanks' balanced salt solution supplemented with 0.5 mM EGTA, 5.5 mM glucose, and penicillin-streptomycin, followed by 40 ml of calcium- and magnesium-free Hanks' balanced salt solution supplemented with 1.5 mMcalcium chloride, 5.5 mM glucose, penicillin-streptomycin, and 0.02 g of Type IV collagenase. Liver was removed, and the digested product was centrifuged at 50 × g for 2 min to pellet the hepatocytes. The hepatocytes were washed three times with Williams' medium E and then cultured in Williams' medium E containing 10% FBS and penicillin-streptomycin. After a 3-h attachment period, the medium with unattached cells was removed, and fresh medium was added. The viability of isolated hepatocytes was >90% by the criterion of trypan blue exclusion. Cells were cultured for ∼16 h before the addition of drugs.

Animals housing

Mice were housed in polycarbonate cages (four per cage), provided normal diet and water ad libitum, and maintained on a 12–12 h light-dark cycle at 22 ± 5°C and 50 ± 20% relative humidity.

Animals' treatment

Study 1: Nrf2 gene-deleted mice (*Nrf2*$^{-/-}$) was a kind gift from Dr. Curtis Klaassen (Professor, Department of Medicine-Gastroenterology, University of Kansas Medical Center, USA). Wild-type mice were C57BL/6J from Jackson's Laboratory. At 8 weeks of age, *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (four mice per group) were fed sodium arsenite (10 ppm) in drinking water for 24 h.

Study 2: Effect of sodium arsenite on Abcb6 expression was measured in 8-week-old C57BL/6J mice. Mice (four per group) were fed sodium arsenite (0, 1, 10, or 100 ppm; As(III)) in drinking water for 1 day or with 10 ppm of sodium arsenite for 1, 3, or 5 days. All mice survived sodium arsenite treatment.

Study 3: AhR gene-deleted mice (*AhR*$^{-/-}$) were a kind gift from Dr. Curtis Klaassen (Professor, Department of Medicine-Gastroenterology, University of Kansas Medical Center, USA). At 8 weeks of age, *AhR*$^{+/+}$ and *AhR*$^{-/-}$ (4 mice per group) were given 37 µg/kg TCDD or an equal volume of vehicle (corn oil) intraperitoneally for 4 days.

Study 4: Abcb6$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice (six mice per group) were sacrificed at 8 weeks of age. At the end of treatment, animals were sacrificed, livers were harvested, and RNA, mitochondria or microsomes were isolated immediately as described below.

RNA isolation, reverse transcription and real-time PCR

RNA isolation from liver tissue or cells were done using TRIZOL® reagent (Invitrogen, CA). 1 µg of RNA was used for reverse complementation using iScript™ cDNA synthesis kit according to manufacturer's protocol (BioRad, CA). Real-time PCR was performed using CFX384™ Real Time PCR System as described previously by using primer sets specific for the mouse genes enlisted in Appendix 1.1.

Immunoblot analysis

For Western analysis, cell lysates were prepared as described previously (64) and 50 µg of total protein, mitochondrial protein, or microsomes was analyzed by polyacrylamide gel electrophoresis (PAGE). Blots were probed with mono- and/or polyclonal anti-Abcb6 antibody, monoclonal anti-HO-1 (Assaydesigns, MI), and monoclonal anti-porin (Mitosciences, OR), mouse P450 oxidoreductase (POR); Cyp2e1 (Abcam, MA) Cyp2b10 (Millipore, MA), Gapdh (Cell Signaling, MA). The polyclonal antiserum to mouse Cyp3a and Cyp1a were a kind gift from Dr. Xiaochao Ma (Associate Professor, University of Pittsburgh, USA).

We detected the secondary antibody by using a chemiluminescence detection kit (Amersham Biosciences, NJ). Abcb6 antibodies were generated using a portion of the Abcb6 protein (aa 592–894) that is predicted to localize to the cytosol and is unique among the Abc transporters. The antibody was affinity purified and characterized for its ability to recognize the native Abcb6 protein.

Cell viability studies

Trypan blue exclusion assay was used to determine cell viability. Briefly, we exposed 10^6 HepG2 or Hep3B vector cells or Abcb6-expressing cells to increasing concentration of sodium arsenite in water for 24 or 48 h at 37^0C and then counted the living cells (that were able to exclude the dye). Results are expressed as a percentage of cells surviving from the total plated.

Cytotoxicity assay

For arsenic toxicity studies, in 96-well plates, we incubated Hep3B or HepG2 cells that express either an empty vector or an Abcb6-expressing vector at 37^O C for 24 h. Sodium arsenite in a dilution series was added and incubation continued for 2–4 days.

For TCDD and B[a]P toxicity, cell viability was measured in logarithmically growing mouse primary hepatocytes, HepG2 and Huh7 cells. Cells were plated onto 96-well plates at a starting density of 10^4 cells and treated with increasing concentrations of TCDD, B[a]P, or the respective solvents.

Cell viability was determined at 24, 48, and 72 h using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide. Absorbance at 570 nm was measured with a kinetic microplate reader (BioTek, VT) and was used as a measure of cell viability.

Measurement of ROS

Cells treated with sodium arsenite (25μM) for 24 h were incubated with 3μM 2, 7-dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, OR) for 15 min at 37^0C. After incubation, cells were washed with PBS, trypsinized, and resuspended in PBS solution. DCF fluorescence was measured using FACScan flow cytometer (excitation at 488 nm, emission at 515–545 nm). Data were analyzed with CellQuest software.

Isolation and purification of mitochondria

Cells were pelleted in 13 Hanks buffered saline solution (Life Technologies, CA), resuspended in buffer A (10 mmol/l NaCl, 1.5mM MgCl2, and 10 mmol/l Tris [pH 7.4]) containing 13 protease inhibitor cocktail (Roche Applied Science, IN), swollen on ice, and disrupted with a type B Dounce homogenizer. Buffer B (525 mmol/l mannitol, 175 mmol/l sucrose, 12.5 mmol/l Tris [pH7.4], and 2.5 mmol/l EDTA) was added in a ratio of 4:10 homogenate/buffer B. The supernatant was collected after centrifugation at 1500 x g for 10 min. The supernatant was centrifuged at 17,000 x g for 15 min to pellet mitochondria. The crude mitochondria were purified from the endoplasmic reticulum as previously described (28).

Preparation of microsomes

Microsomes were prepared from *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice liver tissues. Briefly, 0.3-0.5 g of liver tissue was homogenized in 1X PBS and centrifuged at 17000 x g for 30 min at 4°C. The supernatant was subjected to centrifugation at 100000 x g for 90 min at 4°C. The resulting microsome pellet was resuspended in 1X PBS. Microsomal protein concentrations were determined using the Bio-Rad protein assay reagent. Microsomes were stored at –80 °C until used for western blot analysis or P450 activity assays.

Catalase inhibition and catalase activity

Catalase inhibition was achieved by using 3-aminotriazole (3-AT) as described (141). Briefly, cells were exposed to 20 mmol/l of 3-AT in PBS for the duration of the experiments. For catalase activity, cells (5 x 10^5) were washed twice with PBS, resuspended in PBS, sonicated for 10 s, and centrifuged at 14,000 rpm for 10 min. The supernatants from the centrifugation were subjected to the catalase activity assay. Catalase activity was determined by monitoring the rate of decomposition of H_2O_2, as assessed by the decrease in absorbance at 240 nm. One unit of activity represents the consumption of 1 mmol H_2O_2/min/ mg protein. The assay mixture (1 ml) contained 19 mmol/l H_2O_2 and defined amounts of cell extract in 50 mmol/l phosphate buffer (pH 7) at 25^0C.

Cellular protoporphyrin IX measurement

Intracellular PPIX concentration was measured as described previously (28,142). Briefly, cells were harvested and washed once with PBS. PPIX concentration was measured by using a Vantage flow cytometer (BD Biosciences, NJ). To induce PPIX fluorescence, the excitation wavelength was set at 405 nm, and the emission filter was set at 695 nm/40 nm.

Heme measurement

Heme measurement was done using liver tissue from *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice. Briefly, 5 mg of liver tissue was homogenized in 100 µl of 1X phosphate buffer saline (pH 7.4). Heme extraction was performed using double the volume of Ethyl acetate and acetic acid mixture (4:1). Sample was vortexed briefly and centrifuged at 20000x g for 5 min. Each supernatant was diluted 5 times with 50% acetonitrile and transferred to an auto sampler vial, of which 5.0 µl was injected onto the UPLC-TOFMS system for heme analysis.

Mass spectrometry based P450 assay

Microsomal incubation reaction was performed 1X phosphate-buffered saline (pH 7.4), containing 3µM Midazolam (MDZ) or 20µM Chloroxazone with 0.03 mg of mouse liver microsomes. The final volume of reaction was 200 µl. After 5min of pre-incubation at 37°C, the reaction was initiated by adding NADPH (final concentration 1.0mM). The reactions were shaken at 37°C for 10 min for MDZ and 15 min for Chloroxazone. Incubations were terminated by adding 200 µl of ice cold acetonitrile and vortexing for 10 seconds and centrifuging at 18000x g for 10 min. Each supernatant was transferred to an auto sampler vial, and 5.0 µl was injected onto the UPLC-TOFMS (Waters, MA) system for metabolite analysis.

UPLC-TOFMS analysis

Ultra-performance liquid chromatography (UPLC)-time-of-flight mass spectrometry (TOFMS) analyses were performed as described previously (143). A 100mm × 2.1mm (Acquity 1.7 µm) UPLC BEH C-18 column (Waters, MA) was used to separate metabolites. The flow rate of the mobile phase was 0.3ml/min with a gradient ranging from 2 to 98% aqueous acetonitrile, containing 0.1% formic acid in a 10-min run. TOFMS was operated in a positive mode with electrospray ionization. The source temperature and desolvation temperature were set at 120°C

and 350°C, respectively. Nitrogen was applied as the cone gas (10 l/h) and desolvation gas (700 l/h) and argon as the collision gas. TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine enkeophalin in real time. The capillary voltage and cone voltage were set at 3.5kV and 35V in positive ion mode. Identification of major metabolite were performed using MakerLynx software (Waters, MA), based on accurate mass measurement (mass errors less than 10ppm). All the incubations were performed in duplicate.

Pentobarbital induced sleeping time

Abcb6$^{+/+}$ and *Abcb6*$^{-/-}$ mice were used in these studies. The animals were injected with 40 mg/kg body weight of pentobarbital by intra peritoneal route. The time for animals to sleep was noted as sleep latency and time difference between the animal completely losing their reflexes after pentobarbital administration and the time they regained their reflexes was taken as the pentobarbital-induced sleeping time.

ABCB6/Abcb6 promoter analysis

Promoter analysis was performed as described previously (64). Briefly, HepG2 cells at ~60% confluence were transfected with 1.5 μg/well of either pGL2-luciferase, pLightSwitch-Abcb6-luciferase (Switchgear Genomics, CA), pGL2-Abcb6-luciferase, or pGL2-HO-1-luciferase constructs using Lipofectamine reagent following the manufacturer's protocol. All transfections included Renilla luciferase (100 ng/well) as an internal transfection control. In a subset of experiments, we introduced aryl hydrocarbon-responsive element (AhRE) mutations into the Abcb6 promoter by using a site-directed mutagenesis kit (Stratagene, CA). Briefly, two nucleotide mutations (consensus motifs 5′-GCGTG-3′ mutated to 5′-ATGTG-3′) were introduced into the AhR binding site of the Abcb6 promoter at base pairs −102 bp, −115 bp, and −123 bp

relative to the transcription start site (Figure 4.1.5D). All mutations were confirmed by sequencing using pGL2-basic and pLightSwitch-Abcb6-luciferase primers (Promega, WI). The relative activity of the promoter constructs was determined after subtraction of the values obtained for pGL2-luciferase, and the results were expressed in relative terms (ratio of vehicle control value to treatment value). All experiments were performed at least three times with a minimum of four replicates per experiment.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was performed as described previously (64). Briefly, HepG2 cells at ~90% confluence were treated with vehicle or 5 μM B[a]P for 2 h, and nuclear protein was extracted using the NE-PER nuclear extraction kit (Pierce, IL). To detect AhRE interaction, 20 fmol of biotinylated DNA segments containing either 1) the three AhREs of human *Abcb6* (CGTACGTGCCCTGCGTGAGTGCGTGGCGGCGGCGCGTGCG; the core AhRE is underlined (Figure 4.1.5D), 2) only the two distal AhREs (CGTACGTGCCCT**GCGTG**AGT**GCGTG**GCGGCGG; underlined and in boldface type (Figure 4.1.5D and 4.1.6D), or 3) the two distal AhREs where the sequences flanking the core AhRE were changed to random bases (Figure 4.1.5D) were incubated with 2 μg of nuclear extracts with or without excess amounts (4 pmol) of similar or mutant (CGTACGTGCCCT*ATGTG*AGT*ATGTG*GCGGCGGCATGTG; mutations shown in italic type) unbiotinylated DNA oligonucleotides. Rabbit polyclonal antibody against AhR (Santa Cruz Biotechnology, CA) was used to probe the AhRE complexes, and anti-FXR polyclonal antibody (Santa Cruz Biotechnology, CA) was used as a nonspecific negative control in the experiment. A 25-bp biotinylated DNA motif containing human *CYP1A1* AhRE (5′-CGAGTTGCGTGAGAAGAGCCAGATC- 3′; the core AhRE is underlined) and the

mutant *CYP1A1* AhRE (5′-CGAGTT*GATTG*AGAAGAGCCAGATC- 3′; mutations shown in italic type) was used as a positive control in these experiments.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed using HepG2 cells treated with vehicle or B[a]P (5 μM) for 2 h. Approximately 2 × 10^6 cells were cross-linked with 1% formaldehyde for 15 min at room temperature and washed with ice-cold PBS containing a protease inhibitor mixture. Chromatin derived from isolated nuclei was fragmented to an average size of 0.5–2 kb using a Vibra-cell ultrasonic processor (Sonics, CT). Following centrifugation, supernatants containing sheared chromatin were immunoprecipitated overnight at 4 °C with anti-AhR antibody (M-20 from Santa Cruz Biotechnology, CA, and AhR-31635 from Aviva System Biology, CA) or isotype control IgG. The immunocomplex was precipitated using protein A coupled to Sepharose beads and decross-linked for 6 h. The immunoprecipitated DNA fragments were recovered by QIAquick PCR purification kit. Quantitative RT-PCR was performed using a specific set of primers (sense, 5′-CAGAGCCAGCGGGGCCGTGCTG-3′; antisense, 5′-GGCGCGGACATCCGGGTGCC-3′) spanning the region between −20 and −193 bp around the putative AhR binding site within the Abcb6 promoter. PCR products were also resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Purification of Abcb6 from total lysate

HEK 293 cells (70% confluent) were harvested with 1X PBS and lysed (10 min at 4° C with gentle shaking) in buffer A (1X PBS; 0.2% Triton X100) containing EDTA-free complete protease inhibitor mixture (Roche Applied Science, IN). Soluble and insoluble fractions were separated by centrifugation (10 min at 10,000 rpm, 4°C) and the supernatant was incubated with sepharose conjugated anti-M2 beads (EZview™ Red Anti-FLAG M2 affinity gel) overnight

at 4°C with gentle shaking. The supernatant with the sepharose conjugated M2-beads were transferred to handee spin cups (paper filter) and separated by centrifugation (2,000 RPM for 5 min at 4°C). The beads were washed twice with buffer A, twice with buffer B (10 mM Tris; 0.2% Triton-X-100) and five times with Hepes buffer [Hepes-EGTA-1% n-Octyl-β-D-glucopyranoside (OGP)]. Proteins bound to the affinity column were eluted using elution buffer (Hepes-EGTA-1% OGP-0.5 mg FLAG peptide). The eluted protein was identified by immunoblotting using Abcb6 specific and FLAG-epitope specific antibody (Sigma, MO) followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) peptide mass fingerprint analysis after trypsin digestion, using Mascot (data base NCBInr 20040521)

Purification of Abcb6 from mitochondria

Mitochondria were isolated as previously described (11). Isolated mitochondria were solubilized in buffer A (1X PBS; 0.2% Triton X100) containing EDTA-free complete protease inhibitor mixture (Roche Applied Science, IN) for 30 min at 4°C with gentle shaking. Soluble and insoluble fractions were separated by ultracentrifugation (100,000 x g, 30 min, 4°C) and the supernatant was incubated with anti-M2 sepharose beads as described above for whole cell lysate. Proteins bound to the affinity column was washed and eluted as described above for total cell fraction using FLAG-peptide in elution buffer. The bound protein was identified by immunoblotting and matrix-assisted laser desorption/ionization time-of-flight peptide mass fingerprint analysis after trypsin digestion, using Mascot (data base NCBInr 20040521)

Nucleotide binding assay

Nucleotide binding was measured by 8-azido-[α-32P]ATP photo cross-linking experiments. Cross-linking reactions were performed in a 96-well microtiter plate in a final volume of 25 uL/reaction. Purified wild-type Abcb6 (2 μM final concentration) was incubated for

5 min on ice with 8-azido-[α-32P]ATP (0.05 – 16 μM) in reaction buffer (100 mM KCl, 2.5 mM MgCl2, 50 mM Tris-HCl, pH 7.4). For competition experiments, 0.1 μM to 10 mM unlabeled ATP was included in the buffer. Subsequently, samples were irradiated with UV light (254 nm, 8 watts) for 5 min at 4°C, separated by SDS-PAGE, Coomassie blue-stained, dried, and exposed to Kodak SO230 phosphor screen. Photo cross-linked protein was quantified by phosphorimaging (Phosphor-Imager 445Si, Molecular dynamics) and bands were quantified using ImageJ software (version 1.43u). Intensities were plotted against the 8-azido-ATP concentration and apparent Kd values for 8-azido-[α-32P]ATP were obtained from the best fit of the data to a hyperbolic curve using GraphPad Prism software (version 5, CA). The half-maximal inhibitory concentration (IC_{50}) for ATP was derived by plotting labeling intensities corresponding to Abcb6 as a function of unlabeled ATP concentrations. The Kd(azidoATP) values and the IC_{50} values were used to calculate Kd(ATP) by applying the Cheng-Pursoff equation.

ATP hydrolysis assay

ATPase activity was determined essentially as described by measuring the release of inorganic phosphate (Pi). Briefly, purified Abcb6 (5 μM final concentration) was incubated at 37°C in 100 μL of an ATP buffer containing 50 mM Tris-MOPS; 70 mM KCl; and 2 mM EGTA, pH 7.5, and the ATPase reaction was started by the addition of 12 mM MgATP. The reaction was stopped by the addition of 100 μL of 10% SDS solution, and the amount of inorganic phosphate was determined immediately. ATPase activity was estimated by the difference obtained in Pi levels between ATP buffer alone and ATP buffer containing the purified protein. Inorganic phosphate was measured by a sensitive colorimetric reaction. The SDS-containing samples were supplemented with 200 μL of freshly prepared reagent A containing a final concentration of 5.5% ascorbic acid; 5 mM ammonium molybdate and 2 mM zinc acetate and

the reduced complex was measured at an optical density of 850 nM. A similar protocol was used to determine the ATPase activity of liposome reconstituted Abcb6. For substrate stimulated ATPase activity, indicated drugs were added in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the assay medium was 1%. Control experiments indicated that DMSO at this concentration had no appreciable effect on the ATPase activity.

Reconstitution of Abcb6

For reconstitution of functional (WT) and non-functional (mutant; MT) Abcb6 into lipids, protein bound to the FLAG-affinity column as described above was washed with Hepes-EGTA-1% n-Octyl-β-D-glucopyranoside (OGP) 5 times, and eluted using Hepes-EGTA-1% OGP-0.5 mg FLAG peptide. Lipid solutions (5 mg/mL Phosphatidylcholine (PC), 5 mg/mL Phosphatidylserine (PS), 5 mg/mL Phosphatidylethanolamine (PE), and 10 mg/mL Ergosterol (ES) were dissolved in chloroform, and the solvent was evaporated under a gentle stream of argon and evacuated overnight in a desiccator under vacuum to remove any residual chloroform. The lipid film was rehydrated in 1 mL of reconstitution buffer (15 mM HEPES, 0.5 mM EGTA, pH 7.4) to produce a 1 mM lipid solution. FLAG-tagged WT and MT Abcb6 protein at a concentration of 3.3 µg/µL was added to the rehydrated liposomes and incubated for 1 h at 4°C with gentle agitation. The lipid-protein suspension mixture was sonicated using an ultrasonic cleaner (Avanti polar lipids Inc) for 20 sec; 3 times at 4°C. This procedure resulted in a nearly uniform suspension of relatively unilamellar liposomes. Protein-free liposomes were prepared similarly by replacing purified protein with elution buffer. Unincorporated materials and detergent were removed by dialysis using 1L of reconstitution buffer changed every 24 h for a total duration of 48 h. The resultant proteoliposomes were collected, flushed quickly with argon, and stored at -80°C until used.

Liposome flotation assay

Proteoliposomes were floated in a discontinuous Nycodenz density gradient in a buffer containing 100 mM KCl and 10 mM MOPS/Tris, pH 7.0. Nycodenz and proteoliposomes were solubilized in the same buffer and mixed to a final nycodenz concentration of 40%. Subsequent layers of decreasing Nycodenz concentration (20, 10, 5, and 2%) were layered onto the 40% nycodenz-proteoliposomes mixture. Percentages indicated in the figures are the final concentrations of Nycodenz. The gradient was centrifuged at 4°C for 60 min at 100,000 x g. Subsequently, the gradient was fractionated by taking equal volumes from the top. Fractions were analyzed by SDS-PAGE and western blotting.

Hemin agarose affinity chromatography

The assay was performed as described previously. Briefly, Abcb6-FLAG proteoliposomes (5 µg), was incubated at room temperature for 15 min in the presence of 167.5 nM hemin-agarose (Sigma, MO). The reaction mixture was centrifuged at 4°C. The hemin-agarose-Abcb6-FLAG proteoliposome complex was washed thrice with 1 mL lysis buffer, resuspended in 50 µL of 2 X SDS sample-loading buffer, and then centrifuged. The supernatant from the final spin was analyzed on a 4-15% gradient gel, transferred to a nylon membrane, and probed with a monoclonal antibody to the FLAG epitope to identify Abcb6-FLAG.

Coproporphyrinogen (COPIII) transport assays

COPIII transport assays were performed as previously described (28) with slight modification to accommodate the use of liposomes. Control, Abcb6-WT and Abcb6-MT liposomes (25 to 50 µg for Abcb6 liposomes and equal volume of control liposomes) were resuspended in reconstitution buffer (final volume of 50 µL). Transport reaction was started by adding 50 µL of a reaction mixture (50 mM MOPS-Tris, 70 mM KCl, 10mM MgCl2, and either 4

mM AMP, 4 mM AMP:PNP or 4 mM ATP) and incubated at 37°C for the duration of the experiment as described in the respective figures. Reactions were stopped by diluting the reaction with 1 mL of ice-cold reconstitution buffer. In early experiments using nitrocellulose filters (pore size 0.45 μM) to measure COPIII uptake, we observed that a significant proportion of the proteoliposomes passed through the filters. To avoid this problem, an affinity purification method employing FLAG-affinity columns using anti-M2 magnetic beads was used to separate proteoliposomes from free COPIII. The affinity columns were washed three times with cold reconstitution buffer to remove any free COPIII not transported into the proteoliposomes. The affinity-purified proteoliposomes were lysed by incubation for 10 min in 10% SDS with vortexing. COPIII fluorescence was measured at excitation wavelength 405 nm and emission wavelength 630 nm. Concentration of COPIII was determined from a standard curve of COPIII in 10% SDS. The rate of active transport was calculated as the difference between ATP-dependent and independent uptake.

Verteporfin and tomatine transport assays

The assay was performed as previously described (69). Briefly, following uptake of tomatine and verteporfin into proteoliposomes, the reaction was stopped at the indicated time and liposomes were separated by affinity purification employing FLAG-affinity columns as described above (COPIII transport assay). The affinity column was washed three times with cold reconstitution buffer to remove any free tomatine or verteporfin not transported into proteoliposomes. The affinity-purified proteoliposomes were resuspended in 100 μL of acetonitrile and the amount of verteporfin or tomatine taken up into proteoliposomes was analyzed using Ultra Performance Liquid Chromatography-time-of-flight mass spectrometry (UPLC-TOFMS) as previously described (143). Concentration of verteporfin and tomatine was

determined from a standard curve of verteporfin and tomatine in acetonitrile. The rate of active transport was calculated as the difference between ATP-dependent and independent uptake.

Determination of transport kinetics

Transport kinetics was estimated by non-linear curve-fitting using GraphPad Prism 5.0 software. The Michaelis-Menten equation was used to calculate apparent Km and Vmax values.

Microarray analysis

Abcb6$^{+/+}$ and *Abcb6*$^{-/-}$ mice (n=3) liver samples were used for gene expression analysis using the Affymetrix Genechip System (Affymetrix, CA) in collaboration with the Genome sequencing facility, University of Kansas Medical Center. Briefly, 1µg of total RNA was used to synthesize complementary DNA (cDNA) using the One-Cycle cDNA Synthesis kit. Biotinylated complementary RNA (cRNA) was synthesized following the IVT labeling kit and purified using the GeneChip Sample Cleanup Module. Subsequently, Biotinylated cRNA was fragmented and hybridized to the Genechip Mouse430A 2.0 microarrays. Hybridization was followed by washing of the chip and then scanned using the GeneChip Scanner 3000. The fluorescent signal corresponding to the intensity of hybridization for each transcript was determined using the Gene Chip Operating Software (GCOS 1.4).

Functional and pathways analysis by ingenuity pathway analysis (IPA)

The genes with significantly different expression (p value ≤0.05) and fold change upregulated or downregulated by ≥ 1.5 fold were used for IPA. The dataset of such genes containing information about Affymetrix probe ID and fold change was uploaded onto the IPA System. For functional and canonical pathway analysis, Fischer's exact test was used to calculate the p-value determining the probability that the association between the genes in the

dataset and the biological function and the canonical pathway is due to chance alone. P-values ≤ 0.05 were considered as significant. The Biofunction and Canonical Pathways Analysis identified the pathways in our dataset with most significant association to pathways in the Ingenuity Knowledge Base. For Biofunction analysis Activation z-score was calculated as a measure of significance. An absolute z-score of below (inhibited) or above (activated) 2 was considered as significant. For canonical pathway analysis Fischer's exact p- value were used for significance and a ratio measure was used for pathway overlap. A ratio was calculated by the number of differentially expressed genes from our dataset that map to the pathway divided by the total number of molecules that exist in the canonical pathway. A ratio of 1 implies complete overlap.

Statistical analysis

Statistical analysis of the observed values was performed using Student's *t*-test. All calculations were performed with the SPSS statistical software package. All values are expressed as mean ± S.D. Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., IL). A difference was considered significant at the $p < 0.05$ level.

Chapter 4. **XENOBIOTICS MEDIATED REGULATION OF ABCB6**

4.1. Polycyclic aromatic hydrocarbons (PAHs) mediate transcriptional activation of the ATP binding cassette transporter Abcb6 gene via the aryl hydrocarbon receptor (AhR)

4.2. Xenosensor CAR and PXR regulates ATP binding cassette transporter Abcb6 gene

4.3. Arsenic mediated transcriptional activation of Abcb6

4.1. POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) MEDIATE TRANSCRIPTIONAL ACTIVATION OF ABCB6 GENE VIA THE ARYL HYDROCARBON RECEPTOR (AhR)

Abstract

In the present study, we demonstrate that polycyclic aromatic hydrocarbons (PAHs), the widely distributed environmental toxicants up-regulate Abcb6 expression in both mice and humans. Moreover*, in vivo* studies in aryl hydrocarbon receptor (AhR) knock-out mice demonstrate that PAH induction of Abcb6 is mediated by AhR. Promoter activation studies combined with electrophoretic mobility shift assay and chromatin immunoprecipitation assay demonstrate direct interactions between the AhR binding sites in the Abcb6 promoter and the AhR receptor, implicating drug activation mechanisms for Abcb6 similar to those found in inducible P450s. These studies are the first to describe direct transcriptional activation of both mouse and human Abcb6 by xenobiotics.

Introduction

Heme is indispensable for mammalian life. It is an essential component of numerous heme proteins, with functions including oxygen transport, energy metabolism, and drug biotransformation (144-146). Under normal physiological conditions, intracellular free heme levels are extremely low because increased levels of free heme are cytotoxic, and accordingly heme biosynthesis is tightly regulated (5,8,147). However, the rate of heme biosynthesis must also be responsive to increased demands, for instance, during induction of drug-metabolizing P450s, which is required to assure an adequate and apparently coordinated supply of heme for the generation of functional cytochrome holoprotein (40,148,149). Under these conditions, heme biosynthesis is swiftly up-regulated to provide sufficient heme to nascent apocytochromes.

The two major sites of heme synthesis are bone marrow, where hemoglobin is produced, and liver, where various hemoproteins (in particular, microsomal P450s) rely on prosthetic heme to catalyze the oxidation of endogenous and exogenous compounds. The rate of heme synthesis in both the bone marrow and the liver is controlled at the first committed step, the condensation of glycine and succinyl-CoA to 5-aminolevulinate (150). This committed step is catalyzed by ALAS, which exists as two isoforms: ALAS1, which regulates heme synthesis in liver and other organ systems, and ALAS2, which regulates heme synthesis in hematopoietic tissues (44,151,152). The regulatory role of ALAS in heme synthesis is underlined by the fact that ALAS mRNA is markedly increased under physiological conditions demanding more heme, such as exposure to drugs and environmental toxicants, whereas expression levels of the other enzymes in the pathway do not change significantly (153). Recent studies have shown that this increase in ALAS expression in response to heme demand is not a consequence of heme feedback regulation but a direct activation of ALAS transcription by xenobiotic-sensing nuclear receptors similar to those found in inducible P450s (154).

Although ALAS-mediated regulation of heme synthesis is considered the key step in heme biosynthesis, recent reports have identified a second regulatory step in heme biosynthesis mediated by the mitochondrial ATP binding cassette transporter Abcb6 (28). Abcb6 is highly expressed in fetal liver, erythroid cells, and adult tissues that have substantial heme requirements because of their high metabolic activity (*e.g.* heart and skeletal muscle) (28). Further, Abcb6 expression is directly related to enhanced *de novo* porphyrin biosynthesis, and *Abcb6* overexpression activates the expression of genes important for heme biosynthesis (28). Thus, Abcb6 represents a previously unrecognized rate-limiting step in heme biosynthesis. Supporting this hypothesis, recent observations demonstrate that *Abcb6* mRNA, like ALAS mRNA, is markedly increased under physiological conditions demanding more heme (28,155,156). Despite these observations, very little is known about the mechanisms that regulate Abcb6 expression both under normal physiological conditions and conditions of increasing demand for heme.

Results

4.1.1. PAH increases hepatic porphyrin levels in mice and humans

PAHs are potent atmospheric pollutants that are classified as either being nontoxic or being extremely toxic based on their structure (157-159). The three prototypical PAHs used in the studies described here, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[a]pyrene (B[a]P), and 3-methylcholanthrene (3-MC) are classified as teratogens by the environmental protection agency (EPA) and are considered to be extremely toxic (160-162). Thus in these studies, we first established the toxicity profile for TCDD, B[a]P and 3-MC in mouse primary hepatocytes and human hepatoma cells. Mouse primary hepatocytes and human hepatoma cells (HepG2 and Huh7) were treated with increasing concentrations of either B[a]P (0, 2.5, 5 and 10 μM), TCDD (0, 1 and 2 nM) or 3-MC (0, 5, 10 and 20 μM) for 16 hr and viability was evaluated by trypan blue exclusion assay (to measure necrosis) and annexin V staining (to measure apoptosis). We found that B[a]P was non-lethal upto a concentration of 10 μM, TCDD was non-lethal upto a concentration of 2 nM and 3-MC wan non-lethal upto a concentration of 20 μM, for a maximum exposure time of 16 hours (data not shown). Based on these initial observations an exposure regimen of 0, 2.5, 5 and 10 μM for 16 hr for B[a]P, 0, 1 and 2 nM for 16 hr for TCDD, and 0, 5, 10 and 20 μM for 3-MC was considered as non-lethal to both mouse primary hepatocytes and human hepatoma cells, and was employed as treatment regimen in the rest of the studies.

Previous studies have shown that exposure to PAHs such as B[a]P, 3-MC, and TCDD induces the expression and activity of P450s. This increase in P450 expression would require a co-ordinate increase in cellular porphyrin levels to compensate for the increased heme demand required for increased P450 activity (163-166). To test this we evaluated the effect of B[a]P, 3-MC and TCDD on hepatic PPIX levels, as a measure of heme, using the non-lethal

concentrations of B[a]P, 3-MC and TCDD described above. In these studies, both dose and time dependent effects of B[a]P, 3-MC and TCDD on hepatic PPIX levels were evaluated. In the dose dependent studies mouse primary hepatocytes and human hepatoma cells were exposed to increasing concentration of either B[a]P (0 μM to 10 μM), or 3-MC (0 μM to 20 μM), or TCDD (0 nM to 2 nM) for 16 hr. In the time dependent studies mouse primary hepatocytes and human hepatoma cells were exposed to a single concentration of either 10 μM B[a]P, 10 μM of 3-MC, or 2 nM TCDD for 0, 2, 4, 8 and 16 hr. We found that both B[a]P and 3-MC increased cellular PPIX levels in a dose dependent and time dependent manner in both primary hepatocytes and in hepatoma cells (Figure 4.1.1 A-F, data not shown for 3-MC). Maximum increase in cellular PPIX was observed after 16 hr of exposure to 10 μM B[a]P or 20 μM 3-MC. Percent fold increase in PPIX levels in response to B[a]P and 3-MC treatment was comparable between mouse primary hepatocytes and human hepatoma cells suggesting similar effect of B[a]P and 3-MC on protoporphyrin levels in both mice and humans. In contrast to B[a]P and 3-MC, TCDD did not increase hepatic PPIX levels (data not shown) consistent with previous observations (36).

Figure 4.1.1.

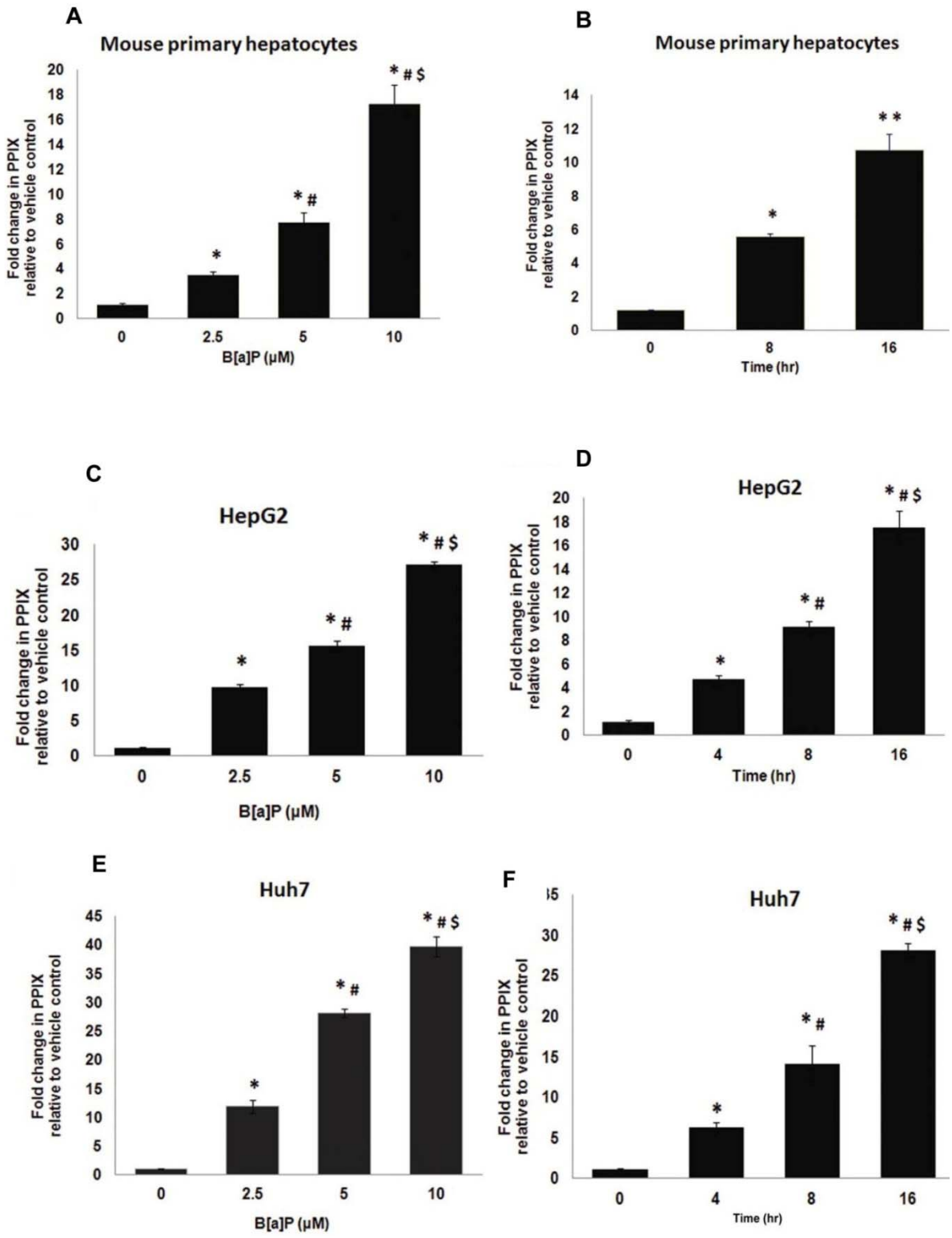

A
Mouse primary hepatocytes
Fold change in PPIX relative to vehicle control
B[a]P (μM)
0
2.5
5
10
B
Mouse primary hepatocytes
Fold change in PPIX relative to vehicle control
Time (hr)
0
8
16
C
HepG2
Fold change in PPIX relative to vehicle control
B[a]P (μM)
D
HepG2
Fold change in PPIX relative to vehicle control
Time (hr)
0
4
8
16
E
Huh7
Fold change in PPIX relative to vehicle control
B[a]P (μM)
F
Huh7
Fold change in PPIX relative to vehicle control
Time (hr)

Figure 4.1.1. (continued)

Polyaromatic hydrocarbon benzo[*a*]pyrene increases hepatic PPIX levels.

Exposure to B[*a*]P increases protoporphyrin IX levels in both mouse primary hepatocytes (*A* and *B*) and human hepatomas (*C*–*F*), in a dose-dependent (*A*, *C*, and *E*) and time-dependent (*B*, *D*, and *F*) manner. Values represent mean ± S.D. (*error bars*) ($n = 4$). Results shown are representative of three independent experiment with $n = 4$ per experiment. *, significantly different from vehicle control ($p < 0.01$); #, significantly different from cells treated with 2.5 μM B[*a*]P in dose-dependent studies and significantly different from 4-h treatment (hepatomas) in time-dependent studies ($p < 0.01$). $, significantly different from cells treated with 5 μM B[*a*]P in dose-dependent studies and significantly different from 8-h treatment (hepatomas) in time-dependent studies ($p< 0.01$). **, significantly different from cells exposed to B[*a*]P for 8 h (mouse primary hepatocytes) ($p < 0.01$).

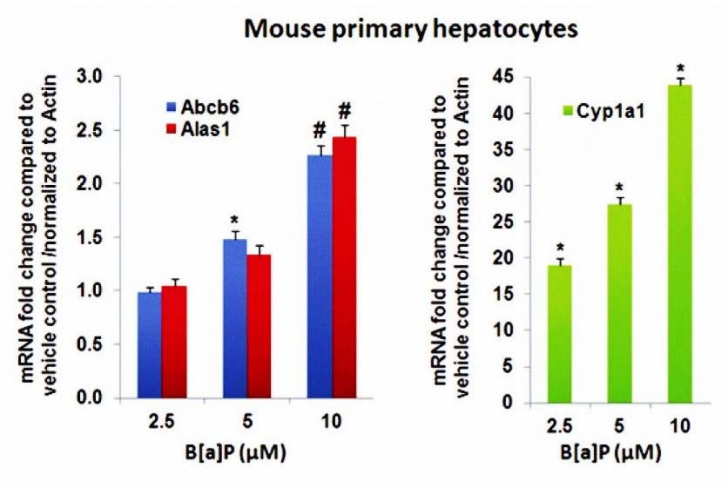
4.1.2. PAH exposure induces the key regulators of hepatic heme synthesis, ALAS1 and Abcb6/Abcb6

B[*a*]P and 3-MC-mediated increase in PPIX could be due to either an increase in porphyrin synthesis or a block in porphyrin metabolism or both. To evaluate this further, we first tested whether B[*a*]P- and 3-MC-induced increase in cellular PPIX levels was a result of increased porphyrin biosynthesis. Because hepatic porphyrin biosynthesis is predominantly regulated by the two key regulatory proteins ALAS1 and Abcb6, we measured the expression of these two genes in response to B[*a*]P and 3-MC as a measure of increased porphyrin biosynthesis. Both time- and dose-dependent effects of B[*a*]P and 3-MC on porphyrin biosynthesis were evaluated in these studies. In the dose-dependent studies, mouse primary hepatocytes and hepatoma cells were treated with increasing concentrations of B[*a*]P or 3-MC for 16 h. In the time-dependent studies, cells were exposed to one single dose of 10 μM B[*a*]P for 0, 2, 4, 8, and 16 h. As shown in Figure 4.1.2, B[a]P induced both Abcb6 and ALAS1 mRNA in a dose- and time-dependent manner in both mouse primary hepatocytes (Figure 4.1.2, A and B) and human hepatoma cells (Figure 4.1.2, C–F). Similarly, 3-MC also induced Abcb6 expression in a dose- and time-dependent manner (data not shown). Significant induction of both of the genes was found at concentrations as low as 5 μM and 16 h of treatment. Further, we found that B[a]P- and 3-MC-mediated increase in Abcb6 mRNA correlated well with B[a]P- and 3-MC-mediated increase in Abcb6 protein expression (Figure 4.1.2G and data not shown for 3.MC). Cytochrome P450 subfamily 1a1 (Cyp1a1) gene known to be induced in response to B[a]P and 3-MC, were used as a positive control in these studies (35). Cyp1a1 expression increased with increasing concentration of B[a]P and 3-MC and with increasing time of exposure (Figure 4.1.2 and data not shown for 3-MC), demonstrating treatment effectiveness. Taken

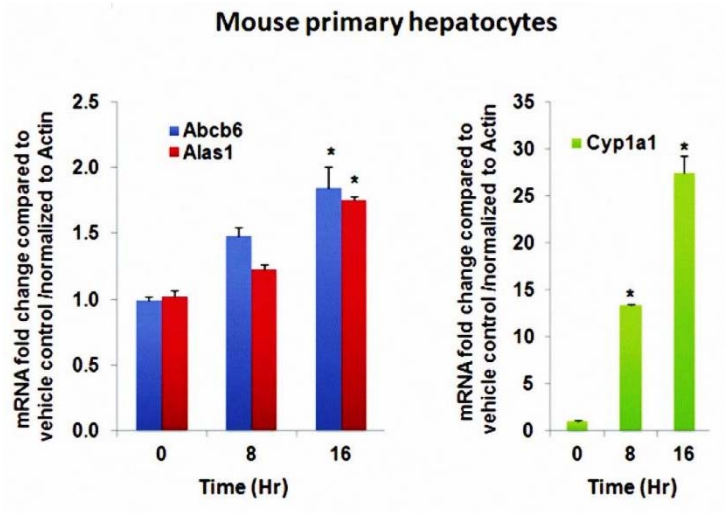
together, results presented in Figure 4.1.2 demonstrate that B[*a*]P- and 3-MC-induced hepatic expression of the key regulators of porphyrin synthesis, ALAS1 and Abcb6.

Figure 4.1.2.

A



B



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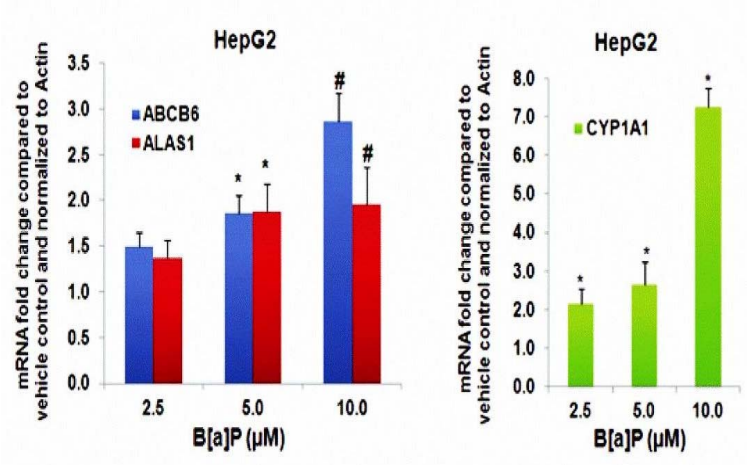
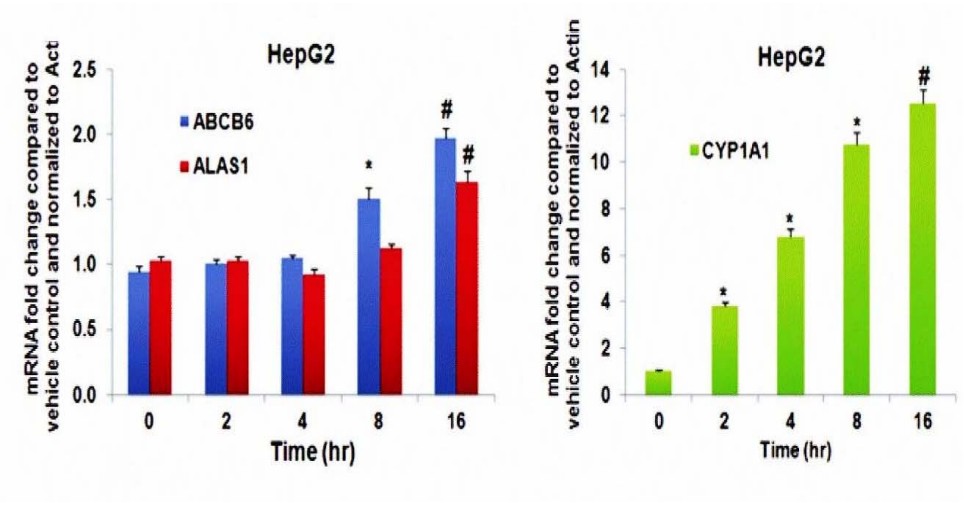
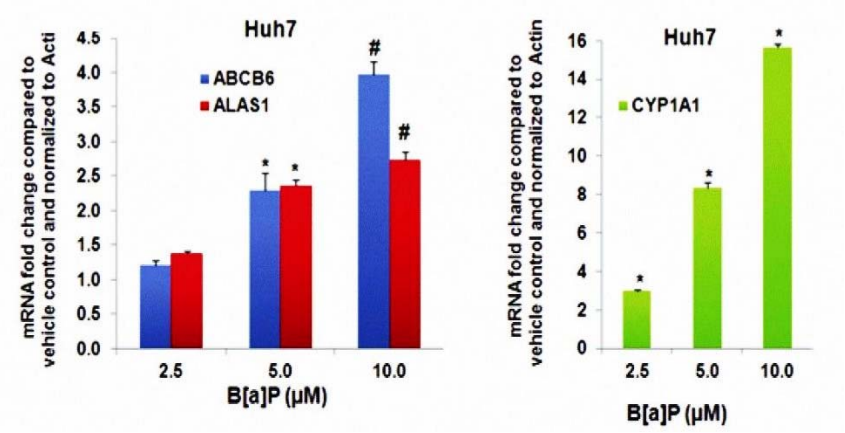


Figure 4.1.2. (continued)

D



E



F

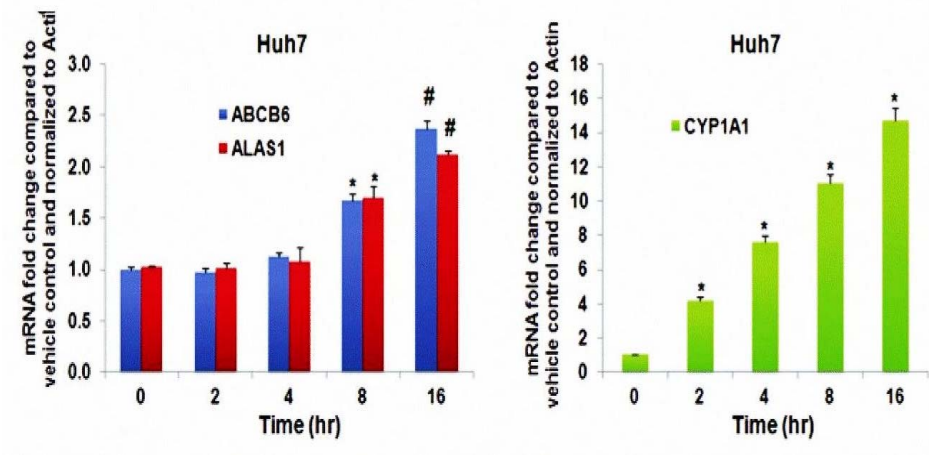
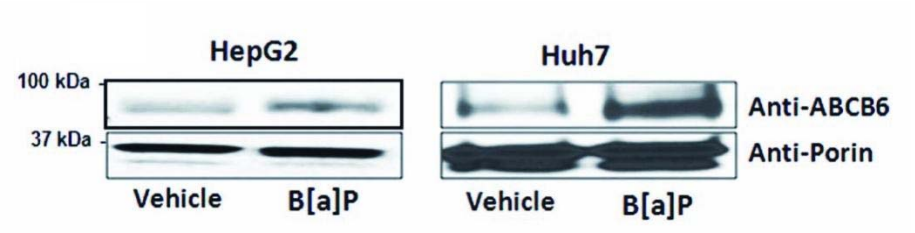


Figure 4.1.2. (continued)

G

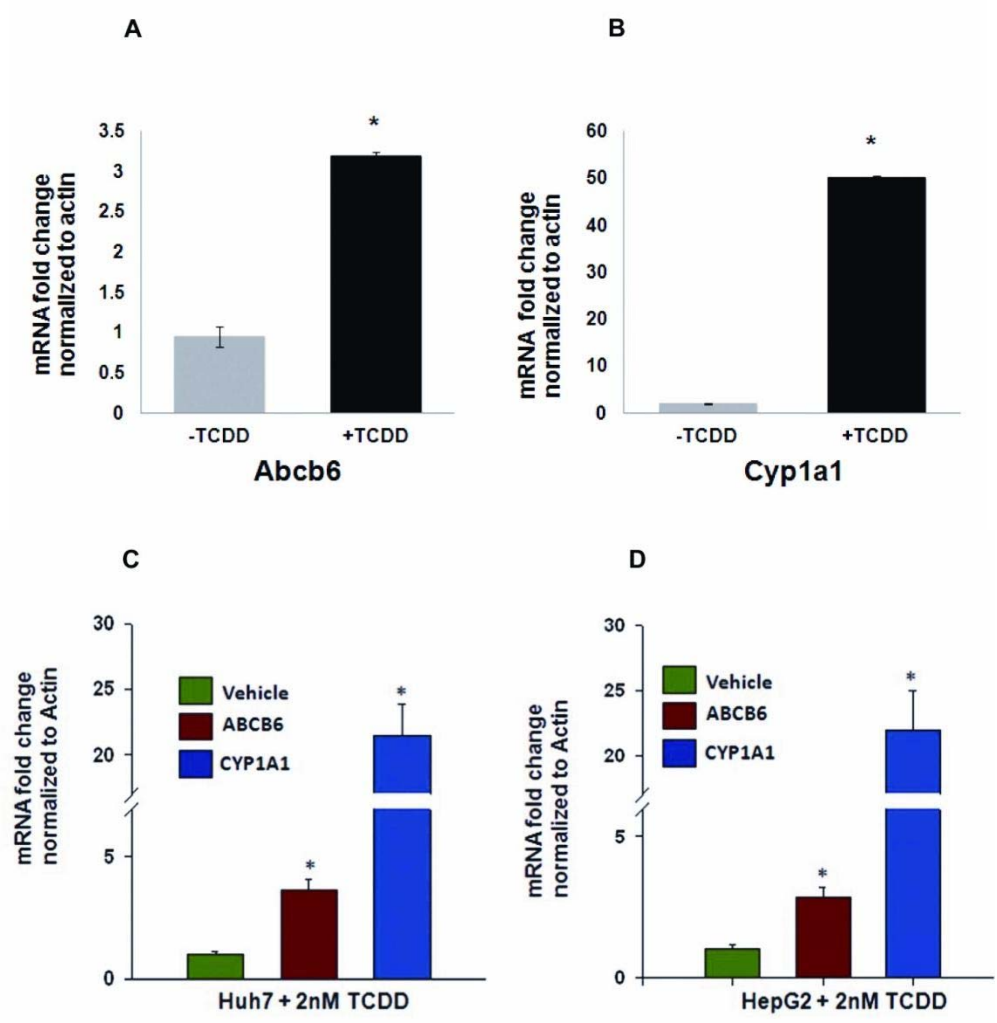


B[a]P induces Abcb6 and ALAS1 expression in a dose- and time-dependent manner.

Exposure to B[*a*]P induces Abcb6 and Alas1 expression in mouse primary hepatocytes (*left panels*; *A* and *B*) and human hepatomas (*left panels*;*C–F*) in a dose-dependent (left panels; *A*, *C*, and *E*) and time-dependent manner (*left panels*;*B*, *D*, and *F*). *Right panels* (*A–F*), B[*a*]P-mediated induction of CYP1A1. CYP1A1, a gene known to be induced by B[*a*]P in liver, is used as a positive control in these experiments. *G*, Abcb6 protein expression in response to B[*a*]P treatment in human hepatomas. Abcb6 expression was measured in isolated mitochondria using an Abcb6-specific antibody. Values represent mean ± S.D. (*error bars*) (*n* = 4). Results shown are representative of three independent experiments with *n* = 4/experiment. *, significantly different from cells exposed to 2.5 μM B[*a*]P in dose-dependent studies and significantly different from cells exposed to B[*a*]P for 4 h (hepatomas) in time-dependent studies ($p < 0.01$). #, significantly different from cells treated with 5 μM B[*a*]P in dose-dependent studies and significantly different from 8-h treatment (hepatomas) in time-dependent studies ($p < 0.01$).

Although TCDD did not increase cellular PPIX levels, previous studies have demonstrated that TCDD treatment induces ALAS1 expression probably as a feedback regulation to increased heme demand required for P450 activity (167-169). Based on these observations, we hypothesized that TCDD might induce Abcb6 expression similar to what has been reported for ALAS1. To test this hypothesis, we exposed both mice and human hepatoma cells to TCDD and measured Abcb6/Abcb6 expression. Mice were treated with 37 μg/kg/day TCDD for 4 consecutive days in corn oil. In contrast, hepatoma cells were treated with a single dose of 2 nM TCDD for 16 h. We found that TCDD induced Abcb6 expression in both mice (Figure 4.1.3A) and hepatoma cells (Figure 4.1.3, C and D). As before, expression of Cyp1a1, a gene known to be induced in response to TCDD (170,171), was used as a positive control in these studies. As with B[a]P and 3-MC treatment, Cyp1a1 expression increased with TCDD treatment (Figure 4.1.3, B–D), demonstrating treatment effectiveness. Together these results suggest that TCDD induces Abcb6 expression in a manner similar to its effect on ALAS1 expression.

Figure 4.1.3.



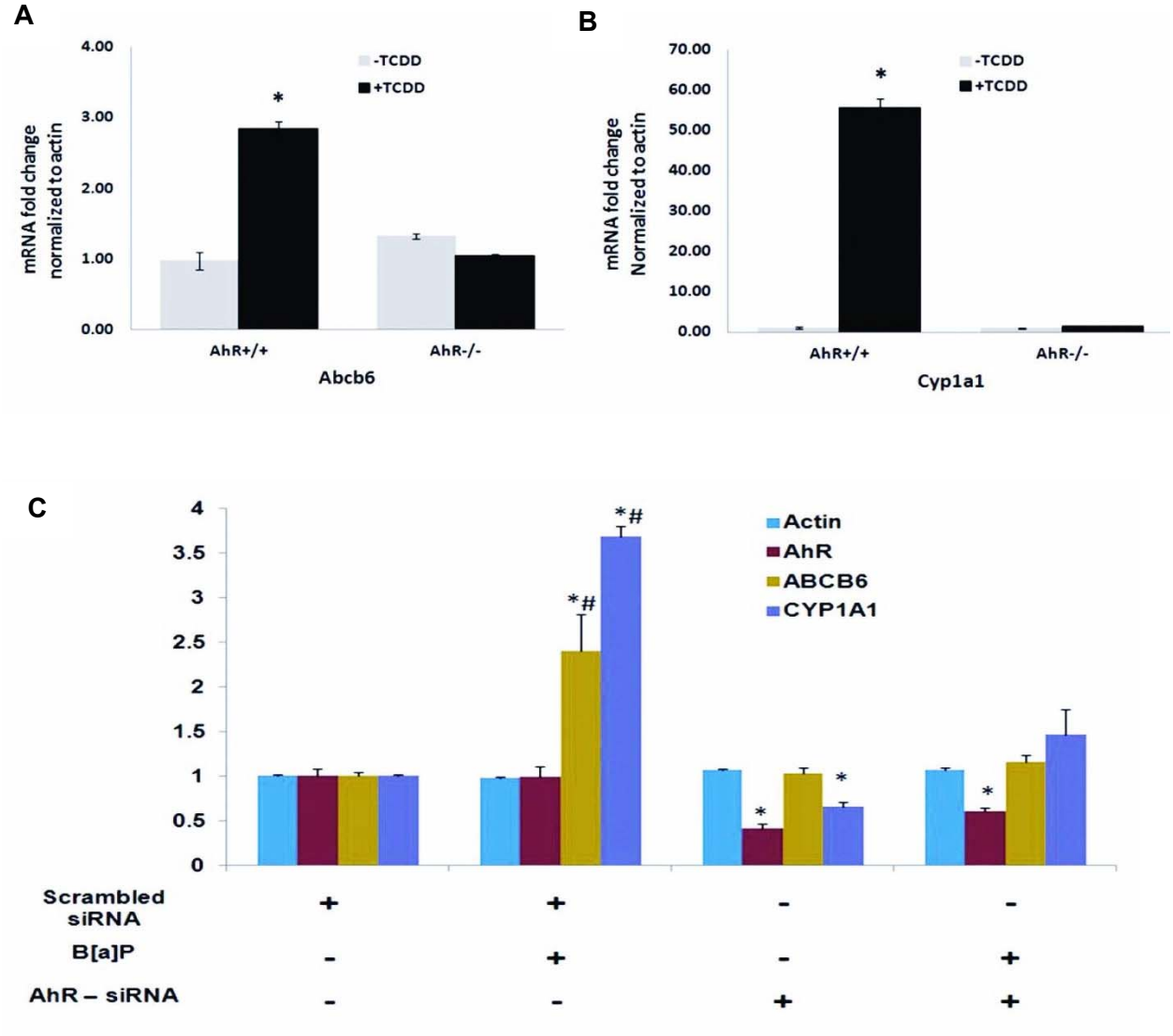
TCDD induces Abcb6 expression in both mice and humans.

Exposure to TCDD induces Abcb6 expression in mouse (A) and human hepatomas (C and D). B, TCDD-mediated induction of Cyp1a1, a gene known to be induced by TCDD in the liver, is used as a positive control in these experiments. Values represent mean ± S.D. (error bars); n = 3 for mice, and n = 4 for cell culture. Results shown are representative of three independent experiments. *, significantly different for vehicle-treated mice in animals and human hepatomas ($p < 0.01$).

4.1.3. PAH-mediated induction of Abcb6/Abcb6 is AhR-dependent

Recent discoveries have implicated the nuclear receptor AhR in PAH-mediated induction of target genes (172,173). Thus, in this study, we evaluated the involvement of the AhR pathway in PAH-mediated up-regulation of Abcb6. Toward this end, we examined Abcb6 mRNA expression in response to TCDD and B[a]P treatment both *in vivo* and *in vitro*. In the *in vivo* studies, AhR wild type and AhR knock-out mice were exposed to TCDD as described under "Experimental Procedures." In the *in vitro* studies, AhR endogenous knock-down and AhR-expressing hepatoma (HepG2) cells were exposed to B[a]P as described under "Experimental Procedures." Loss of AhR expression in the AhR knock-out mouse and AhR knock-down cells was confirmed by real-time PCR analysis of the AhR transcript using gene-specific primers (Figure 4.1.4C). Abcb6 expression was evaluated by real-time PCR using gene-specific primers. As before, CYP1A1 induction in response to AhR ligands was used as a positive control. We found that in both the AhR knock-out mice and in AhR knock-down hepatoma cells, AhR-responsive gene CYP1A1 was not activated, whereas it was activated in the AhR wild type mouse and hepatoma cells (Figure 4.1.4, B and C). More importantly, we found that TCDD and B[a]P induced a marked increase in the expression of Abcb6 mRNA in AhR proficient mice (Figure 4.1.4A) and hepatomas (Figure 4.1.4C), but TCDD and B[a]P had no effect on Abcb6 expression in AhR-deficient mice and hepatomas (Figure 4.1.4, A and C). Taken together, these results suggest that PAH-mediated increase in Abcb6/ Abcb6 transcript is AhR-dependent.

Figure 4.1.4.



A functional AhR pathway is required for B[*a*]P-mediated up-regulation of Abcb6.

Mouse (*A*) and HepG2 (*C*) cells lacking AhR are unable to up-regulate Abcb6 expression, whereas mouse (*A*) and HepG2 (*C*) cells with functional AhR up-regulate Abcb6 expression in response to TCDD and B[*a*]P. Cyp1a1, a gene known to be induced by TCDD and B[*a*]P, is used as a positive control in these experiments. Values represent mean ± S.D. (*error bars*); n = 4 for mice, and n = 3 for HepG2. Results representative of three independent experiments. *, significantly different from scrambled siRNA transfected cells; $p < 0.01$ in hepatomas and *AhR*$^{+/+}$ vehicle-treated mice. #, significantly different from AhR siRNA-transfected cells treated with B[*a*]P. $p < 0.01$.

4.1.4. AhR activates transcription of Abcb6 by binding to the AhR response element (AhRE) in the Abcb6/Abcb6 promoter

Previous studies have demonstrated that in the absence of its ligand, AhR is localized to the cytoplasm. However, upon binding the ligand, AhR translocates from the cytoplasm to the nucleus, where it interacts with specific DNA response elements in target gene promoters and activates gene transcription (174,175). Thus in these studies, we first confirmed that B[a]P treatment results in the redistribution of AhR from the cytoplasm to the nucleus (Data not shown). We next tested whether PAH-induced expression of Abcb6/Abcb6 is mediated by AhR-dependent activation of the Abcb6 promoter. For this purpose, both human and mouse Abcb6 promoters were cloned in frame in a luciferase reporter vector, and the promoter-luciferase-reporter constructs were transfected into HepG2 cells as described (64). Using the heme oxygenase-1 (HO-1) promoter luciferase- reporter construct, known to be activated by AhR, as a positive control, we evaluated the effect of PAH on the transcriptional activation of the Abcb6/Abcb6 promoter. Because both TCDD and B[a]P are known to activate target genes in a similar manner, via the AhR receptor (174,175), only B[a]P treatment was used in the promoter activation studies. We found that B[a]P activated both the human and mouse Abcb6 promoter (Fig. 4.1.5, A and B) in a manner that corresponded well with Abcb6 transcriptional up-regulation seen in Figure 4.1.2. Together, these results suggest that the B[a]P-mediated increase in Abcb6 expression is mediated by activation of the Abcb6 promoter by AhR.

The apparent similarity in AhR-mediated activation of both the mouse and human Abcb6 promoter suggests similarities in the genomic organization of their AhR-binding response elements. In an effort to identify the existence of such elements in the Abcb6/Abcb6 promoter, we used a computer algorithm (Transfac) to analyze the 5′-flanking sequence of the human and mouse gene encoding Abcb6. Transfac analysis found three putative AhREs located at 102,

115, and 123 bp upstream of the transcription start site in the human and at 168, 185, and 195 bp upstream of the transcription start site in the mouse Abcb6 promoter (Figure 4.1.5C). For clarity, the three AhR binding sites are labeled according to their occurrence in the gene, with the farthest upstream from the transcription start site referred to as Abcb6-ARE1 (123 bp in the human and 195 bp in the mouse promoter) and the one closest to the start site referred to as Abcb6-ARE3 (102 bp in the human and 168 bp in the mouse promoter (Figure 4.1.5D).

The existence of three putative AREs in the 5′-flanking region of both the human and mouse Abcb6 promoter suggests that AhR could interact with any of these AREs to induce Abcb6 expression. To test this assertion and to assess the relative contribution of the three AREs to Abcb6 promoter transactivation by B[a]P, we generated mutations in each of the three AREs by site-directed mutagenesis (Figure 4.1.5D) and transfected HepG2 cells with these promoter constructs followed by treatment with B[a]P. Because the three AREs appeared to be conserved in both the mouse and human promoter, and because the activation of these mouse and human promoters appear to be similar (Figure 4.1.5, A and B), only the human promoter was used to explore the relative contribution of the three AREs to B[a]P-mediated activation. We found that mutations in the two proximal AREs at 115 and 102 bp (Abcb6-M2 and Abcb6-M3, respectively) did not affect B[a]P-mediated activation of the human Abcb6 promoter (Figure 4.1.5E). In contrast, mutation of the distal ARE at123 bp (Abcb6-M1) or mutation of all the three AREs (Abcb6-M1M2M3) completely abolished B[a]P-mediated activation of the human Abcb6 promoter (Figure 4.1.5E). Taken together, these results suggest that the most distal ARE in the human Abcb6 promoter (Abcb6- ARE1; at 123 bp) is responsible for B[a]P-mediated induction of Abcb6, whereas the two proximal AREs (Abcb6-ARE2 and Abcb6-ARE3, at 115 and 102 bp) are dispensable for B[a]P-mediated induction of Abcb6. In all of these studies, the heme oxygenase-1 promoter-luciferase-reporter construct was used as a positive control to confirm treatment effectiveness (Figure 4.1.5).

Figure 4.1.5.

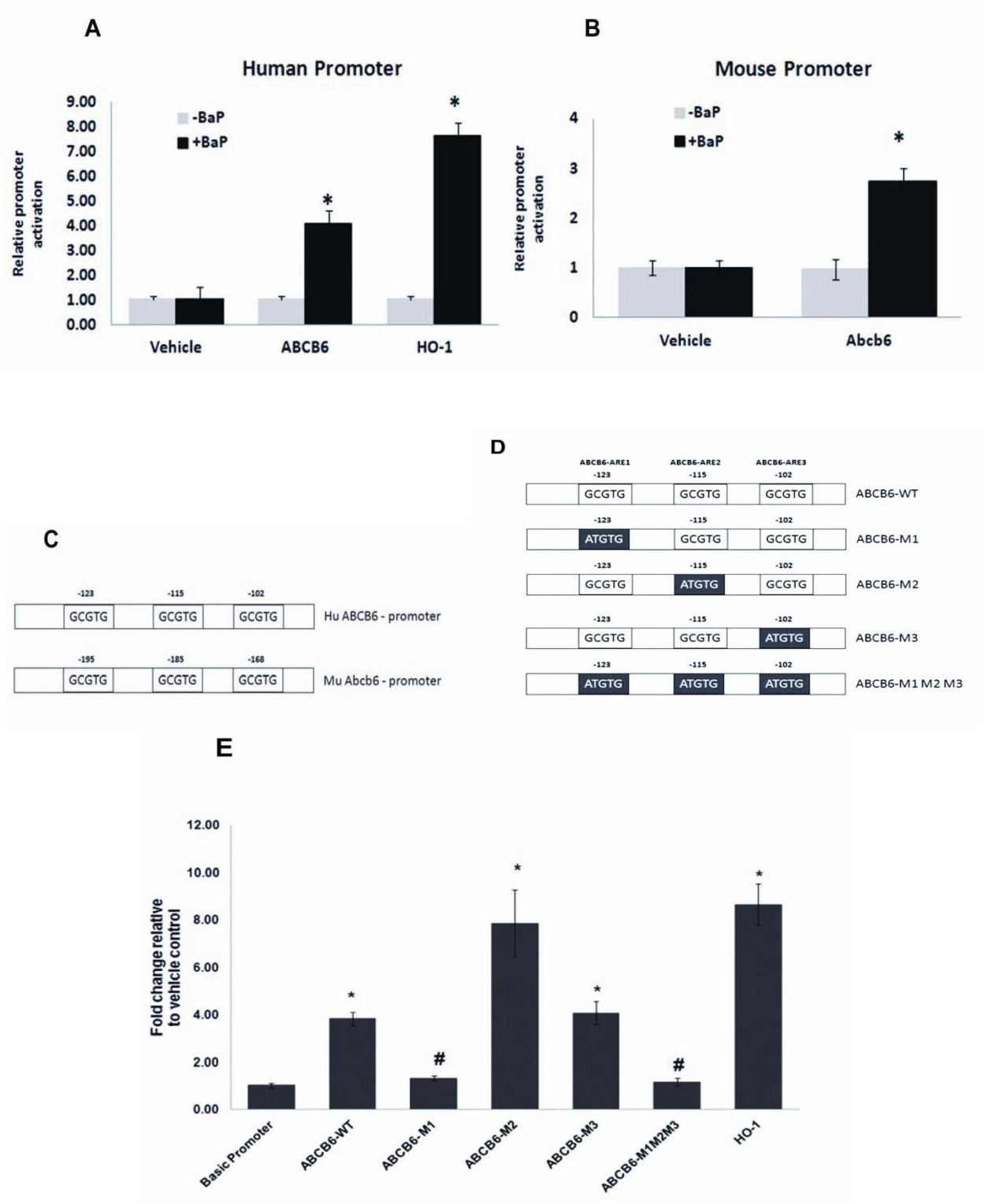

A
Human Promoter
-BaP
+BaP
Relative promoter activation
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Vehicle
ABCB6
HO-1
B
Mouse Promoter
-BaP
+BaP
Relative promoter activation
4
3
2
1
0
Vehicle
Abcb6
C
-123
-115
-102
GCGTG
Hu ABCB6 - promoter
-195
-185
-168
Mu Abcb6 - promoter
D
ABCB6-ARE1
ABCB6-ARE2
ABCB6-ARE3
ABCB6-WT
ABCB6-M1
ABCB6-M2
ABCB6-M3
ABCB6-M1 M2 M3
ATGTG
E
Fold change relative to vehicle control
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Basic Promoter
ABCB6-WT
ABCB6-M1
ABCB6-M2
ABCB6-M3
ABCB6-M1M2M3
HO-1

Figure 4.1.5. (continued)

Active AhR response element in human and mouse 5′-flanking region.

Shown is activity of human (*A*) and mouse (*B*) 5′-flanking sequence in the Abcb6 gene in response to B[*a*]P. *C*, schematic representation of the 5′-flanking region of human and mouse Abcb6 genes. Core AREs are shown as *boxes* with respective locations of the 5′ end base from the reported transcription start sites. *D*, schematic representation of the human Abcb6 promoter, used in the transactivation studies, showing the introduction of mutations in the ARE. *Gray shaded boxes* show the AhR sequence motif that was mutated by site-directed mutagenesis on the human Abcb6 promoter.*E*, mutation of either the distal ARE or all of the three AREs does not activate the promoter-luciferase reporter in response to B[*a*]P. Values represent mean ± S.D. (*error bars*) (*n* = 3). Results representative of six independent experiments. *, significantly different from empty vector-transfected cells treated with B[*a*]P and Abcb6 promoter-transfected cells treated with vehicle. $p < 0.01$. #, significantly different from Abcb6-WT, Abcb6-M1, and Abcb6-M2 promoter-transfected cells treated with B[*a*]P. $p < 0.01$.

4.1.6. AhR binds and interacts with the distal AhR response element in the Abcb6 promoter

To confirm and extend our promoter transactivation analysis and to demonstrate that AhR interacts with the distal promoter element of the human Abcb6 promoter, we performed an EMSA with oligonucleotide probe containing the human Abcb6 AREs. Although both AREs at 102 and 115 bp failed to activate the Abcb6 promoter in response to B[a]P (Figure 4.1.5E), we decided to include these in our EMSA studies to confirm and extend the results from the promoter transactivation studies (Figure 4.1.5). Further, because of the close proximity of the AREs at 115 and 123 bp, a single oligonucleotide probe carrying both of these two sites were used in EMSA. Following EMSA, we found that nuclear extracts of HepG2 cells exposed to B[a]P caused an intense band with the 123 to 115 bp Abcb6-ARE oligonucleotide probe, retarded on the gel, indicative of a ARE-AhR protein complex at either 123 or 115 bp (Figure 4.1.6C). In contrast, ARE at 102 bp did not demonstrate any retarded bands on the gel, suggesting the absence of ARE-AhR protein complex interaction at the 102 bp site (data not shown). Further, we found that the binding affinity of B[a]P-treated nuclear extract with the oligonucleotide probe (carrying both 123 and 115 bp) could be competed with the use of either excess unlabeled oligonucleotide probe (carrying 123 and 115 bp) or excess unlabeled oligonucleotide probe carrying mutations in the 115 bp AhRE (Abcb6-M2) but not with unlabeled oligonucleotides carrying mutations in Abcb6-M1 (123 bp) or mutations in both 115 and 123 bp (Abcb6-M1M2) (Figure 4.1.6C). These results suggest that an intact core AhRE at 123 bp in the Abcb6 promoter is required to form this DNA-protein complex. The fact that co-incubation with an antibody specific for AhR was capable of reducing the intensity of the 123 bp retarded band on the gel confirms the presence of AhR in this DNA-protein complex (Figure 4.1.6C). Instead of a supershifted band, studies have shown a reduction of AhR/ARNT-response element complex formation in the presence of an AhR antibody (176). Previous studies have demonstrated B[a]P-

mediated interaction of AhR with the CYP1A1-ARE and the ability of AhR-specific antibody to disrupt this interaction. Thus, in our studies, CYP1A1-ARE interaction with AhR was used as a positive control (Fig. 4.1.6A).

Although the EMSA studies demonstrate AhR interaction with the AhRE in the Abcb6 promoter, we observed a significant difference in the migration pattern of Abcb6 promoter- AhR protein complex compared with CYP1A1 promoter-AhR protein complex (Figure 4.1.6, A and C). Further, the intensity of Abcb6 promoter-AhR protein complex on the EMSA was noticeably lighter compared with the interacting complex seen with CYP1A1 (Figure 4.1.6, A and C). We hypothesized that the difference in the migration pattern of the Abcb6-AhR protein complex could be due to DNA binding proteins, activated in response to B[a]P, that interact with the Abcb6 promoter at sites independent of AhR interaction with the ARE. To test this hypothesis, we synthesized a Abcb6-AhRE oligonucleotide probe where the sequence flanking the AREs (123 and 115 bp) was changed to random bases (Figure 4.1.6D) while keeping the core Abcb6-ARE intact (Figure 4.1.6D). EMSA analysis of the core sequence and the two randomly altered probes demonstrates that changing the ARE flanking sequence to random bases restores the migration pattern of the Abcb6-ARE protein complex similar to what was seen with the CYP1A1-AhR protein complex (Figure 4.1.6A and E). Further, these studies also demonstrate that the ARE in the Abcb6 promoter is sufficient for AhR interaction with the Abcb6 promoter because changing the flanking sequence did not alter overall AhR binding to the Abcb6-oligonucleotide probe (Figure 4.1.6E). These studies were confirmed further by competition assays using unlabeled and mutant oligonucleotide probes as described above (Figure 4.1.6F).

Taken together, our findings suggest that the Abcb6 promoter is directly activated by AhR and that the ARE at 123 bp (Abcb6-ARE3) is essential and sufficient for the transcriptional activation of the human Abcb6 promoter. The predicted AhR response element at AREs in the Abcb6 promoter was further evaluated for its ability to bind AhR in the physiologically relevant

cellular environment. For this purpose, we performed chromatin immunoprecipitation experiments in HepG2 cells treated with B[a]P (5 μM). As shown in Figure 4.1.6, G and H, AhR protein was recruited to the ARE-containing regions of the Abcb6 promoter. Further, the AhR protein interaction with the Abcb6 promoter was significantly increased after B[a]P treatment (Figure 4.1.6, G and H). These results indicate that AhR activates the Abcb6 promoter through direct interaction with the AREs in the presence of B[a]P *in vivo*.

Figure 4.1.6.

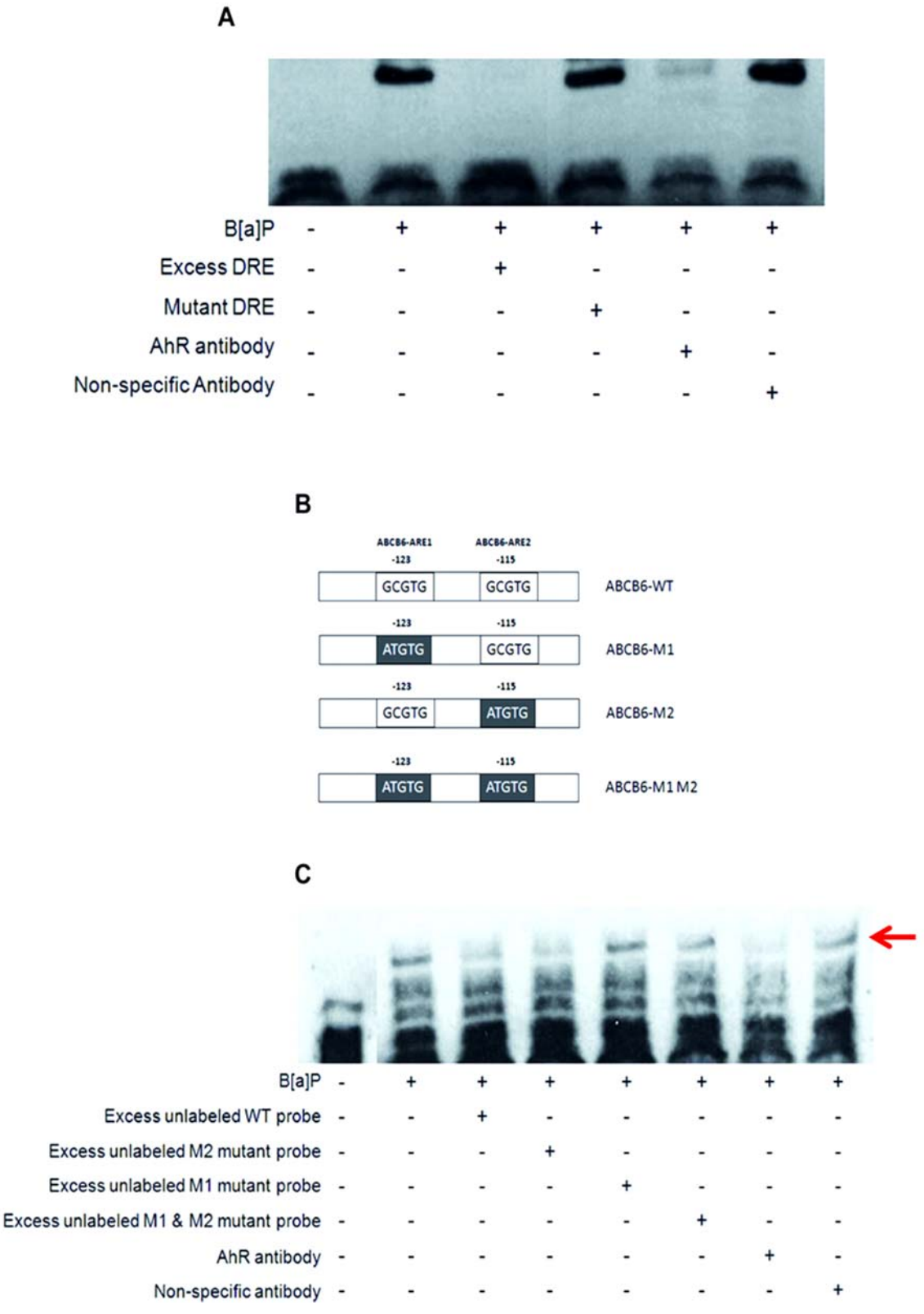
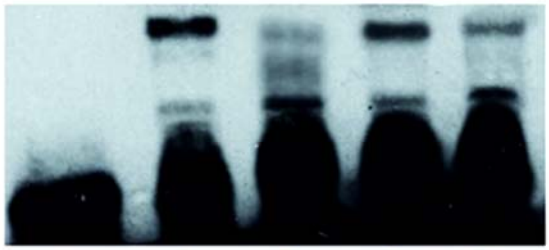

A
B[a]P - + + + + +
Excess DRE - - + - - -
Mutant DRE - - - + - -
AhR antibody - - - - + -
Non-specific Antibody - - - - - +
B
ABCB6-ARE1
-123
ABCB6-ARE2
-115
GCGTG
GCGTG
ABCB6-WT
ATGTG
GCGTG
ABCB6-M1
GCGTG
ATGTG
ABCB6-M2
ATGTG
ATGTG
ABCB6-M1 M2
C
B[a]P - + + + + + + +
Excess unlabeled WT probe - - + - - - - -
Excess unlabeled M2 mutant probe - - - + - - - -
Excess unlabeled M1 mutant probe - - - - + - - -
Excess unlabeled M1 & M2 mutant probe - - - - - + - -
AhR antibody - - - - - - + -
Non-specific antibody - - - - - - - +

Figure 4.1.6. (continued)

D

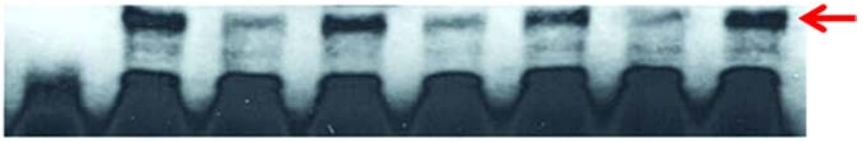
ABCB6 CORE SEQUENCE	5′ GTGCCCTGCGTGAGTGCGTGGCGGCGG 3′
ABCB6 Probe 1	5′ AAAACCTGCGTGAGTGCGTGGCGTTTT 3′
ABCB6 Probe 2	5′ CAATATCGCGTGAGTGCGTGGATATTG 3′

E



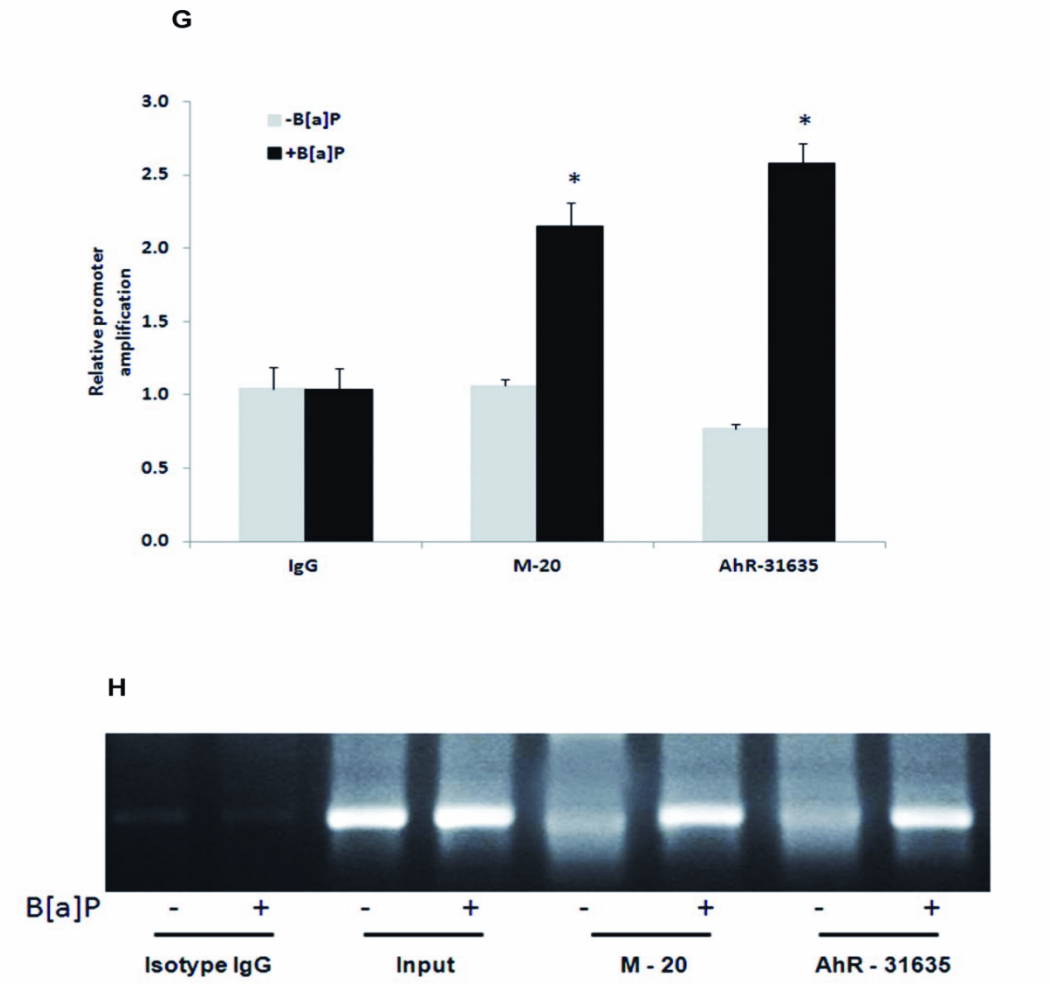
B[a]P	-	+	+	+	+
CYP1A1 probe	-	+	-	-	-
ABCB6 Core sequence probe	-	-	+	-	-
ABCB6 Probe 1	-	-	-	+	-
ABCB6 Probe 2	-	-	-	-	+

F



B[a]P	-	+	+	+	+	+	+	+
Excess unlabeled WT probe	-	-	+	-	-	-	-	-
Excess unlabeled M1 mutant probe	-	-	-	+	-	-	-	-
Excess unlabeled M2 mutant probe	-	-	-	-	+	-	-	-
Excess unlabeled M1 & M2 mutant probe	-	-	-	-	-	+	-	-
AhR antibody	-	-	-	-	-	-	+	-
Non-specific antibody	-	-	-	-	-	-	-	+

Figure 4.1.6. (continued)



AhR is recruited to the distal AhR response element in the human Abcb6 promoter.

A, electrophoretic mobility shift assay of AhR complex binding to the ARE element in the CYP1A1 promoter. B, schematic representation of the human Abcb6 promoter used in EMSA studies. C, electrophoretic mobility shift assay of AhR complex binding to the ARE element in the human Abcb6 promoter. D, schematic representation of the changes introduced in the ARE flanking sequence. Letters highlighted in blue show the altered bases introduced in the ARE flanking sequence, and letters in red show the core ARE. E and F, electrophoretic mobility shift assay of AhR complex binding to the human Abcb6-WT promoter and to the human Abcb6 promoter carrying changes to the ARE flanking sequence. In all EMSA assays (A, C, E, and F), biotinylated DNA probes containing ARE were incubated with nuclear extracts of HepG2 cells

treated with either vehicle or 5 μM B[a]P in the presence or absence of excess unlabeled probe or the mutant AREs. Polyclonal antibody against AhR was used to show antibody-induced reduction in the intensity of the AhR-protein complex band. Incubation with equal amounts of a nonspecific polyclonal antibody did not reduce the band intensity. Results are representative of four independent experiments. G and H, chromatin immunoprecipitation analysis shows recruitment of AhR to the ARE in the Abcb6 promoter. Histograms (G) represent real-time PCR values of promoter amplification, whereas H shows conventional PCR analysis (28 cycles) for AREs of Abcb6 to confirm the specificity of PCR amplification and quantitation in SYBR green real-time PCR. Immunoprecipitation was carried out with two anti-AhR antibodies (M-20 and AhR-31635) or isotype control IgG. Values represent mean ± S.D. (error bars) of three independent experiments. Arrows indicates desired band. *, significantly different from B[a]P-treated isotype IgG control values. $p < 0.01$.

Discussion

All PAHs are potent inducers of microsomal monooxygenases, which require heme for their activity (5,177). Hence, heme demand is higher in animals exposed to PAH (42,154,178). When heme demand is high, the preferred response of cells to increased heme demand is to increase heme synthesis. Abcb6 is a mitochondrial transporter that regulates heme synthesis (28). Thus, in this study, we investigated the role of Abcb6 in PAH-mediated increase in heme synthesis and characterized the mechanism that regulates Abcb6 expression under these conditions.

Previous studies have shown that exposure to TCDD induces the expression of ALAS1, the rate-limiting enzyme in heme biosynthesis, suggesting a feedback regulation of heme synthesis to compensate for the increased heme demand (154). However, this increase in ALAS1 does not result in an increase in heme or its precursor PPIX but results in the accumulation of uroporphyrins (167,169). This is because TCDD inhibits UROD, the enzyme that catalyzes the conversion of uroporphyrinogen to COPIII (167). Our studies confirm these observations showing no increase in PPIX levels in mice or tissue culture cells treated with TCDD. However, as with ALAS1 expression, both mouse and human Abcb6 expression were induced in response to TCDD treatment. This response appears to be a coordinated induction mediated by AhR to support the increased expression and activity of induced monooxygenases.

In contrast to TCDD, the effect of B[a]P and 3-MC exposure on hepatic porphyrin levels is not known. We show here that exposure to B[a]P or 3-MC results in increased PPIX levels in both mouse primary hepatocytes and human hepatoma cells, suggesting that the B[a]P and 3-MC effect on UROD activity is not the same as that of TCDD. Our results confirm previous observations in leukocytes, where B[a]P has been show to increase PPIX accumulation (179). Again, in response to B[a]P and 3-MC exposure, both ALAS1 and Abcb6 expression were

induced, supporting the hypothesis that in response to cellular heme demands, the heme biosynthetic pathway is up-regulated.

Recent studies have demonstrated that the induction of P450s by drugs is mediated by several orphan nuclear receptors, members of a superfamily of DNA-binding proteins that act as transcription factors (163). With respect to PAH, the key transcription factor is AhR, which binds the DNA consensus sequence 5-GCGTG-3 (also known as ARE) in the gene promoters, thus inducing transcription. Here we demonstrate that B[a]P-mediated induction of Abcb6 expression is regulated by the AhR pathway. The up-regulation of Abcb6 promoter by AhR and the requirement that a functional AhR be present to ensure up-regulation of Abcb6 expression reveal a novel mechanism whereby a cell can, on demand, induce Abcb6 expression to promote heme biosynthesis to meet the increasing heme demands under these conditions.

In our studies, although three potential ARE elements were identified by algorithm-based analysis of the Abcb6 promoter, only one of these ARE elements appeared to be important for AhR-mediated activation of Abcb6. Interestingly, mutation of the ARE element at 115 bp (Abcb6-M2) showed an increase in promoter activation in response to B[a]P that was significantly higher than the one seen with the unmutated native sequence. We speculate that this observation suggests the potential existence of repressor motifs in close proximity to the ARE motif at 115 bp. Observations such as these are not uncommon and have given rise to the idea that enhancer and repressor motifs often exist in close proximity to one another and might serve as crucial recruitment sites for transcriptional activation machinery that functions to either promote or suppress gene activation in response to external stimuli. In this context, it is interesting to note that the ARE sequence 5-GCGTG-3 is similar to the HIF-1response element sequence (consensus 5-RCGTG-3), suggesting a potential role of HIF and hypoxia in regulating Abcb6 expression.

It is interesting to note that in our studies treatment with B[a]P led to additional DNA-protein complex formation in the Abcb6 promoter. However, this interaction appeared to be independent of AhR interaction with the Abcb6 promoter. At present, it is not clear what these interacting proteins are and what if any is the significance of these interactions to Abcb6 expression and function.

Previous studies have shown that the expression of membrane- bound efflux transporters mediated by AhR in response to environmental contaminants is not uniform between mice and humans (176). For example, breast cancer resistance protein, a transporter important in cellular detoxification and multidrug resistance, is induced in an AhR-dependent manner in humans but not in mice (176). However, in our studies, we found that TCDD and B[a]P induced both mouse and human Abcb6 in an AhR-dependent manner that appears to be comparable, suggesting a common underlying mechanism of PAH-mediated activation of Abcb6 in humans and mice.

The results from our studies presented in this work have both pharmacological and toxicological significance. In addition to their ability to precipitate porphyria, PAHs are carcinogenic in many animal species (158,180-182). Following their conversion to dihydrodiol epoxides and epoxide hydrolase by CYP1A1, these compounds interact with DNA and form PAH-DNA adducts (183,184). These epoxides are highly reactive electrophiles and cause mutations and cytotoxicity in both prokaryotic and eukaryotic cells. In this context, it is interesting to note that Abcb6 expression promotes cell growth and proliferation in hepatoma cells, and Abcb6 expression is induced during the development of hepatocellular carcinoma (185-187). Thus, Abcb6 induction by PAHs could be a potential contributing factor in PAH carcinogenicity. In addition, it is also possible that PAH-mediated activation of Abcb6 could enhance CYP1A1 activity and accelerate the metabolism of PAHs to epoxide hydrolase, thus promoting carcinogenesis.

In conclusion, this is the first report to illustrate that an important regulator of heme synthesis, Abcb6, is transcriptionally regulated by AhR. The resultant enhancement of Abcb6 function represents an essential requirement coordinated by AhR to support the increased heme demand that occurs when animals are exposed to xenobiotics and therapeutic drugs. The observation that this AhR-induced expression of Abcb6 is common to both mice and humans highlights the importance and significance of this process.

4.2. XENOSENSOR CAR AND PXR REGULATES ATP BINDING CASSETTE TRANSPORTER ABCB6 GENE

Abstract

In the present study, we demonstrate that 1, 4-bis [2-(3, 5-dichloropyridyloxy)] benzene (TCPOBOP) a prototypical inducer of CAR and 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) a prototypical ligand of PXR, up-regulate Abcb6 expression. Moreover, *in vivo* studies in CAR null mice demonstrate that TCPOBOP induction of Abcb6 is mediated by CAR. Similarly, studies with PXR null mice demonstrate that PCN mediated induction of Abcb6 is mediated through PXR. *In silico* analysis of Abcb6 promoter demonstrates the existence of CAR and PXR response elements. However, the significance of these elements to CAR and PXR mediated activation of Abcb6 promoter needs further investigation.

Introduction

P450s constitute a superfamily of heme-proteins that play an important role in the activation of chemical carcinogens, detoxification of numerous xenobiotics as well as in the oxidative metabolism of endogenous compounds such as steroids, fatty acids, prostaglandins, and leukotrienes. In addition, some P450s have important roles in physiological processes, such as steroidogenesis and the maintenance of bile acid and cholesterol homeostasis. Given their importance, the molecular mechanisms of P450 gene regulation have been intensely studied. Direct activation by nuclear receptors represents one of the primary means by which the expression of these genes is controlled. However, because P450 are heme dependent enzymes that require heme for their activity, the availability of heme represents an important factor in the regulation of P450 activity. This requires coordinate synthesis of heme and a P450 apoprotein moiety. Despite these observations our understanding of the role of nuclear receptors in the regulation of heme synthesis is not completely understood.

The xenosensing nuclear receptors CAR and PXR have been previously shown to regulate heme synthesis by regulating ALAS1 (188). ALAS1 regulates the rate of heme synthesis by controlling the first committed step, the condensation of glycine and succinyl-CoA to 5-aminolevulinate. Although ALAS-mediated regulation of heme synthesis is considered the key step in heme biosynthesis, recent reports have identified a second regulatory step in heme biosynthesis mediated by the mitochondrial ATP binding cassette transporter Abcb6. Abcb6 regulates heme biosynthesis by controlling the transport of COPIII, an intermediate in heme synthesis, from the cytoplasm into the mitochondria to complete heme synthesis. However, despite this knowledge little if any information is available on the role of nuclear receptors, the predominant regulators of P450s, in the regulation of Abcb6.

In the present study, we demonstrate that Abcb6 expression is induced by both TCPOBP and PCN, two of the most common canonical activators of CAR and PXR,

respectively. We show that TCPOBOP and PCN mediated induction of Abcb6 is transcriptionally regulated and requires functional CAR and PXR.

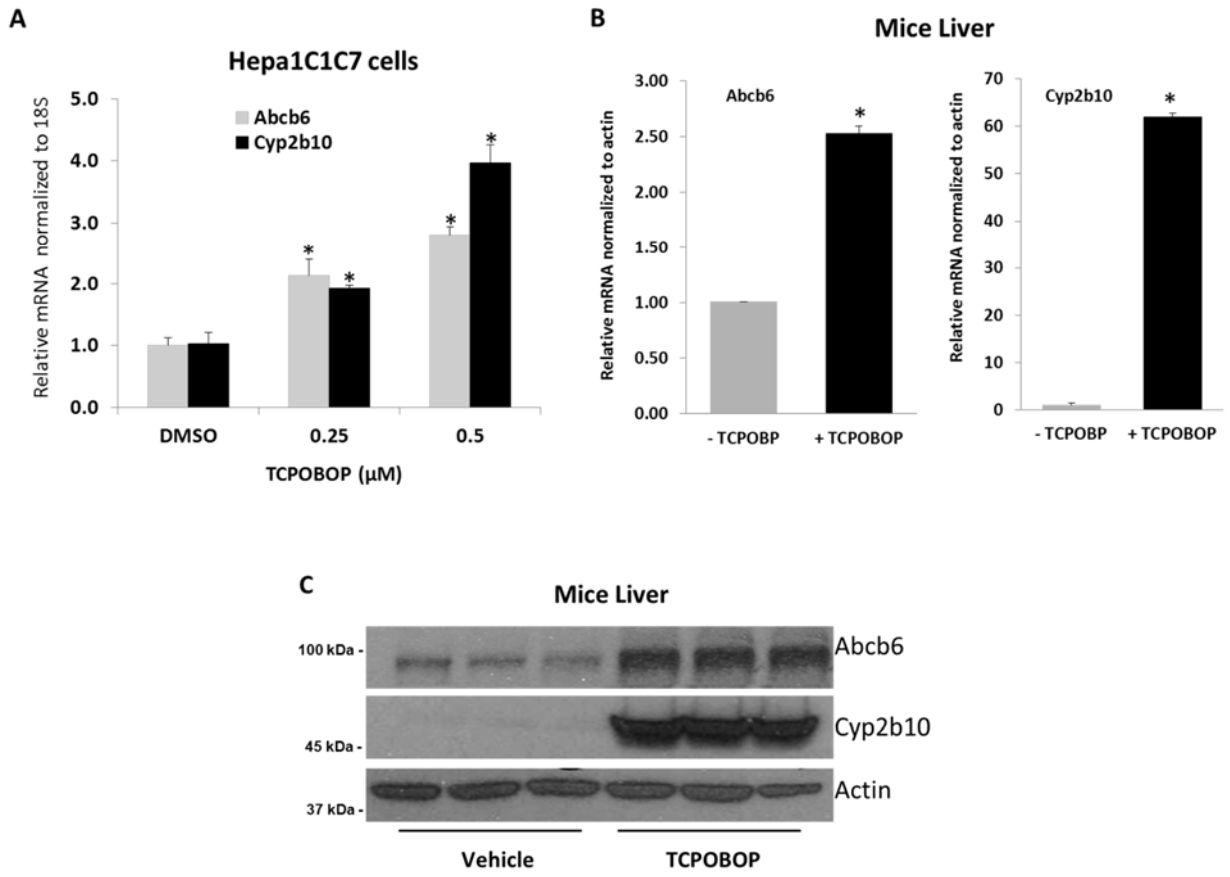
Results

4.2.1. Abcb6 expression in mouse hepatoma cells and mice treated with TCPOBOP

Mouse hepatoma cells (Hepa1c1c7) were treated with increasing concentration of CAR ligand, TCPOBOP (0, 0.25, and 0.5μM) for 24 h. TCPOBOP treatment resulted in a dose-dependent induction of Abcb6 mRNA (Figure 4.2.1A). Cyp2b10, a gene whose expression is induced by TCPOBOP, was used as a positive control (Figure 4.2.1A).

We next evaluated if TCPOBOP induces Abcb6 expression in mice (C57BL/6J). Mice were exposed to TCPOBOP (0.3 mg/kg/day in corn oil) intra peritoneal for 4 days. Change in hepatic Abcb6 expression was determined by quantitative real time PCR and western blot analysis. TCPOBOP induced Abcb6 mRNA and protein levels in mice liver (Figure 4.2.1 B – C). Expression of Cyp2b10, a gene known to be induced in response to TCPOBOP, was used as positive control. Cyp2b10 expression increased with TCPOBOP treatment, demonstrating treatment effectiveness (Figure 4.2.1 B – C). Actin was used as a loading control in these experiments. Taken together, results presented in Figure 4.2.1 demonstrate that TCPOBOP induced hepatic expression of Abcb6.

Figure 4.2.1.



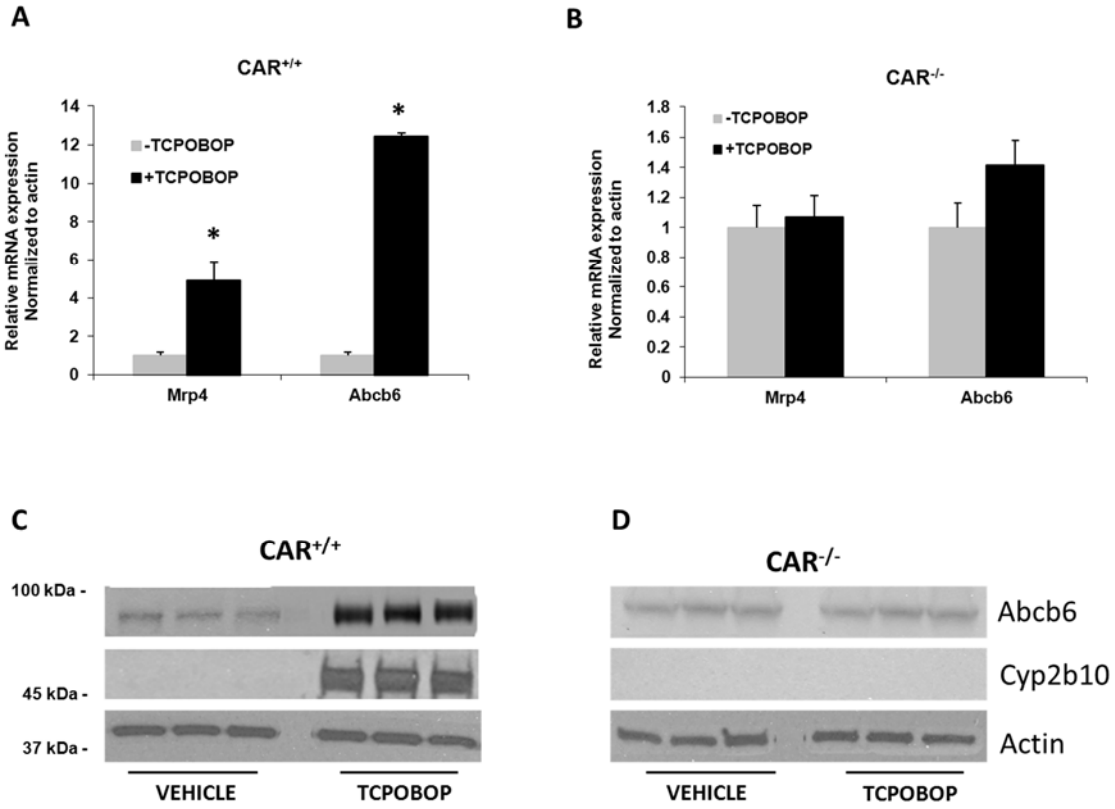
TCPOBOP induces Abcb6 expression.

Exposure to TCPOBOP induces Abcb6 expression in mouse hepatoma (*A*) in a dose-dependent manner and in mice liver (*B*). Abcb6 protein expression in response to TCPOBOP treatment in mice livers *(C).* Abcb6 expression was measured using an Abcb6-specific antibody. TCPOBOP-mediated induction of Cyp2b10, a gene known to be induced by TCPOBOP in liver, is used as a positive control in these experiments. Values represent mean ± S.D. (*error bars*) (*n* = 4). Results shown are representative of three independent experiments with *n* = 4/experiment. *, significantly different from vehicle treated control ($p < 0.01$).

4.2.2. TCPOBOP mediated Induction of Abcb6 is CAR-dependent

Recent discoveries have implicated the nuclear receptor CAR in TCPOBOP-mediated induction of target genes. Thus, in this study, we evaluated the involvement of the CAR pathway in TCPOBOP-mediated up-regulation of Abcb6. CAR wild type and CAR knock-out mice were exposed to TCPOBOP (0.3 mg/kg/day in corn oil) intra peritoneal for 4 days. Abcb6 expression was evaluated by real-time PCR using gene-specific primers and western blot analysis using protein specific antibody. Mrp4 and Cyp2b10, genes known to be induced in response to CAR activation, were used as positive controls. We found that in CAR knock-out mice CAR-responsive genes Mrp4 and Cyp2b10 were not activated, whereas they were activated in the CAR wild type mouse (Figure 4.2.2 A – D). More importantly, we found that TCPOBOP induced a marked increase in the expression of Abcb6 mRNA and protein in CAR wild type mice (Figure 4.2.2 A and C), but TCPOBOP had no effect on Abcb6 expression in CAR-deficient mice (Figure 4.2.2 B and D). Taken together, these results suggest that TCPOBOP-mediated increase in Abcb6 transcript and protein is CAR-dependent.

Figure 4.2.2.



TCPOBOP mediated Induction of Abcb6 is CAR-dependent.

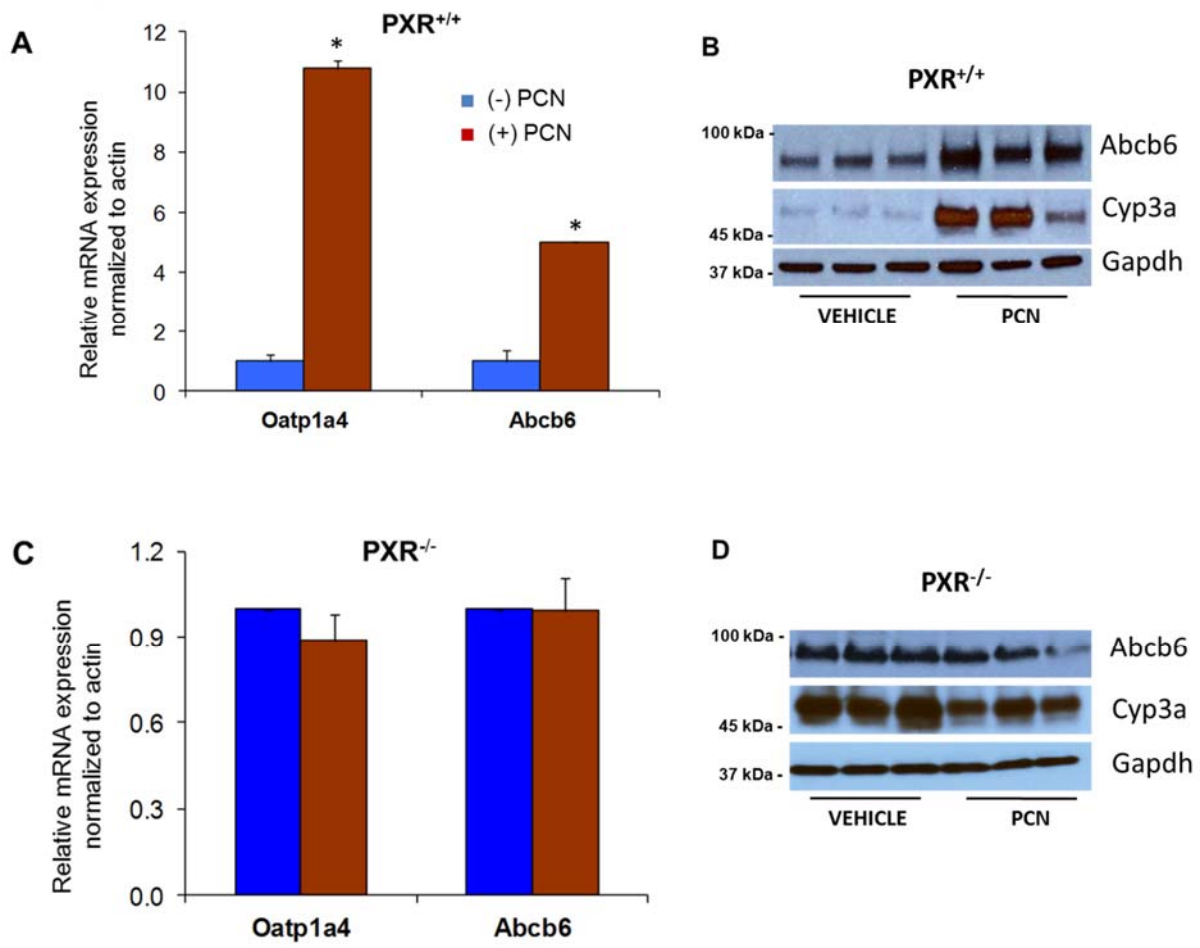
Real time PCR analysis for Abcb6 expression in mouse with functional CAR (A) and mouse lacking CAR (B), TCPOBOP up-regulates Abcb6 expression in CAR wild type mouse and not in CAR null mouse. Immunoblot analysis for Abcb6 expression in mouse with functional CAR (C) and mouse lacking CAR (D), TCPOBOP up-regulates Abcb6 expression in CAR wild type mouse and not in CAR null mouse. CAR dependent and TCPOBOP-mediated induction of Mrp4 and Cyp2b10 genes is used as a positive control in these experiments. Values represent mean ± S.D. (*error bars*); *n* = 4 for mice. Results representative of three independent experiments. *, significantly different from mice exposed to vehicle treatment ($p < 0.01$).

4.2.3. PCN induces Abcb6 expression in a PXR-dependent manner

Recent studies demonstrate that nuclear receptors, chicken CXR, human PXR and mouse PXR regulate ALAS1 gene expression, the rate-limiting enzyme in heme biosynthesis, in response to xenobiotics. In the present study, we investigated the role of PXR in the regulation of Abcb6, the newly discovered rate-limiting protein in heme biosynthesis.

PXR wild type and PXR knock-out mice were exposed to PCN (200 mg/kg/day in corn oil) intra peritoneal for 4 days. Abcb6 expression was evaluated by real-time PCR using gene-specific primers and western blot analysis using protein specific antibodies. Oatp1a4 and Cyp3a, genes known to be induced in response to PXR activation, were used as positive controls. We found that in PXR knock-out mice PXR-responsive genes Oatp1a4 and Cyp3a were not activated, whereas they were activated in the PXR wild type mouse (Figure 4.2.3 A – D), demonstrating treatment effectiveness. More importantly, we found that PCN induced a marked increase in the expression of Abcb6 mRNA and protein in PXR wild type mice (Figure 4.2.3 A and B), but PCN had no effect on Abcb6 expression in PXR-deficient mice (Figure 4.2.3 C and D). Taken together, these results suggest that PCN-mediated increase in Abcb6 transcript and protein is PXR-dependent.

Figure 4.2.3.



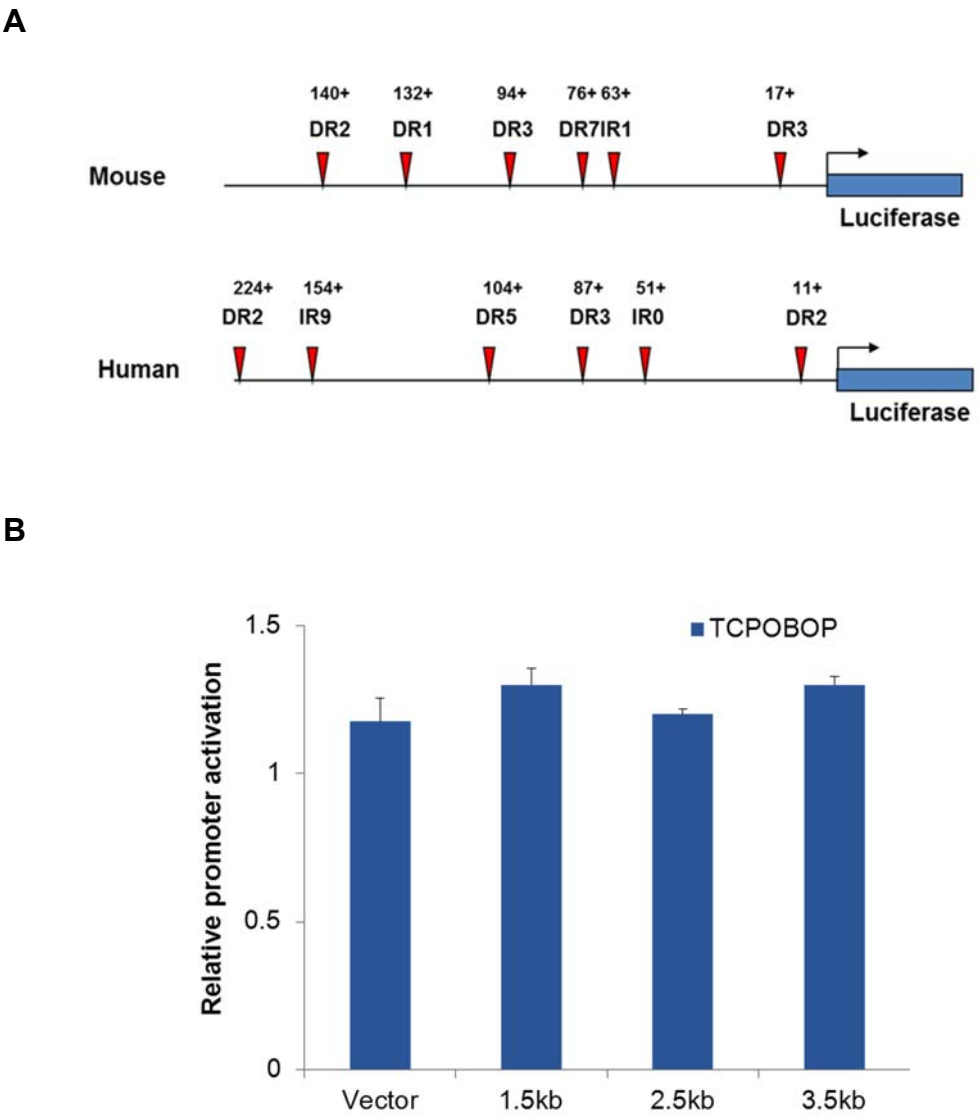
PCN induces Abcb6 expression in PXR-dependent manner.

Real time PCR analysis for Abcb6 expression in mouse with functional PXR (A) and mouse lacking PXR (C), Pregnenolone-16α-carbonitrile (PCN) up-regulate Abcb6 expression in PXR wild type mouse and not in PXR null mouse. Immunoblot analysis for Abcb6 expression in mouse with functional PXR (B) and mouse lacking PXR (D), PCN up-regulates Abcb6 expression in PXR wild type mouse and not in PXR null mouse. PXR dependent and PCN-mediated induction of Oatp1a4 and Cyp3a genes is used as a positive control in these experiments. Values represent mean ± S.D. (*error bars*); n = 3 for mice. *, significantly different from mice exposed to vehicle treatment ($p < 0.01$).

4.2.4. CAR and PXR response element in the Abcb6 Promoter

Our results demonstrate transcriptional activation of Abcb6 in the presence of ligands for nuclear receptors CAR and PXR. To test whether ligand-induced expression of Abcb6 is mediated by CAR or PXR-dependent manner, mouse Abcb6 promoters were cloned in frame in a luciferase reporter vector. First we did *in silico* analysis for potential *cis*-elements that may be capable of binding PXR or CAR using 'Nubiscan' which uses the known consensus sequences for nuclear receptor binding sites. Further, the promoter-luciferase-reporter constructs were transfected into Hepa1c1c7 cells as described (64). We evaluated the effect of CAR – ligand TCPOBOP on the transcriptional activation of the Abcb6 promoter (Figure 4.2.4). Although nubiscan analysis found several putative CAR and PXR binding sites (Figure 4.2.4A) in the Abcb6 promoter, we did not see any activation of Abcb6 promoter (1.2 kb upstream of the transcription start site) (Figure 4.2.4B). These results suggest that the CAR activation sites could be upstream of the 1.2 kb promoter region used in these studies. Analysis of PXR mediated activation of Abcb6 promoter will be done in future studies.

Figure 4.2.4.



Nuclear receptors PXR and CAR response elements in Abcb6 Promoter.

A, The consensus sequences for nuclear receptor binding sites were searched using 'Nubiscan' which identified potential cis-elements that may be capable of binding PXR or CAR. 'number +' for example '140+' represents the nucleotide base and the strand orientation (+strand). B, activity of mouse 5′-flanking sequence in the Abcb6 gene in response to TCPOBOP. Values represent mean ± S.D. (*error bars*) (*n* = 3).

Discussion

In response to endo and xenobiotic exposure, hepatic detoxification mechanisms are activated to prevent liver injury that accompanies such exposure. The efficient functioning of this detoxification mechanism requires fully active P450s, the predominant detoxification system in the liver. However, because P450s are heme dependent enzymes we envisioned a pathway in which P450 induction is coordinated with heme synthesis to support P450 function. We had previously proposed that this coordinated induction of heme synthesis could involve mechanisms that complement P450 induction. Consistent with this proposal we demonstrate that Abcb6 expression is transcriptionally induced by TCPOBOP and PCN the two prototypical inducers of P450s, potentially to support the increased heme demand.

In our studies TCPOBOP and PCN mediated regulation of Abcb6 appear to require functional CAR and PXR, as Abcb6 expression was not induced in CAR and PXR knockout animals. In contrast, although PCN mediated induction of ALAS the rate limiting enzyme in heme synthesis is dependent on PXR, TCPOBOP mediated induction of ALAS appears to be independent of CAR (42,178). The basis for this differential regulation of Abcb6 and ALAS by TCPOBOP remains unclear.

An evaluation of the Abcb6 5' flanking region revealed several nuclear response elements (DR1-5, ER6) within the first 1.2-kb upstream of the Abcb6 transcription start site, nevertheless Abcb6 promoter was not activated by either CAR or PXR ligands. However, this is not unusual as other genes that are CAR and PXR targets (CYP2B6, CYP3A4, MDR1 and Mrp4) all have distal (>7 kb) upstream nuclear receptor binding sites. Again consistent with this observation a nuclear receptor site computer scanning algorithm (Nubiscan, www.nubiscan.unibas.ch) used to probe for putative upstream nuclear receptor binding sites

(upstream of 1.2 kb) of the Abcb6 gene identified several nuclear receptor sites preferred by CAR (*i.e.* DR3, DR4, and ER6). Therefore, future studies using this region between 1.2 and 10 kb upstream of the transcription start site in promoter activation studies should help identify the response elements responsible for CAR mediated activation of Abcb6.

CAR and PXR ligands include endogenous bile acids, therapeutic drugs, dietary constituents, and environmental pollutants. All or some of these chemicals are substrates for P450s. Thus through CAR activation, xenobiotics can induce Abcb6 expression that can increase heme synthesis to support increased expression and function of P450 activity. However, variation in Abcb6 expression either due to non-functional mutations in the gene or existence of splice variants of Abcb6 with altered function could affect P450 function and might have a significant impact on the metabolism of a wide range of pharmacological agents and other foreign compounds. Thus, identifying and defining Abcb6 mutations, splice variants, and the specific effect of these changes to Abcb6 expression and function in the human population should provide insight into this important xenobiotic response.

Although CAR and PXR were initially characterized as xenosensors that coordinated hepatic responses to xenobiotics, recent evidence indicates that they are also important regulators of hepatic energy metabolism. In liver, both receptors regulate expression of genes in pathways that control lipid and carbohydrate metabolism. Both lipid and carbohydrate metabolism require a functional mitochondria with optimally functioning mitochondrial respiratory complexes. Given that most if not all mitochondrial respiratory complexes are hemoproteins and require heme for their activity, CAR and PXR mediated regulation of Abcb6 might have broader implications in mitochondrial function and cellular energy metabolism.

In summary, we demonstrate that the ABC transporter, Abcb6, a regulator of heme synthesis, is induced by the nuclear receptors CAR and PXR, as is ALAS1, the rate-limiting

enzyme in heme synthesis. The up-regulation of Abcb6 and ALAS1 by CAR and PXR, the two canonical inducers of P450, suggests coordinate regulation of heme synthesis with P450 expression. The upregulation of Abcb6 and ALAS1 by CAR and PXR also implicates them as *bona fide* members of hepatic detoxification cascade. Moreover, because hepatic hemoproteins include not only P450s but also enzymes involved in oxidative stress response, the results suggest that Abcb6 and ALAS1 expression might be induced in response to oxidative stress and might play a potentially important role in oxidative stress response.

4.3. ARSENIC MEDIATED TRANSCRIPTIONAL ACTIVATION OF ABCB6

Abstract

Arsenic is an environmental carcinogen and a major health hazard. Inspite of extensive efforts, the mechanism of arsenic induced toxicity and tumorigenicity is not yet clearly understood. Of importance, exposure to pro-oxidants such as arsenic, also results in the induction of a gene expression program, whose primary function is to protect cells from oxidative stress. However, little is known about the components of arsenic induced mammalian oxidative stress response and its regulatory logic. In the present study, we show that sodium arsenite, a major biohazard following environmental or occupational exposure, induces Abcb6 expression in a dose-dependent manner both in mice fed sodium arsenite in drinking water and in cells exposed to sodium arsenite *in vitro*. Arsenite-induced Abcb6 expression was transcriptionally regulated, but this induction was not mediated by the redox-sensitive transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2). Collectively, these results, suggest that Abcb6 expression is transcriptionally regulated by sodium arsenite in a Nrf2 independent manner.

Introduction

Arsenic is a ubiquitous environmental pollutant. Chronic exposure of humans to inorganic arsenic results in formation of tumors of the liver and kidney and probably skin, lung, and urinary bladder (101,189). In addition to its carcinogenic effect, epidemiological studies have associated chronic arsenic exposure with an increased risk in many human nonmalignant diseases, such as peripheral vascular disease, diabetes, and chronic lung disease (190). Evidence suggests that arsenic exerts its chronic toxicity by interacting with sulfhydryl groups and generating reactive oxygen species (ROS) that cause damage to cellular macromolecules via oxidative stress (191-195). Of importance, exposure to pro-oxidants such as arsenic also results in the induction of a gene expression program whose primary function is to protect cells from oxidative stress (196-199).

The 97-kDa Abcb6 protein is a member of the ATP-binding cassette (ABC) superfamily of transport proteins that was originally identified while screening for novel drug-resistant genes in the liver (118). Initial cloning and homology-modeling studies revealed that Abcb6 is related to the heavy metal tolerance factor 1 (HMT1) of *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (200-202). HMT1 is a half transporter that confers tolerance to heavy metal toxicity in *S. pombe* (200) and tolerance to cadmium in *C. elegans* (202). These homology-modeling studies suggest a possible role for Abcb6 in metal tolerance. However, Abcb6 expression and metal tolerance in cell culture is conflicting. Recent studies by *Annereau et al.* and *Paterson et al.* suggest a direct correlation between arsenic resistance and Abcb6 expression in various human and mouse cell lines (203,204). They suggest that cell lines selected for resistance to arsenite have elevated levels of Abcb6 messenger RNA (mRNA) and protein. In contrast, studies by *Gebel et al.* demonstrate no increase in Abcb6 mRNA in HepG2 cells that were selected for arsenic resistance (205).

Thus, the present study was conducted to determine whether arsenic could induce Abcb6 expression. We report that sodium arsenite induces upregulation of Abcb6. Abcb6 expression appears to be transcriptionally regulated, but this expression is not mediated by the redox-sensitive transcription factor Nrf2.

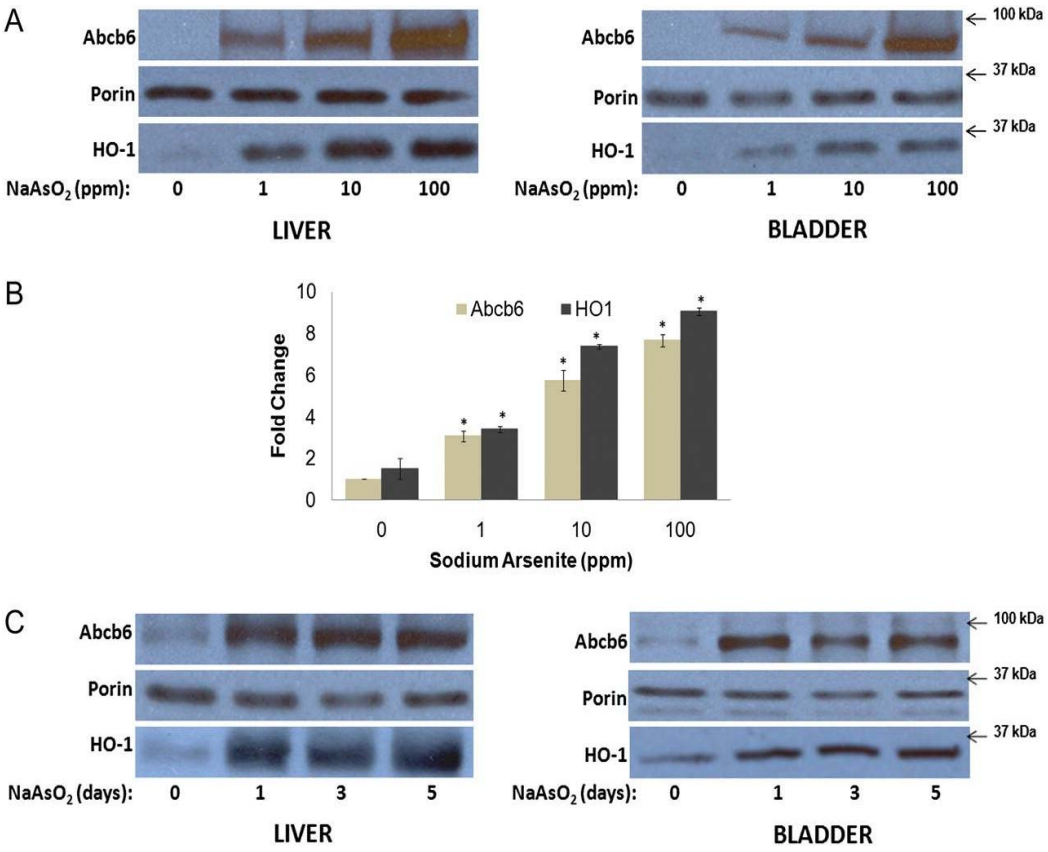
Results

4.3.1. Abcb6 expression in mice fed sodium arsenite

Abcb6 expression was measured in mice (C57BL/6J) fed increasing concentrations of sodium arsenite (0, 1, 10, and 100 ppm) in drinking water for 24 h. Sodium arsenite induced both Abcb6 mRNA and protein levels in a dose-dependent manner (Figure 4.3.1, A – B). Highest induction of Abcb6 was seen in mice fed 100 ppm of sodium arsenite. Expression of heme oxygenase 1 (*HO-1*), a gene known to be induced in response to sodium arsenite, was used as positive control. HO-1 expression increased with increasing concentration of sodium arsenite, demonstrating treatment effectiveness (Figure 4.3.1, A – B). Mitochondrial protein porin whose expression is not altered in response to sodium arsenite was used as loading control in these experiments.

We next evaluated whether Abcb6 expression was regulated by sodium arsenite in a time-dependent manner. Mice were exposed to 10 ppm of sodium arsenite for 1, 3, or 5 days. Sodium arsenite exposure induced Abcb6 expression throughout the 5-day treatment period (Figure 4.3.1C). However, the magnitude of Abcb6 expression on day 3 and day 5 following sodium arsenite treatment was similar to expression levels seen on day 1 (Figure 4.3.1C). As in the dose-dependent studies, expression of HO-1 and porin were used as positive and negative controls, respectively. Cumulatively results presented in Figure 4.3.1 demonstrate that sodium arsenite induces Abcb6 expression in a dose-dependent manner in mice fed sodium arsenite in drinking water.

Figure 4.3.1.



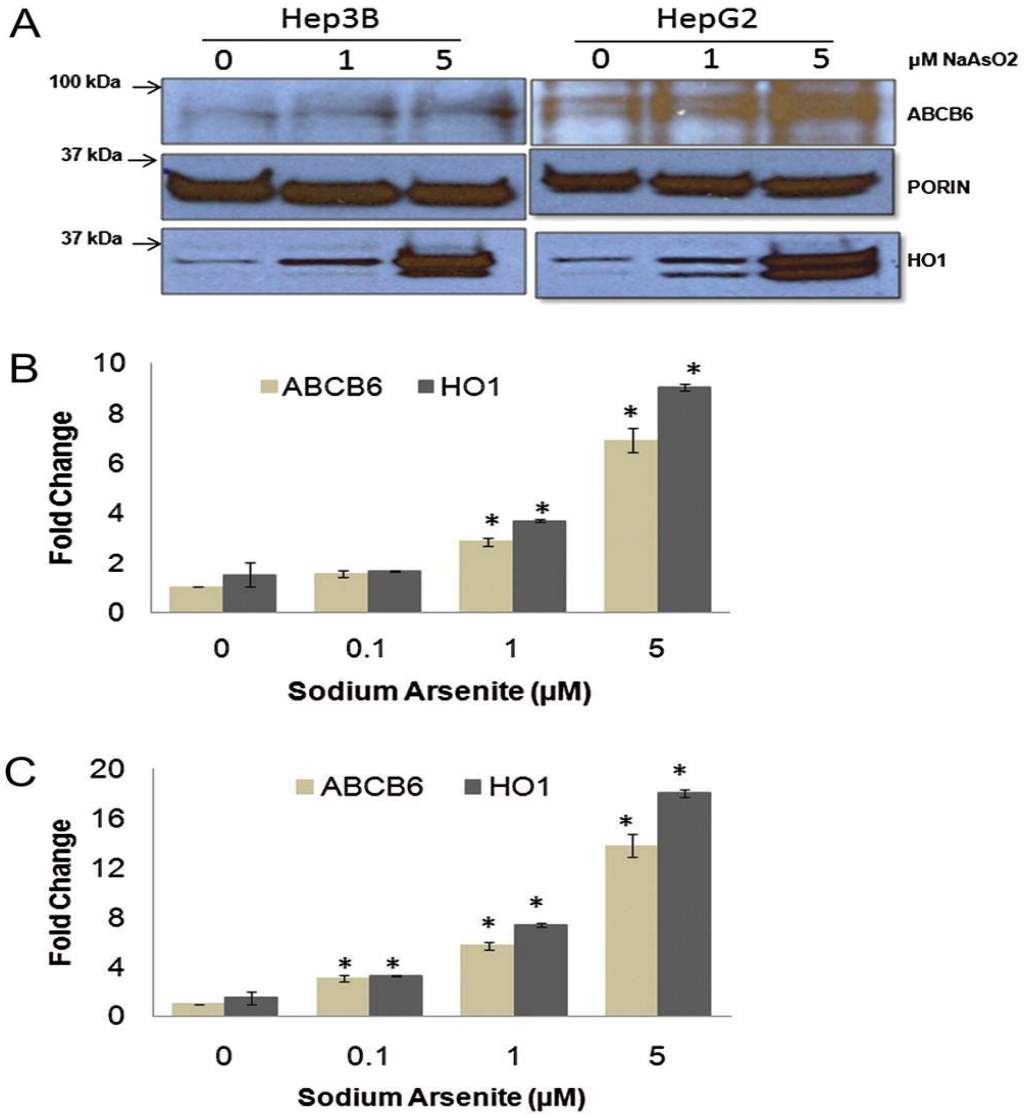
Abcb6 expression in mice fed sodium arsenite in drinking water.

Abcb6 expression in mice fed sodium arsenite in drinking water. (A) Immunoblot analysis of Abcb6 expression in mice (C57BL/6J) fed increasing concentrations (0, 1, 10, and 100 ppm) of sodium arsenite for 24 h. Abcb6 expression was measured in isolated mitochondria (100 µg) using Abcb6-specific antibody. Results are representative of three independent experiments with n = 4 mice per treatment group. (B) Real-time PCR analysis of Abcb6 mRNA levels in livers of mice fed increasing concentrations (0, 1, 10, and 100 ppm) of sodium arsenite for 24 h. Abcb6 expression was measured using gene-specific primers. Values represent mean ± SD; n = 6. Results are representative of three independent experiments. "*" Significantly different from untreated controls. $p < 0.01$. (C) Immunoblot analysis of Abcb6 expression in mice fed sodium arsenite (10 ppm) for 1, 3, or 5 days. Abcb6 expression was measured in isolated mitochondria (100 µg) using Abcb6-specific antibody. Results are representative of three independent experiments with n = 4 mice per treatment group.

4.3.2. Abcb6 expression in human hepatoma cells treated with sodium arsenite

Human hepatoma cells (HepG2 and Hep3B) were treated with increasing concentration of sodium arsenite (0–5μM) for 24 h. These cells were selected because sodium arsenite elicits stress response, initiates ROS production, and induces apoptosis in these cells (205-207). Sodium arsenite treatment resulted in a dose-dependent induction of *Abcb6* mRNA (Figure 4.3.2, B – C) and Abcb6 protein (Figure 4.3.2A) in both HepG2 and Hep3B cells.

Figure 4.3.2.



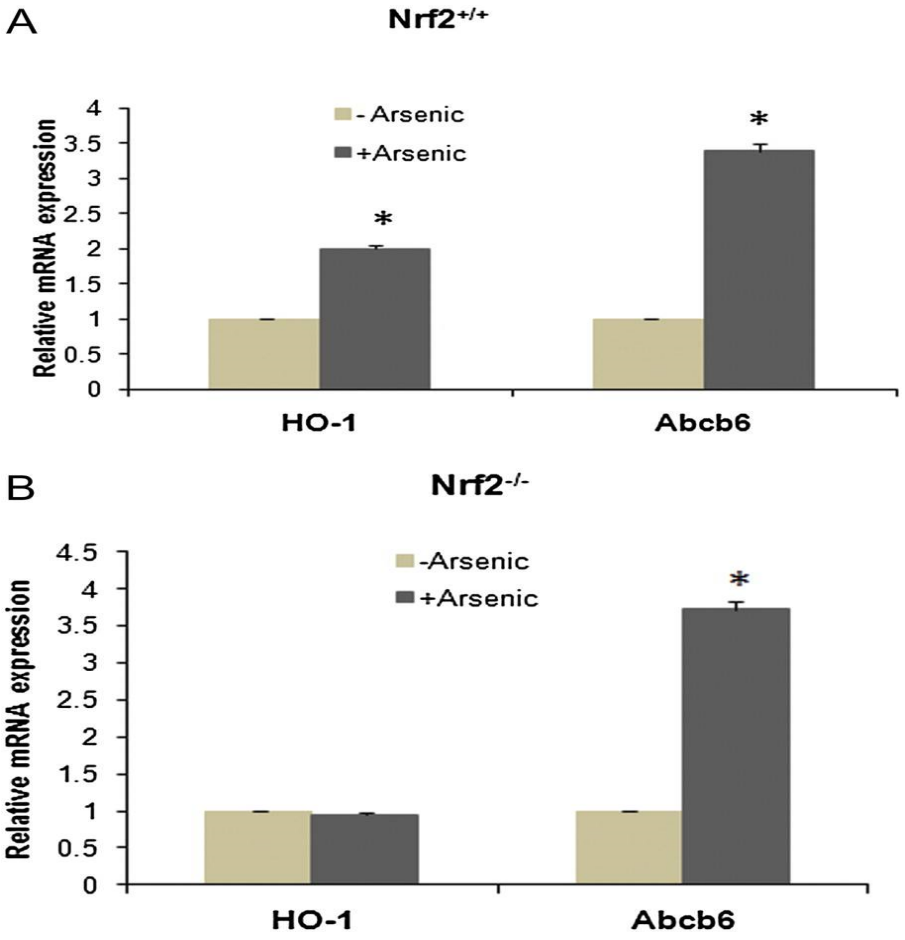
Abcb6 expression in hepatoma cells treated with sodium arsenite.

(A) Immunoblot analysis of Abcb6 expression in HepG2 and Hep3B cells treated with increasing concentrations (0, 1, and 5μM) of sodium arsenite for 24 h. Abcb6 expression was measured in isolated mitochondria (100 μg) using Abcb6-specific antibody. Results are representative of three independent experiments. (B and C) Real-time PCR analysis of Abcb6 mRNA levels in (B) Hep3B and (C) HepG2 cells treated with increasing concentrations (0, 0.1, 1, and 5μM) of sodium arsenite for 24 h. Abcb6 expression was measured using gene-specific primers. HO-1 expression was used as positive control. Values represent mean ± SD. Results are representative of three independent experiments. "*" Significantly different from untreated controls. $p < 0.01$.

4.3.3. Abcb6 expression in *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice fed sodium arsenite in drinking water

Arsenic-mediated regulation of gene transcription is believed to be governed by redox-sensitive transcription factors that are activated in response to oxidative stress. Among the many redox-sensitive transcription factors, Nrf2 (208) appears to play a key role in arsenic-mediated activation of downstream genes (196,209,210). Because Abcb6 transcript was found in our studies to be upregulated by sodium arsenite, we tested whether this induction was mediated by Nrf2. We used the Nrf2 wild-type (*Nrf2*$^{+/+}$) and Nrf2 gene-deleted (*Nrf2*$^{-/-}$) mice for these studies. We did not see any difference in basal Abcb6 expression between *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (Data not shown). Sodium arsenite treatment induced *Abcb6* expression to a similar extent in both *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (Figure 4.3.3, A – B; ~3.5-fold increase in *Abcb6* mRNA in arsenite-treated vs. vehicle control mice), suggesting that sodium arsenite–dependent induction of *Abcb6* is not mediated by Nrf2. *HO-1*, a gene whose expression is activated by Nrf2 (101,189,211), was used as a positive control (Figure 4.3.3, A – B). *HO-1* expression was induced approximately two fold in arsenite-treated *Nrf2*$^{+/+}$ mice but not in *Nrf2*$^{-/-}$ mice.

Figure 4.3.3.



Abcb6 expression in the liver of Nrf2 wild-type (*Nrf2*$^{+/+}$) and Nrf2 gene-deleted (*Nrf2*$^{-/-}$) mice fed sodium arsenite in drinking water.

(A) Nrf2 wild-type (*Nrf2*$^{+/+}$) and (B) Nrf2 gene-deleted (*Nrf2*$^{-/-}$) mice were treated with 10 ppm sodium arsenite for 24 h. Abcb6 expression was measured by real-time PCR using gene-specific primers. Expression of HO-1 was used as positive control in these studies. Values represent mean ± SD with n = 4 mice per treatment group. Results are representative of three independent experiments. "*" Significantly different from control mice (vehicle treated). $p < 0.01$.

Discussion

Inorganic arsenic is a ubiquitous environmental pollutant, and chronic exposure in humans has devastating health effects, including the formation of tumors of the liver, kidney, skin, lung, and bladder (101,189). Its ubiquity in the environment has led to the evolution of arsenic defense mechanisms in all living organisms studied, from *Escherichia coli* to man (212). These defense mechanisms work either alone or in concert to reduce cellular oxidative stress. In this study, we determined whether arsenic can induce Abcb6 expression and further investigated the role of Abcb6 to arsenic toxicity (Chapter 5.2). We find that *in vitro* sodium arsenite induces Abcb6 expression in hepatoma cells at a concentration as low as 1 μM. This low-dose induction suggests that Abcb6 expression is an adaptive response to arsenite toxicity, a phenomenon commonly termed as preconditioning that allows cells and tissues to resist future toxic insults to similar stressors (213). *In vivo* Abcb6 expression is induced in a dose-dependent manner within a day following arsenite treatment, which suggests that Abcb6 is an early response gene activated in response to injury.

As noted earlier, the redox-sensitive transcription factor Nrf2 provides a predominant pathway of oxidant-mediated induction of downstream genes. In our initial analysis, Abcb6 expression did not appear to be regulated by Nrf2. This suggests that pathways independent of Nrf2 might be involved in regulating Abcb6 expression. Alternatively, arsenite-induced Abcb6 expression might be regulated posttranscriptionally. Interestingly, recent studies by *Hubner et al.* (214) demonstrated induction of Abcb6 mRNA in humans in response to cigarette smoking. In these studies, Nrf2 did not mediate Abcb6 expression induced by smoking-related oxidative stress. Our studies support these findings, demonstrating that Nrf2 does not regulate Abcb6 expression. In addition to Nrf2, arsenic exposure has been shown to activate several transcription factors including p53, Sp1, activator protein 1 (AP-1), and NF-kappaB (215-220).

Preliminary analysis of the human and mouse Abcb6/Abcb6 promoter using a pattern search program (nubiscan) reveals several putative *cis*-elements that may be capable of binding AP-1, NF-kB, and p53. Our future studies will evaluate the mechanism of sodium arsenite–mediated activation of Abcb6 and will address the role of these transcription factors in the activation of Abcb6.

In summary, the present studies demonstrate that sodium arsenite induces Abcb6 expression both *in vitro* and *in vivo.* Abcb6 expression is transcriptionally regulated by sodium arsenite in an *Nrf2* independent manner. Future studies will focus on the mechanisms by which arsenic induces Abcb6 expression.

Chapter 5. **SIGNIFICANCE OF ABCB6 TO XENOBIOTIC INDUCED PORPHYRIA AND OXIDATIVE STRESS – *IN VITRO***

5.1. Role of Abcb6 in PAH mediated porphyria

5.2. Role of Abcb6 in arsenic cytotoxicity

5.1. ROLE OF ABCB6 IN PAH MEDIATED PORPHYRIA

Abstract

Porphyrias are inherited or acquired disorders in which clinical manifestations are attributable to a disturbance in heme synthesis. Porphyrias are characterized by elevation in heme synthesis accompanied by elevations of heme precursors (porphyrins) in blood, urine and/or stool. A number of chemicals, particularly metals and halogenated hydrocarbons, induce disturbances of heme synthesis in experimental animals leading to porphyria. In the present study, we demonstrate that Abcb6 the second rate limiting protein in heme synthesis is an important component of environmental toxicant induced hepatic porphyria. Using a model of environmental toxicant induced hepatic porphyria we demonstrate that loss of Abcb6 expression *in vitro* in tissue culture or *ex vivo* in primary hepatocytes (isolated from Abcb6 knockout mouse) results in decreased accumulation of porphyrins, suggesting a role for Abcb6 in the manifestation of porphyria due to prolonged exposure to environmental toxicants.

Introduction

The acute porphyrias are a group of disorders that result from inherited defects in the enzymes of the heme biosynthetic pathway, which lead to accumulation of porphyrin intermediates. The main clinical manifestations of porphyrias are intermittent attacks of neuropsychiatric dysfunction and/or sensitivity of the skin to sunlight (221,222). Affected patients are prone to potentially fatal acute attacks, which are frequently precipitated by exposure to pharmacological chemicals and/or environmental contaminants (222,223). It has been observed that for several of these pharmacological chemicals and/or environmental contaminants soon after their administration or exposure there is an overproduction of heme precursors and their accumulation in certain tissues. A sequence of events has been suggested in which the chemical decreases the activity of specific enzyme(s) in the heme synthesis pathway, usually in association with stimulation of heme synthesis.

In the body, heme synthesis is regulated at two steps. The rate-limiting enzyme ALAS, which catalyzes the condensation of Glycine and Succinyl CoA to produce delta-Aminolevulinic acid, mediates the first rate-limiting step (21,22). The second rate-limiting step is mediated by the mitochondrial ABC transporter Abcb6 that regulates the energy dependent transport of COPIII from the cytoplasm into the mitochondria. It is now well established that induction of ALAS1 following exposure to environmental contaminants plays an important role in porphyrogenicity. Our studies described in Chapter 4.1 demonstrate that similar to ALAS, Abcb6 expression is induced following exposure to environmental contaminants. However, the significance of such induction to drug-induced porphyria is not known.

PAHs are potent environmental pollutants and have been shown to be associated with the induction of porphyria in humans and in experimental animals. In the present study, we used

PAH exposure as a model of porphyria to examine the relationship between Abcb6 induction and accelerated heme synthesis to manifestation of porphyria.

Results

5.1.1. Abcb6 expression is required for PAH-mediated porphyrin synthesis

We observed increased hepatic porphyrin levels in mouse primary hepatocyte and human hepatoma cells with PAH treatment (Figure 4.1.1). To evaluate the functional importance and the relative contribution of Abcb6 expression to B[a]P induced porphyrin synthesis, we blocked endogenous Abcb6 expression in HepG2 and Huh7 cells and measured porphyrin synthesis following exposure to B[a]P. Reduced Abcb6 expression was achieved in these cells using Abcb6-specific shRNA, which resulted in greater than 80% reduction of endogenous Abcb6 relative to shRNA vector cells (185). PPIX levels were measured in these cells by flow cytometry as described under "Experimental Procedures" (28). We found that reduced Abcb6 expression *per se* did not decrease PPIX levels under physiological conditions, confirming our earlier observations (Figure 5.1.1, A and B) (20). Interestingly, in the presence of 5 μM B[a]P, cellular protoporphyrin IX levels were decreased significantly in both of the hepatoma cells (Figure 5.1.1, A and B).

Although a significant decrease in B[a]P-mediated cellular PPIX levels was observed in the hepatoma cells in the absence of Abcb6, this decrease was not as dramatic as one would have expected if Abcb6 were to play a significant role in porphyrin biosynthesis. To test this further and to evaluate the full impact of Abcb6 on B[a]P-induced porphyrin biosynthesis, we isolated primary hepatocytes from *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$ mice and measured protoporphyrin levels in these hepatocytes in the presence and absence of B[a]P. We found that complete loss of Abcb6 expression significantly compromised the ability of *Abcb6*$^{-/-}$ primary hepatocytes to respond to B[a]P-mediated increase in cellular PPIX levels (Figure 5.1.1E).

Figure 5.1.1.

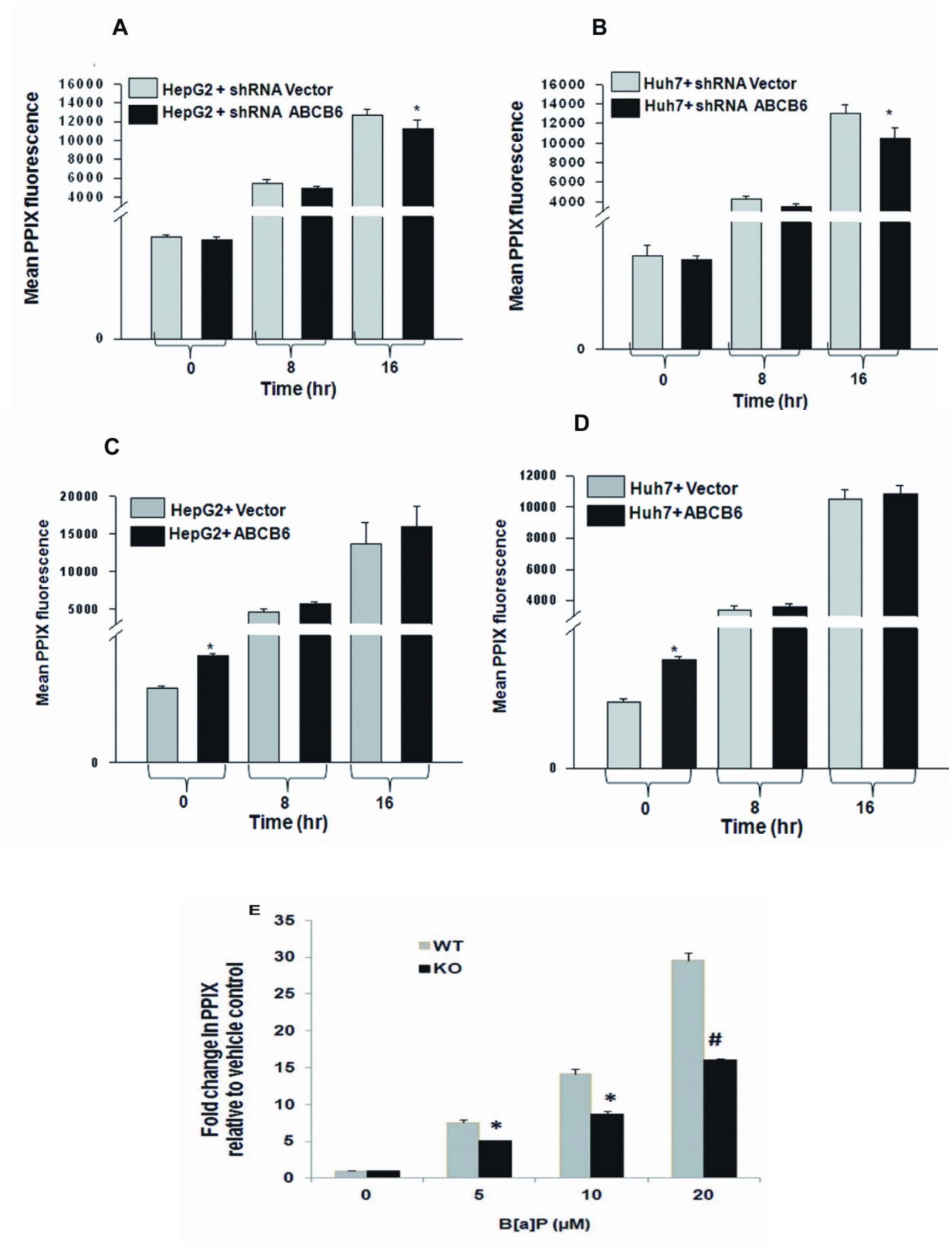

A
HepG2 + shRNA Vector
HepG2 + shRNA ABCB6
Mean PPIX fluorescence
Time (hr)
B
Huh7+ shRNA Vector
Huh7+ shRNA ABCB6
C
HepG2+ Vector
HepG2+ ABCB6
D
Huh7+ Vector
Huh7+ ABCB6
E
WT
KO
Fold change in PPIX relative to vehicle control
B[a]P (μM)

Figure 5.1.1. (continued)

Loss of Abcb6 expression compromises B[*a*]P-induced hepatic porphyrin levels.

Endogenous knockdown of Abcb6 expression results in decreased PPIX levels in both HepG2 (*A*) and Huh7 (*B*) cells exposed to B[*a*]P. In contrast, exposure to B[*a*]P does not affect PPIX levels in HepG2 (*C*) and Huh7 (*D*) cells overexpressing Abcb6. *E*, loss of Abcb6 expression results in decreased PPIX levels in mouse primary hepatocytes isolated from *Abcb6*$^{-/-}$ mice exposed to B[*a*]P. Values represent mean ± S.D. (*error bars*) (n = 3). Results are representative of three independent experiments with n = 3 per experiment. *, significantly different from shRNA vector control cells exposed to B[*a*]P for 16 h in (*A* and *B*) and significantly different from vector control cells (*C* and *D*). $p < 0.05$. *E*, *, significantly different from B[*a*]P-treated wild-type mouse primary hepatocytes. $p < 0.01$. #, significantly different from B[*a*]P-treated wild-type mouse primary hepatocytes. $p < 0.001$.

We next evaluated the effect of B[a]P treatment on porphyrin biosynthesis in cells engineered to overexpress Abcb6. Abcb6 overexpression was achieved by engineering stable expression of Abcb6 under a constitutively active CMV promoter as described previously (28,185). In contrast to the porphyrin phenotype seen in response to B[a]P treatment of Abcb6-depleted cells, in Abcb6-overexpressing cells, B[a]P treatment did not increase porphyrin biosynthesis beyond the levels already exhibited by increased Abcb6 overexpression in these cells (Figure 5.1.1, C and D).

Based on the results presented in Figure 5.1.1, A, B, and E, which demonstrate significant decrease in hepatic porphyrin levels in the absence of Abcb6 in response to B[a]P treatment, we hypothesized that B[a]P-induced CYP1A1 activity might be altered in cells that have a loss of Abcb6 expression. However, despite our efforts, we were not able to detect any CYP1A1 activity in these cell culture systems. Thus, evaluation of this aspect requires *in vivo* studies using *Abcb6* gene-deleted mouse.

Discussion

A number of halogenated hydrocarbons have been experimentally demonstrated to be porphyrinogenic in exposed animals, and in *in vitro* tests. There is little question that individuals who are genetically predisposed to porphyria can have both neurological and cutaneous manifestations of porphyria triggered by exogenous chemicals. It is also clear that certain exogenous chemicals activate mechanisms that regulate heme synthesis. However, it is not apparent as to the degree that clinical manifestations of environmentally induced porphyria might be attributable (if at all) to the associated induction in heme synthesis, apart from potential direct toxic effects of inhibition of enzymes in the heme synthesis pathway. The results presented here provide evidence suggesting that disturbed expression or function of proteins that regulate heme synthesis may have a contributing role in chemical induced porphyria.

A basic pathogenic event in PAH-induced porphyria is the accumulation of porphyrins. In many cases, the porphyria remains latent until clinical manifestations (phototoxicity, neural or visceral symptoms, elevation in urinary porphyrins) occur because of pharmacological exposure to chemicals and therapeutic drugs, which precipitates an acute attack because of increased heme synthesis (13,40,148,168). In this regard, increased Abcb6 expression in response to environmental contaminants and therapeutic drugs might contribute to the manifestation of porphyria. In this scenario, increased Abcb6 expression in response to environmental contaminants could enhance heme synthesis in patients compromised in the activity of enzymes of the heme synthetic pathway. This would result in a progressive increase in the accumulation of porphyrin intermediates that leads to clinical manifestation of the latent disease. Consistent with this observation in our studies loss of Abcb6 expression resulted in decreased accumulation of porphyrins in response to PAH exposure.

Porphyrias have historically been classified based on the specific enzyme principally involved and the principal origin(s) of the excess heme precursors. Among these is hereditary coproporphyria (HCP), which results in the accumulation of coproporphyrinogen. (224). However, it has to be acknowledged that loss of Abcb6 function could also be predicted to precipitate coproporphyria. Abcb6 has been proposed to promote heme synthesis by regulating the energy dependent transport of coproporphyrinogen from the cytoplasm into the mitochondria to complete heme synthesis (224). Ironically, in the absence of Abcb6 loss of coproporphyrinogen transport into the mitochondria could lead to accumulation of coproporphyrinogen in the cytosol precipitating coproporphyria. In this context, it is interesting to note that pesticides such as oxadiazon or oxyfluorfen induce hepatic porphyria whose etiology is not clearly understood. One could speculate that oxadiazon or oxyfluorfen induced hepatic porphyria could potentially involve interference with Abcb6 mediated porphyrin transport.

It has recently been proposed that a variety of chemical-associated illnesses for which there are no widely accepted specific diagnostic tests or etiologic explanations – such as multiple chemical syndrome, Persian gulf war illnesses, conditions associated with silicone breast implants, and various fatigue syndromes – may represent either mild chronic cases of porphyria, and in part could be manifestations of acquired abnormalities in heme synthesis. Thus in cases involving these otherwise unexplained illnesses; it would be interesting to test whether Abcb6 expression and/or function is compromised. As we gain additional knowledge, therapeutic manipulation of Abcb6 could provide an alternative way of treating these porphyric phenotypes of unknown etiology.

5.2. ROLE OF ABCB6 IN ARSENIC CYTOTOXICITY

Abstract

Arsenic is an inorganic carcinogen and a major hazard after environmental or occupational exposure. Current treatments for arsenic toxicity are limited. Consideration of endogenous protective pathways activated by cells in response to arsenic will potentially reveal novel pharmacological targets. In the present study, we evaluated the significance of Abcb6 expression to arsenic toxicity in cell culture. We demonstrate that, in HepG2 and Hep3B cells, knockdown of Abcb6 expression using Abcb6-specific small interfering RNA sensitized the cells to arsenite toxicity. In contrast, stable overexpression of Abcb6 conferred a strong survival advantage toward arsenite-induced oxidative stress. Collectively, these results, obtained by both loss of function and gain of function analysis, suggest that Abcb6 expression might be an endogenous protective mechanism activated to protect cells against arsenite-induced oxidative stress.

Introduction

Arsenic is a ubiquitous environmental pollutant. Chronic exposure of humans to inorganic arsenic results in formation of tumors of the liver and kidney and probably skin, lung, and urinary bladder. In addition to its carcinogenic effect, epidemiological studies have associated chronic arsenic exposure with an increased risk in many human nonmalignant diseases, such as peripheral vascular disease, diabetes, and chronic lung disease. Evidence suggests that arsenic exerts its chronic toxicity by interacting with sulfhydryl groups and generating reactive oxygen species (ROS) that cause damage to cellular macromolecules via oxidative stress. Of importance, exposure to pro-oxidants such as arsenic also results in the induction of a gene expression program whose primary function is to protect cells from oxidative stress. Understanding this protective program of adaptation and acquired tolerance to arsenic could potentially open up new therapeutic avenues for prevention and intervention strategies against the toxic effects of arsenic.

We recently characterized Abcb6 and found that this transporter localizes to the mitochondria and regulates *de novo* porphyrin biosynthesis (28). Abcb6 overexpression increases the concentration and activity of hemoproteins, including the heme-dependent antioxidant defense enzyme catalase. Abcb6's ability to increase catalase activity protects cells against hydrogen peroxide (H_2O_2)–mediated oxidative stress (225). Thus, Abcb6 expression protects against oxidative stress. However, based on the studies reported by *Paterson et al.* and *Gebel et al.*, it is not clear whether Abcb6 can protect against arsenic-mediated oxidative stress (204,205).

Thus, the present study was conducted to determine whether arsenic could protect cells against arsenic-induced oxidative stress. We demonstrate that loss of Abcb6 expression sensitizes cells to the toxic effects of arsenite. Finally, we provide convincing evidence that

Abcb6's ability to reduce arsenite-induced oxidative stress is a contributing mechanism by which Abcb6 protects against arsenite toxicity.

Results

5.2.1. Effect of sodium arsenite on cell survival in the presence and absence of Abcb6

The functional importance of Abcb6 to arsenite toxicity was evaluated by blocking endogenous Abcb6 expression in two human cell lines (hepatoma cells Hep3B and HepG2) (Figure 5.2.1). Reduced Abcb6 expression was achieved in these cells using Abcb6-specific siRNA, which resulted in greater than 70% reduction of endogenous Abcb6 (Figure 5.2.1, A and B, bottom panel) compared with scrambled siRNA. Arsenic toxicity was evaluated in these cells by measuring cell viability. Reduced Abcb6 expression in Abcb6-specific siRNA-treated cells sensitized both HepG2 and Hep3B cells to arsenite toxicity compared with vector control cells or cells transfected with the scrambled siRNA (inhibitor concentration IC_{50} of 19.6 and 18.7μM in the presence of Abcb6-specific siRNA vs. 27.3 and 27.4μM in the absence of siRNA in Hep3B and HepG2 cells, respectively (Figure 5.2.1 A and B).

Based on the results presented in Figures 5.2.1A and B, it is conceivable that Abcb6 overexpression is cytoprotective against arsenite toxicity. To test this hypothesis, we developed Abcb6-overexpressing cell lines using HepG2 and Hep3B cells as described (28). Abcb6 expression in these cells was confirmed by immunoblot analysis using Abcb6-specific antibody (Figure 5.2.1C and D, bottom panels). Abcb6's ability to protect against arsenite toxicity was evaluated by measuring cell viability. We found that both HepG2 and Hep3B cells that overexpress Abcb6 demonstrate better survival in the presence of sodium arsenite compared with vector control cells (IC_{50} of 211μM vs. 15.8μM for Abcb6-expressing and empty vector Hep3B cells, respectively, and 236μM vs. 12.9μM for Abcb6-expressing vs. empty vector HepG2 cells, respectively; Figures 5.2.1C and D). More importantly, knockdown of Abcb6

expression using Abcb6-specific siRNA resulted in sensitization of Abcb6-overexpressing HepG2 and Hep3B cells to arsenite toxicity (Figure 5.2.1C and D).

Figure 5.2.1.

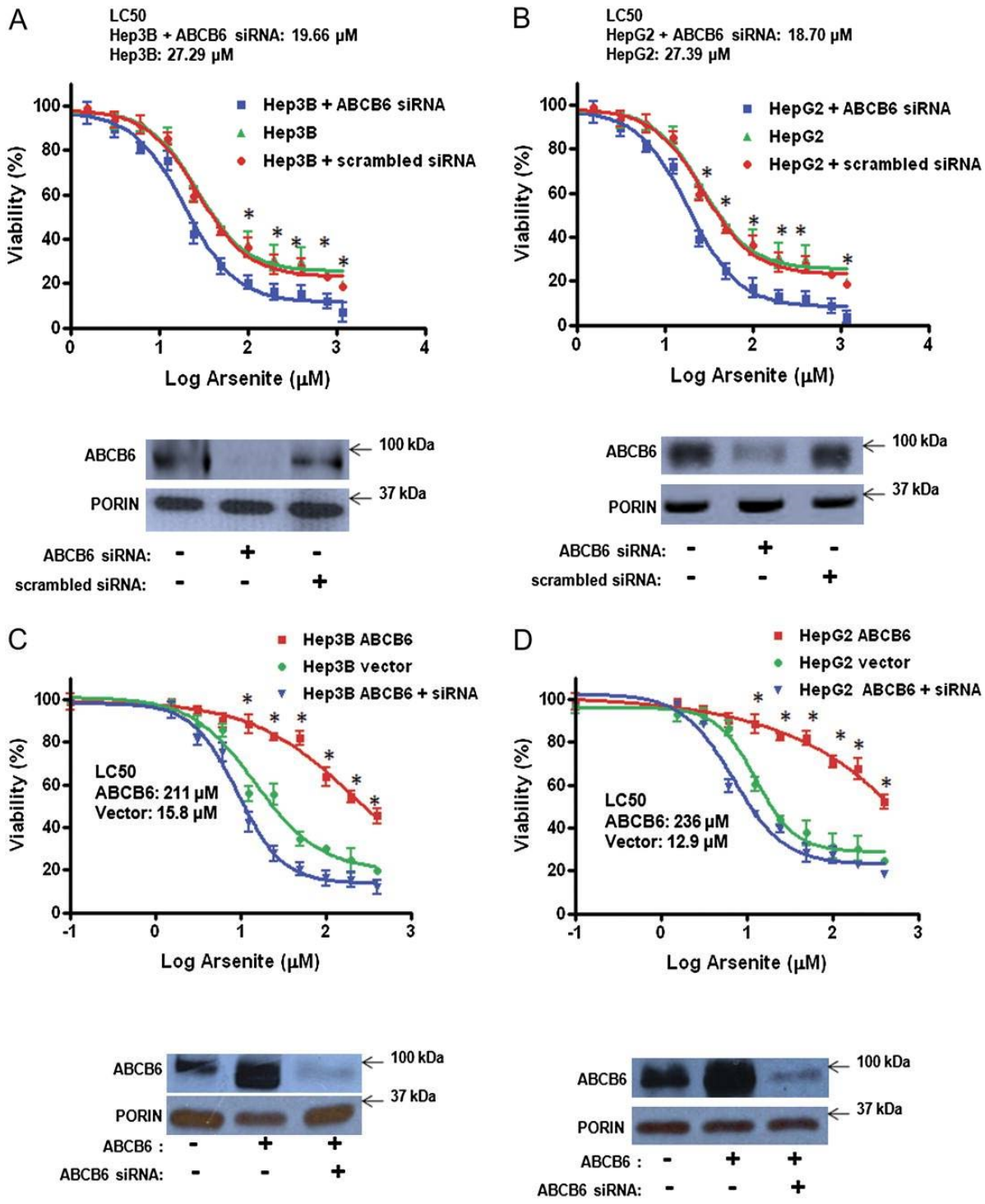

A
LC50
Hep3B + ABCB6 siRNA: 19.66 μM
Hep3B: 27.29 μM
Hep3B + ABCB6 siRNA
Hep3B
Hep3B + scrambled siRNA
Viability (%)
Log Arsenite (μM)
ABCB6
PORIN
100 kDa
37 kDa
ABCB6 siRNA:
scrambled siRNA:
B
LC50
HepG2 + ABCB6 siRNA: 18.70 μM
HepG2: 27.39 μM
HepG2 + ABCB6 siRNA
HepG2
HepG2 + scrambled siRNA
Viability (%)
Log Arsenite (μM)
ABCB6
PORIN
100 kDa
37 kDa
ABCB6 siRNA:
scrambled siRNA:
C
Hep3B ABCB6
Hep3B vector
Hep3B ABCB6 + siRNA
LC50
ABCB6: 211 μM
Vector: 15.8 μM
Viability (%)
Log Arsenite (μM)
ABCB6
PORIN
100 kDa
37 kDa
ABCB6 :
ABCB6 siRNA:
D
HepG2 ABCB6
HepG2 vector
HepG2 ABCB6 + siRNA
LC50
ABCB6: 236 μM
Vector: 12.9 μM
Viability (%)
Log Arsenite (μM)
ABCB6
PORIN
100 kDa
37 kDa
ABCB6 :
ABCB6 siRNA:

Figure 5.2.1. (continued)

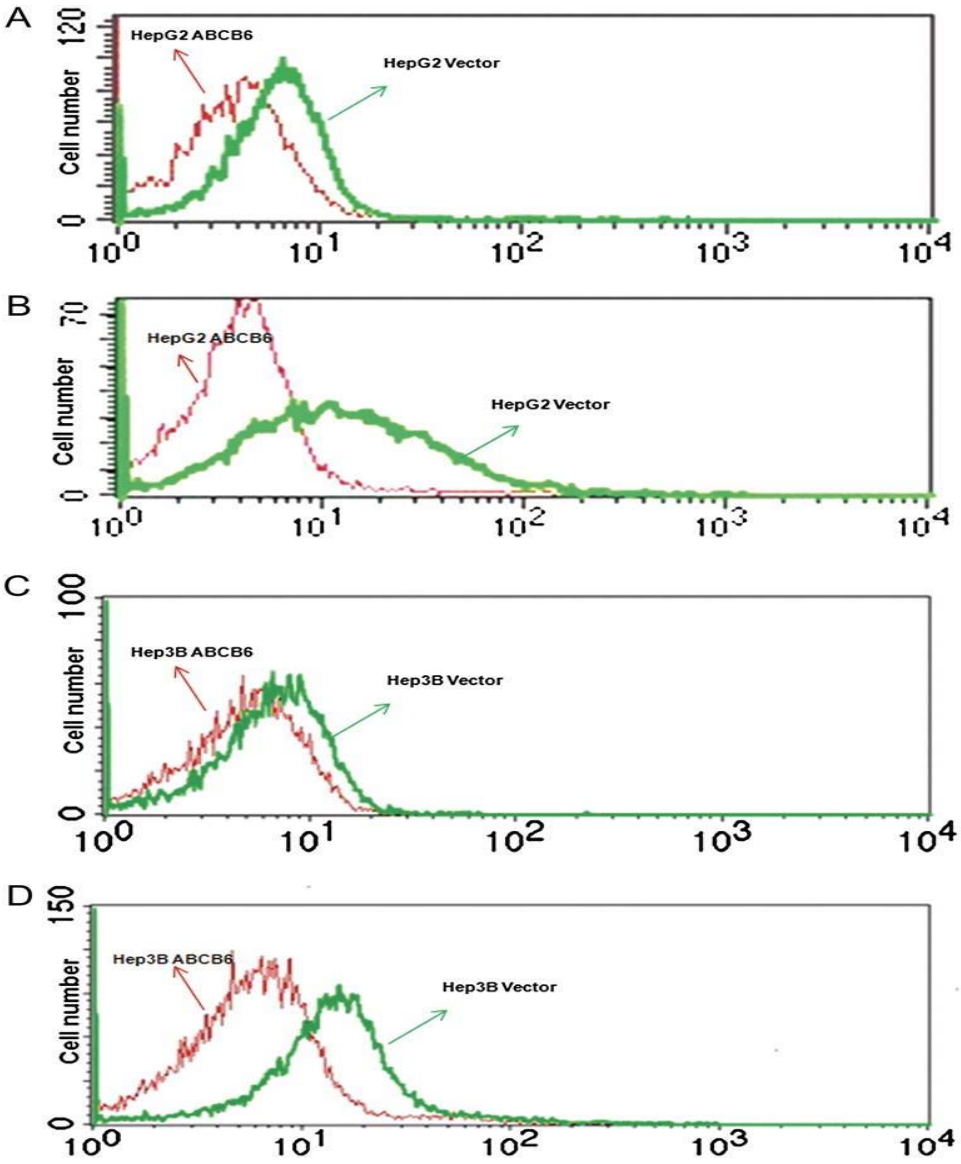
Effect of sodium arsenite on cell survival in Abcb6-expressing and Abcb6 knockdown cells.

Survival of (A) Hep3B and (B) HepG2 cells treated with increasing concentrations of sodium arsenite for 48 h. Immunoblot analysis of Abcb6 expression in Hep3B and HepG2 mitochondria in cells transfected with Abcb6-specific and scrambled (nonspecific) siRNA is presented in the bottom panel (A and B). Survival of (C) Abcb6-overexpressing Hep3B and (D) Abcb6-overexpressing HepG2 cells treated with increasing concentrations of sodium arsenite for 48 h. Immunoblot analysis of Abcb6 expression in Abcb6-expressing and siRNA knockdown cells is presented in the bottom panel (C and D). Porin expression is used as loading control in these experiments. Values represent the mean ± SD; n = 5. Results representative of three independent experiments; "*" Significantly different from siRNA knockdown cells and vector control cells at the respective time points. $p < 0.01$.

5.2.2. Effect of sodium arsenite on oxidative stress in cells expressing Abcb6

It has been reported that arsenic toxicity is in part attributed to increased oxidative stress (191-195). To test the hypothesis that Abcb6 protects against arsenite-induced oxidative stress, we measured ROS in Abcb6-overexpressing HepG2 and Hep3B cells in response to sodium arsenite. We first evaluated the basal level of ROS in Abcb6-overexpressing and vector control cells. We found reduced basal ROS in Abcb6-overexpressing cells compared with vector control cells (Figure 5.2.2A and C). We next evaluated sodium arsenite–induced oxidative stress in the vector control and Abcb6-overexpressing cells. Sodium arsenite induced oxidative stress in both vector control and Abcb6-overexpressing cells. However, the magnitude of oxidative stress in Abcb6-overexpressing cells was significantly lower than that observed in vector control cells. (Figure 5.2.2B and D). These results suggest that Abcb6 expression reduces arsenite-induced oxidative stress.

Figure 5.2.2.

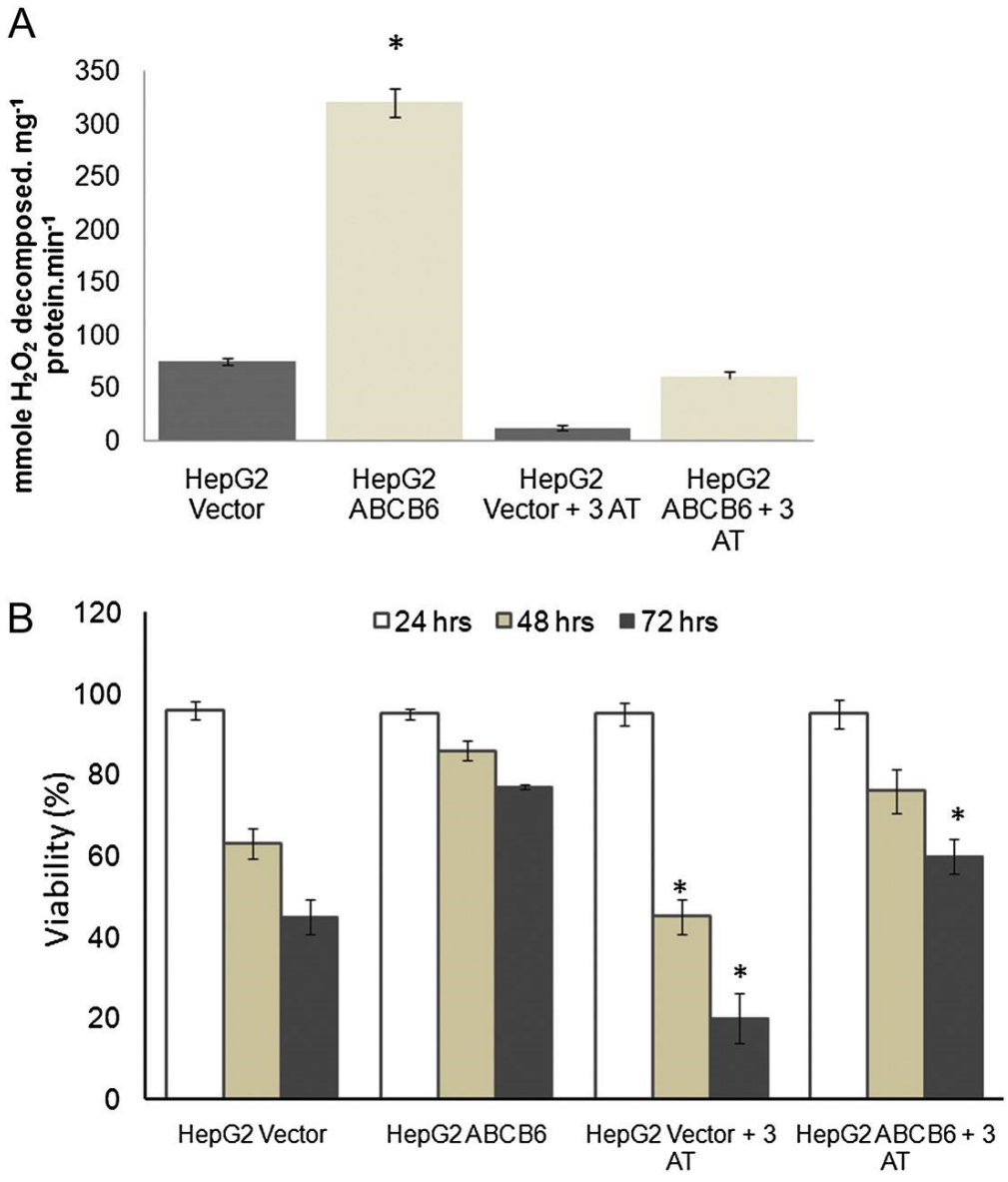


Sodium arsenite–induced oxidative stress in Abcb6-overexpressing and vector control cells.

Fluorescence-activated cell-sorting analysis of DCF fluorescence as a measure of oxidative stress in (A) Abcb6-overexpressing HepG2 and (C) Abcb6-overexpressing Hep3B cells in the absence of sodium arsenite treatment. Arsenite-induced oxidative stress in (B) Abcb6-overexpressing HepG2 and (D) Abcb6-overexpressing Hep3B cells. Results are representative of three independent experiments.

We have previously demonstrated that the expression and activity of catalase is increased in cells that overexpress Abcb6. Furthermore, this increased catalase activity in Abcb6-expressing cells protects these cells against H_2O_2-mediated oxidative stress (156). Because arsenic toxicity is in part attributed to increased oxidative stress mediated by H_2O_2 (199,226,227), we tested the relative contribution of increased catalase activity in Abcb6-overexpressing cells in protecting against arsenite toxicity. In the studies reported here, we first evaluated the ability of 3-Aminotriazole (3-AT) to inhibit catalase activity in Abcb6-expressing and nonexpressing cells. 3-AT at a concentration of 20 mmol/l was capable of inhibiting > 80% of Abcb6-induced catalase activity (Figure 5.2.3A). We next evaluated the contribution of catalase to Abcb6-mediated protection against sodium arsenite. Following pretreatment with the catalase inhibitor (3-AT), Abcb6-overexpressing and nonexpressing cells were exposed to sodium arsenite for 48 h. Pretreatment with the catalase inhibitor was essential to eliminate most of the steady-state catalase protein that is induced in Abcb6-overexpressing cells (156). Survival was evaluated at the end of 48 h by measuring cell viability. We found that, in vector control cells, blocking catalase activity increased arsenite-mediated cell death (63 and 45% at 48 and 72 h, respectively, in the absence of 3-AT compared with 45 and 20% in the presence of 3-AT; Figure 5.2.3B), which was statistically significant ($p < 0.05$). In contrast, in Abcb6-expressing cells, blocking catalase activity increased significant ($p < 0.05$) arsenite-mediated cell death only after 72 h of treatment. Abcb6-overexpressing HepG2 cells showed 85 and 76% survival (48 and 72 h, respectively) in response to sodium arsenite in the absence of 3-AT compared with 76 and 60% survival in cells that were pretreated with 3-AT (Figure 5.2.3B).

Figure 5.2.3.



Effect of sodium arsenite on cell survival in Abcb6-expressing cells in the presence and absence of the catalase inhibitor 3-AT.

(A) Effect of 3-AT (20 mmol/l) on Abcb6-induced catalase activation in human hepatoma cells HepG2 in the absence of sodium arsenite treatment. (B) Effect of 3-AT on cell death in Abcb6-expressing HepG2 cells treated with sodium arsenite (25μM) for 48 h. Values represent the mean ± SD; $n = 5$. Results are representative of three independent experiments. "*" Significantly different from their respective untreated groups. $p < 0.05$.

Discussion

In our studies, Abcb6 expression protected cells against acute arsenic toxicity. These results are in agreement with the studies reported by (204) but are in contrast to the studies reported by (205). Paterson *et al.* suggest a direct correlation between Abcb6 expression and arsenic resistance, whereas Gebel *et al.* demonstrate no increase in Abcb6 expression in HepG2 cells that were selected for arsenic resistance (204,205). One clear distinction between the studies reported in the manuscript and the studies reported by Gebel *et al.* (205) is that Gebel *et al.* evaluated Abcb6 expression in arsenic-resistant and arsenic-susceptible cells, whereas studies presented in the manuscript use Abcb6 knockdown cells that could make these cells acutely susceptible to arsenic toxicity. Thus, taken together, the results from these studies suggest the possibility that Abcb6 expression is induced in response to short-term low-dose exposure to arsenic and that this increased expression could protect against short-term acute arsenic toxicity. In contrast, long-term exposure to acute arsenic toxicity might not induce Abcb6 expression. In this context, additional studies are required to evaluate if Abcb6 expression is induced in response to long-term acute arsenic toxicity and if Abcb6 expression can provide prolonged protection against acute arsenic toxicity.

Although our studies demonstrate protection against arsenic toxicity in *in vitro* cell culture, they do not provide any relevant information regarding Abcb6 expression to chronic arsenic–induced diseases in humans. The high dose of arsenic used in our study causes acute toxicity, whereas moderate doses of repetitive exposure are associated with human diseases such as cancer, diabetes, and cardiovascular diseases. Thus, the role of Abcb6 in arsenic-induced diseases of humans requires confirmation *in vivo.*

Arsenic is known to cause cellular injury by several mechanisms including the generation of ROS (191-194), and the metabolism of arsenic has an important role in this

process (228,229). Although high concentrations of inorganic arsenic can stimulate ROS production, most of the absorbed inorganic arsenic is biomethylated to its methylated species, which appear to stimulate ROS production more efficiently than inorganic arsenic (193,230,231). Thus, one adaptive mechanism that protects against arsenic toxicity is increase in the expression of stress-response genes that reduce cellular oxidative stress (197-199). Because we have previously demonstrated Abcb6-mediated protection against oxidative stress, we first evaluated whether Abcb6's ability to protect against arsenic toxicity was associated with its ability to reduce oxidative stress. We found reduced arsenite-induced oxidative stress in Abcb6-expressing cells. Thus, one potential mechanism by which Abcb6 protects against arsenite toxicity is by reducing oxidative stress.

Results from both *in vivo* and *in vitro* studies show that both superoxide and H_2O_2 are produced in humans and animals exposed to arsenite (198,227,232). The production of H_2O_2 appears to be involved in the induction of apoptosis by arsenite in cell culture (196,199,226). It is important to note that preincubation with catalase ameliorated arsenite-induced ROS in human bladder cell culture (232), whereas treatment with 3-AT, an inhibitor of catalase, increased arsenite-induced micronuclei (226). We have previously demonstrated that Abcb6 expression protects cells against the toxic effects of H_2O_2, which is mediated by Abcb6's ability to potentiate the expression and activity of catalase (156). In the studies described in the manuscript, we tested whether Abcb6 protection against sodium arsenite is mediated by its ability to potentiate catalase activity. Treatment of cells with the catalase inhibitor 3-AT blocked Abcb6-mediated activation of catalase but had only a modest effect on the survival of cells exposed to sodium arsenite. Thus, although H_2O_2 is reported to be involved in arsenite toxicity, this does not appear to be the sole mechanism by which Abcb6 protects against arsenite toxicity.

Increased Abcb6 expression occurs in drug-resistant cell lines (203,233,234). Elevated Abcb6 mRNA levels were reported in adriamycin, camptothecin, paclitaxel, and 5-fluorouracil–resistant tumor cell lines (235). Based on these observations, it was speculated that Abcb6 could be a multidrug resistance gene and that the multidrug resistance phenotype could be attributed to Abcb6's ability to function as a transporter to reduce intracellular concentrations of the respective drug compounds. It is quite possible that a similar transport mechanism mediated by Abcb6 for inorganic arsenic or its metabolite could reduce cellular arsenic concentrations. Supporting this hypothesis in cells that are engineered to overexpress Abcb6, the transporter was shown to localize to the plasma membrane (204). However, in our studies reported here, we were not able to detect endogenous Abcb6 expression at the plasma membrane. It is quite possible that either the concentration of arsenite used in these studies was not sufficient or the analytical techniques used were not sensitive enough to detect endogenous Abcb6 expression at the plasma membrane. Thus, additional studies are required to evaluate endogenous expression of Abcb6 at the plasma membrane and its potential contribution to arsenic toxicity. Alternatively, because most of the chemotherapeutic drugs used in the studies described by *Annereau et al., Szakacs et al.,* and *Yasui et al.* cause cellular stress (203,204,234) and based on the results presented in the manuscript, one could hypothesize that the mode of protection against drug-induced toxicity may be associated with Abcb6's ability to protect against oxidative stress.

In summary, the present studies demonstrate that sodium arsenite induces Abcb6 expression both *in vitro* and *in vivo.* Moreover, this induction of Abcb6 is likely responsible for protection against arsenite toxicity *in vitro*. Future studies will focus on both the mechanisms by which arsenic causes induction of Abcb6 and the *in vivo* significance of Abcb6 expression to protection against oxidative stressors.

Chapter 6. **SIGNIFICANCE OF ABCB6 TO XENOBIOTIC INDUCED PORPHYRIA AND OXIDATIVE STRESS – *IN VIVO***

6.1. Generation and initial characterization of Abcb6 knockout mouse

6.1. GENERATION AND INITIAL CHARACTERIZATION OF ABCB6 KNOCKOUT MICE

Abstract

Abcb6 is a mammalian mitochondrial ATP-binding cassette (ABC) transporter that is suggested to regulate *de novo* porphyrin synthesis. However, the role of Abcb6 *in vivo* remains undefined. To explore this role, we generated Abcb6 deficient mice. Unexpectedly, these mice showed altered metabolism of the classic P450 substrate pentobarbital. Further studies demonstrated that Abcb6 deficient mice have decreased expression and activity of specific P450 enzymes including Cyp2b10 and Cyp3a11, the two predominant detoxification enzymes in the liver. In addition, hepatic gene expression analysis revealed that loss of Abcb6 expression also results in altered expression of genes involved in cell death and survival, lipid metabolism and embryonic development. These results suggest that Abcb6, which was initially thought to play a role in heme synthesis, might have additional biological functions. Development of the Abcb6 deficient mice should provide an opportunity to explore the role of Abcb6 in normal and patho-physiology.

Introduction

ATP binding cassette transporters belong to a family of integral membrane proteins that play a major role in many cellular processes and mediate the active transport of a vast variety of solutes across cellular membranes. Although many cellular ABC proteins have been characterized, mitochondrial ABC proteins are only beginning to emerge as important regulators of this organelle's function. To date, four members of the ABC transport proteins have been identified in the mitochondria, however, only a fraction of them have a known function. ABCB7 and ABCB8 are thought to play a role in the maturation of iron-sulfur clusters and iron homeostasis, while ABCB10 is thought to play a role in heme synthesis (94,236,237). Dysfunction of ABCB7 is involved in cellular iron homeostasis and causes a rare type of X-linked sideroblastic anemia (114). Disruption of ABCB8 in mice leads to cardiomyopathy (236), while loss of ABCB10 expression results in oxidative stress and cell death (94). Abcb6 is a half-molecule ABC protein that localizes to the outer mitochondrial membrane, and contains single transmembrane- and nucleotide binding-domains. Abcb6 has been suggested to play a role in heme and porphyrin transport and protection against oxidant induced cell death *in vitro* (132,156); however, its primary function *in vivo* is not known.

To characterize the function of Abcb6 *in vivo,* we generated a mouse model of whole body Abcb6 deficiency, and studied the effects of this deficiency on hepatic heme homeostasis, and hepatic global gene expression changes. We demonstrate that deletion of Abcb6 in mice results in mild hepatic heme deficiency confirming its role in regulating heme homeostasis. More importantly, we demonstrate that Abcb6 is required for maintenance of normal function of a specific set of P450s, influences cholesterol and lipid homeostasis, and plays a role in growth and proliferation.

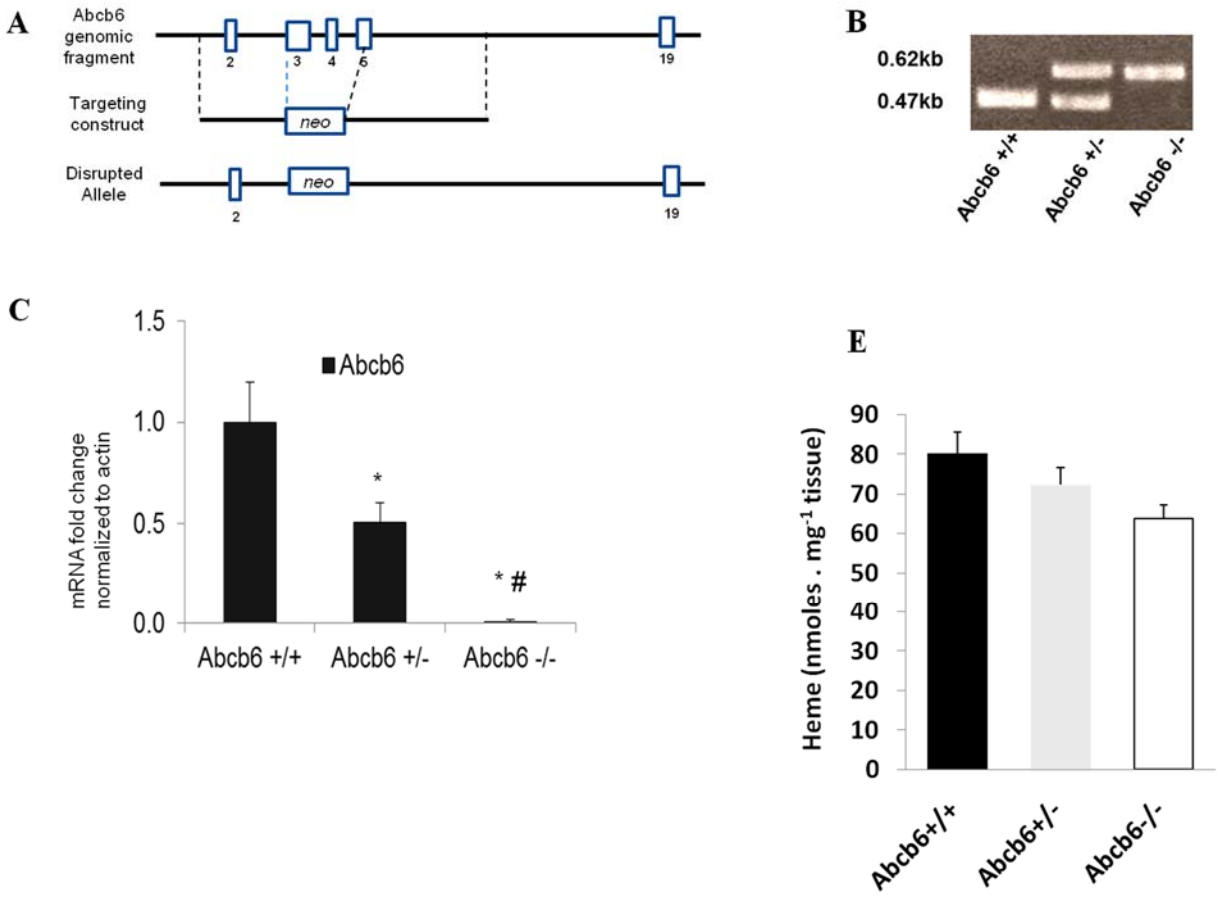
Results

6.1.1. Abcb6 knockout and heme content

The murine Abcb6 knockout mice were generated on a C57BL/6N background. To disrupt the Abcb6 gene the region extending from exons 3 to 5 of Abcb6 was replaced with a neomycin-containing cassette by homologous recombination (Figure 6.1.1A). Gene disruption was confirmed by genotyping (Figure 6.1.1B), Real Time PCR analysis of RNA transcript (Figure 6.1.1C) and immunoblotting (Figure 6.1.1D). The amount of immunodetectable Abcb6 was proportional to the amount of the Abcb6 transcript (Figure 6.1.1C and D). Aside from the Abcb6 monomer band of approximately 90 kDa, we observed 2 additional immunoreactive bands at ~ 80 and ~60 kDa (Figure 6.1.1D). The amount of all three immunodetectable proteins was proportional to the amounts of the Abcb6 transcript (Figure 6.1.1C and D). No immunoreactive protein band was detected in the *Abcb6*$^{-/-}$ mice (Figure 6.1.1D), suggesting that all three protein bands contain Abcb6.

The *Abcb6*$^{-/-}$ mice were fertile, and litter sizes were normal. However, the Abcb6 null allele showed a non-mendelian inheritance with 10 % fewer mice of the *Abcb6*$^{-/-}$ genotype than expected. We also observed random events of relatively small *Abcb6*$^{-/-}$ mice among the littermates but this physical phenotype was inconsistent. Abcb6 has been proposed to play a role in heme synthesis. Thus, the significance of Abcb6 expression to hepatic heme synthesis was evaluated in *Abcb6*$^{-/-}$ mice. As shown in Figure 6.1.1E heme levels decreased in *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice in a manner that was consistent with a gene dose effect but the values were not statistically significantly.

Figure 6.1.1.



	Abcb6$^{+/+}$	*Abcb6*$^{+/-}$	*Abcb6*$^{-/-}$	Total
Expected % (n)[a]	103 (25%)	206 (50%)	103 (25%)	413
Observed % (n)	118 (29%)	231 (56%)	64 (15%)	413

Figure 6.1.1. (continued)

Generation of *Abcb6*$^{-/-}$ mice and heme measurement.

Construct used to generate *Abcb6*$^{-/-}$ mice (A). qPCR analysis of genomic DNA from *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice (B). The wild-type allele amplifies a 0.47 kb fragment and the null allele a 0.62 kb fragment. Real-time PCR analysis of Abcb6 from liver tissue of 8 week old *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice (C). Western blot analysis (D, upper panel) of Abcb6 in total cell lysate from liver tissue of 8 week old *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice and non-Mendelian inheritance (D, lower panel) of Abcb6 gene in mice. E, Heme content measured using total liver cell lysate from *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice and normalized to protein. Values represent mean ± S.D. (error bars); n = 3 mice per experiment. Results shown are representative of three independent experiments. *, significantly different from *Abcb6*$^{+/+}$ (p < 0.01). #, significantly different from *Abcb6*$^{+/-}$ (p < 0.01).

6.1.2. Global gene expression changes in *Abcb6*$^{-/-}$ mice

To determine the effect of Abcb6 expression *in vivo* we compared transcript expression in the liver of *Abcb6*$^{-/-}$ and *Abcb6*$^{+/+}$ by using an Affymetrix microarray. We focused on 496 genes that were up- or down- regulated by a fold change of 1.5 with p value ≤ 0.05 in *Abcb6*$^{-/-}$ mice liver (317 down regulated and 179 upregulated genes). Functional networks and pathways analyses were generated through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems®, www.ingenuity.com).

IPA analysis gave insights into the molecular processes and pathways altered in Abcb6 knockout mice. The highest altered pathway according to IPA is organismal survival with increased prediction of organismal, perinatal and neonatal death based on 92-gene set altered in *Abcb6*$^{-/-}$ mice (Table 6.1). The increased organismal death could in part explain the reduced inheritance of the Abcb6-null allele (Figure 6.1.1D, lower panel). Additional important biological pathways that were altered in our gene set were related to lipid metabolism, steroid homeostasis, embryonic development and carbohydrate metabolism (Figure 6.1.2A and Table 6.1). Consistent with the lipid metabolism pathway prediction we observed increased serum cholesterol levels in Abcb6 null mice fasted for 16 hr (Figure 6.1.3C).

Further analysis of the data for alteration in canonical pathways (Figure 6.1.2B) showed subtle differences in UDP-N-acetyl-D-galactosamine Biosynthesis I/II, PXR/RXR Activation, Prolactin Signaling, Endoplasmic Reticulum Stress Pathway, Retinol Biosynthesis, Thyroid Cancer Signaling, RAR Activation, IL-9 Signaling, Colanic Acid Building Blocks Biosynthesis, N-acetylglucosamine Degradation I, Type II Diabetes Mellitus Signaling and Glutathione Biosynthesis.

Table 6.1. List of activated and inhibited functions by functional annotation analysis

Category	Functions Annotation	p-Value	Activation z-score	# genes
Activated Functions				
Organismal Survival	organismal death	1.57E-02	3.924	92
	perinatal death	9.99E-04	3.831	35
	neonatal death	5.23E-03	3.113	25
Small Molecule Biochemistry	concentration of cholesterol	1.45E-02	2.774	13
Molecular Transport		1.45E-02	2.774	13
Lipid Metabolism		1.45E-02	2.774	13
Small Molecule Biochemistry	quantity of steroid	1.49E-02	2.221	20
Molecular Transport		1.49E-02	2.221	20
Lipid Metabolism		1.49E-02	2.221	20
Cell Death and Survival	cell death of Neuroblastoma cell lines	8.97E-03	2.219	5
Metabolic Disease	adiposity	1.54E-02	2.213	8
Connective Tissue Disorders		1.54E-02	2.213	8
Small Molecule Biochemistry	concentration of phospholipid	4.80E-02	2.213	7
Molecular Transport		4.80E-02	2.213	7
Lipid Metabolism		4.80E-02	2.213	7
Small Molecule Biochemistry	concentration of lipid	7.47E-03	2.110	32
Molecular Transport		7.47E-03	2.110	32
Lipid Metabolism		7.47E-03	2.110	32
Cell Death and Survival	cell death of neuroblasts	1.67E-05	2.000	4
Embryonic Development		1.67E-05	2.000	4
Inhibited Functions				
Carbohydrate Metabolism	synthesis of carbohydrate	6.58E-04	-2.180	13
	synthesis of D-glucose	7.23E-04	-2.200	6
Small Molecule Biochemistry	synthesis of D-glucose	7.23E-04	-2.200	6

Figure 6.1.2.

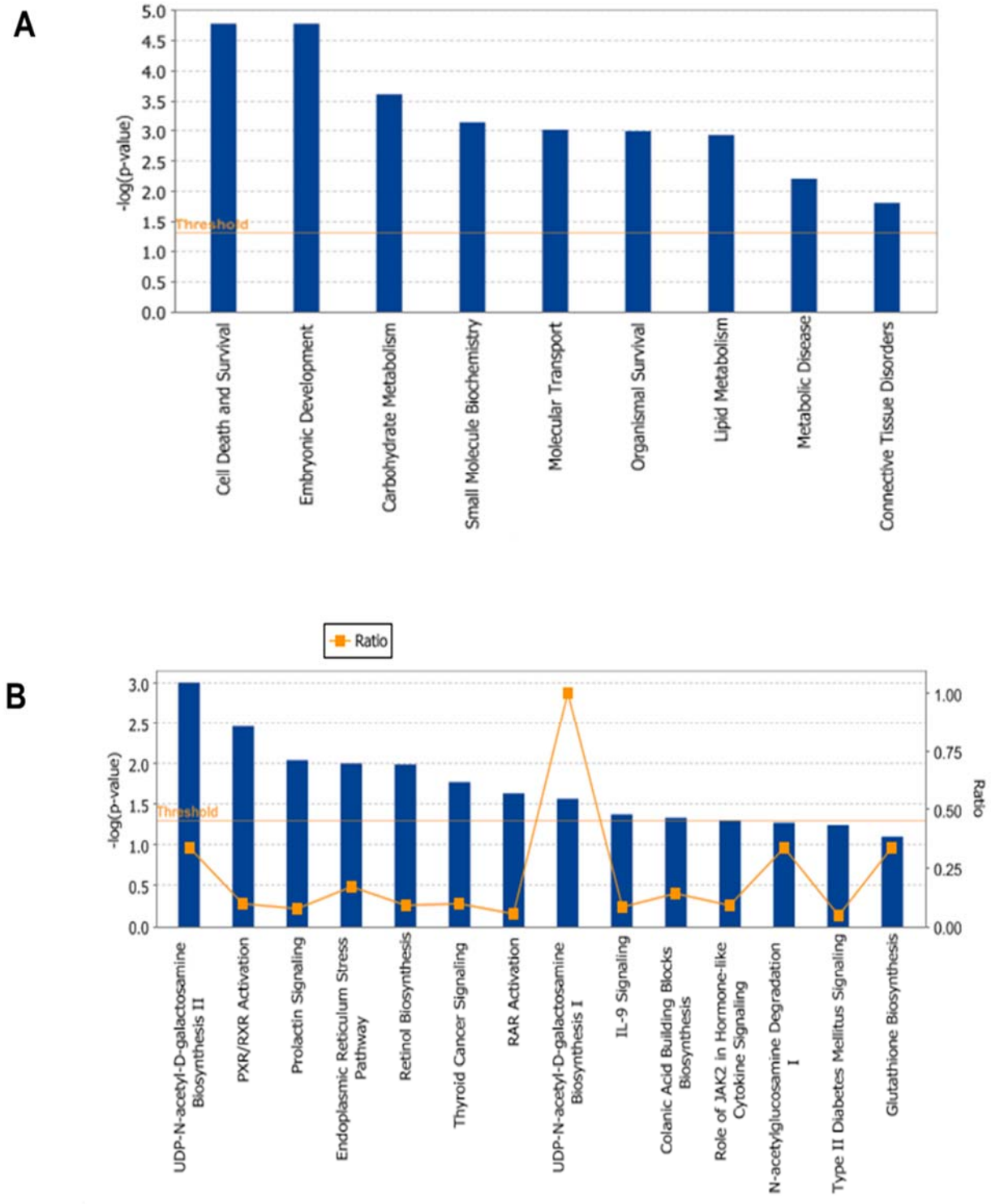
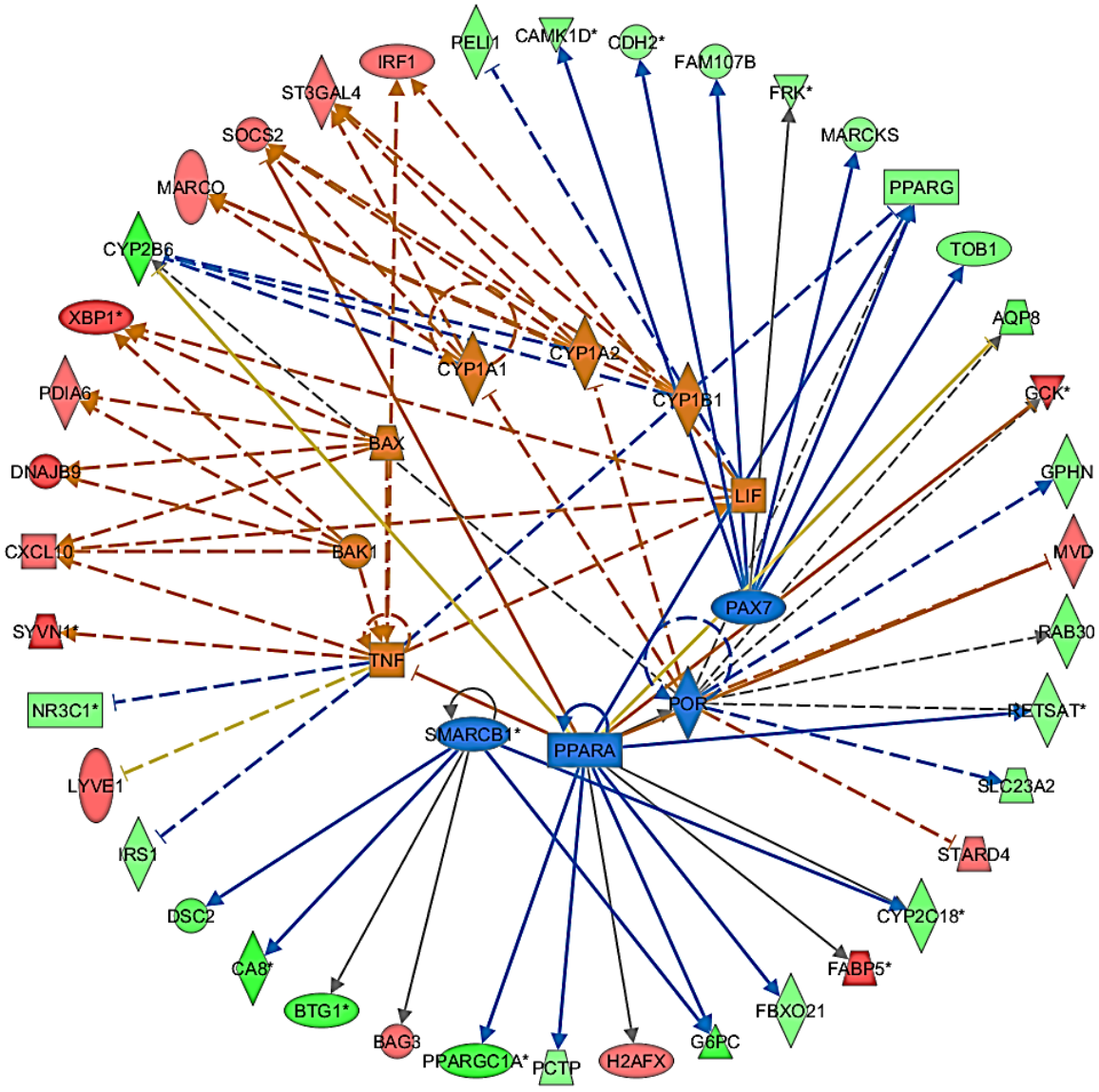

A
-log(p-value)
Threshold
Cell Death and Survival
Embryonic Development
Carbohydrate Metabolism
Small Molecule Biochemistry
Molecular Transport
Organismal Survival
Lipid Metabolism
Metabolic Disease
Connective Tissue Disorders
B
Ratio
-log(p-value)
Threshold
UDP-N-acetyl-D-galactosamine Biosynthesis II
PXR/RXR Activation
Prolactin Signaling
Endoplasmic Reticulum Stress Pathway
Retinol Biosynthesis
Thyroid Cancer Signaling
RAR Activation
UDP-N-acetyl-D-galactosamine Biosynthesis I
IL-9 Signaling
Colanic Acid Building Blocks Biosynthesis
Role of JAK2 in Hormone-like Cytokine Signaling
N-acetylglucosamine Degradation I
Type II Diabetes Mellitus Signaling
Glutathione Biosynthesis

Figure 6.1.2. (Continued)

C

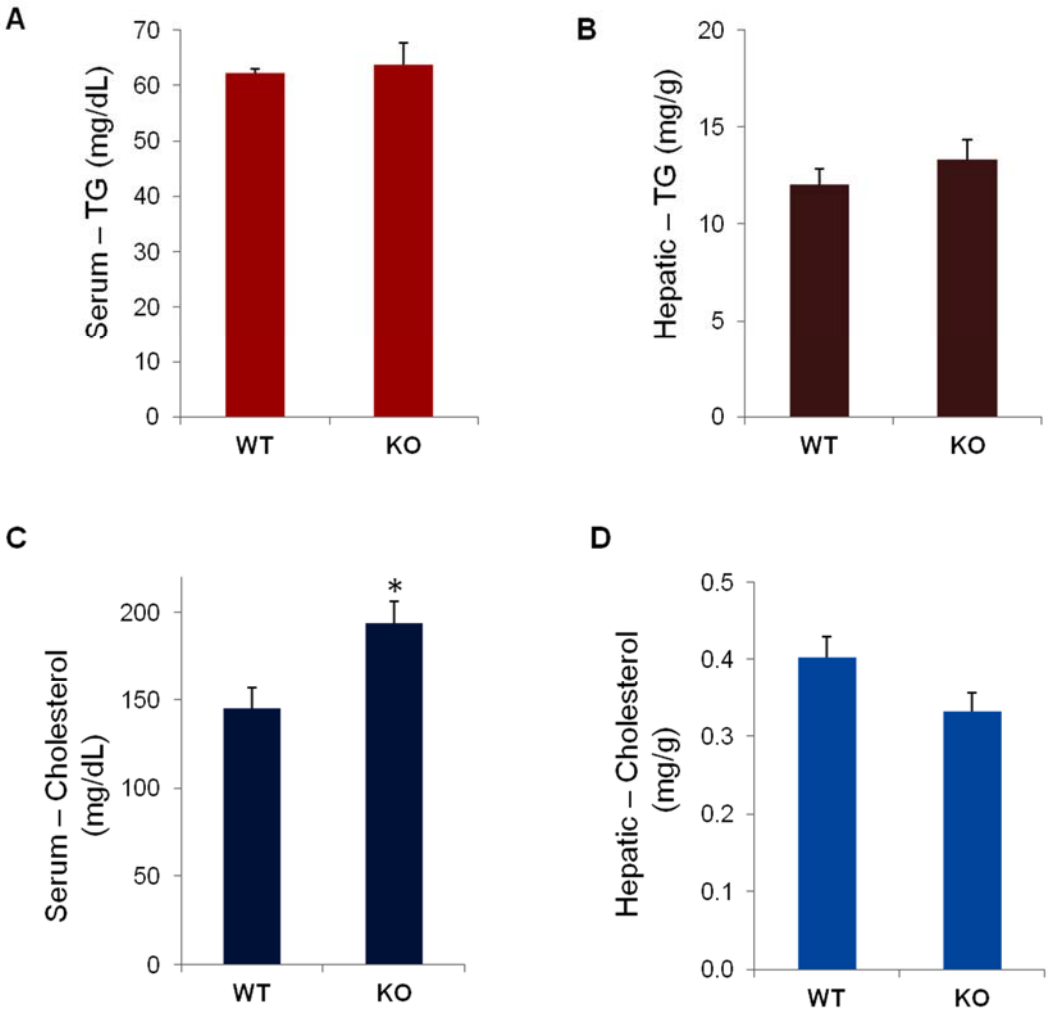


Biofunction, Canonical pathway and Upstream regulators analysis by IPA software based on the Abcb6 null mice dataset.

Comparative Biofunction analysis (A), Comparative canonical pathway analysis (B), Upstream regulators analysis (C) of *Abcb6+/+* and *Abcb6-/-* group. Figure shows the top (most significant) bio-functional or canonical groups. Threshold is set at *p*= 0.05. Significance is expressed as a p-value (≤ 0.05), which was calculated using the right-tailed Fisher's exact test. In canonical

pathways a ratio of the number of differentially expressed genes from our dataset that map to the pathway divided by the total number of molecules that exist in the canonical pathway was used for determining which pathways overlap the most. In upstream regulator analysis each ode represents a gene; red color denotes over-expressed genes and green color denotes down-regulated genes. The color intensity appears according to the related expression level by fold change. Solid connections indicate direct regulatory interactions. Arrows are colored differently to ease the identification of each connection.

Figure 6.1.3.



Increased serum cholesterol in Abcb6 knockout mice.

Loss of Abcb6 expression results in increased serum cholesterol in mice fasted for 16 hr. (A & B) Triglyceride content in serum (A) and liver (B) of *Abcb6*$^{-/-}$ mice. (C & D) Cholesterol measurement in serum (C) and liver (D) of *Abcb6*$^{-/-}$ mice. Values represent mean ± S.D. (error bars); n = 3 per experiment. *, significantly different from WT control mice ($p < 0.01$).

6.1.3. Altered expression of specific P450 enzymes in the liver of *Abcb6*$^{-/-}$ mice

One of the most interesting results from the affymetrix microarray analysis was the observation that loss of *Abcb6* expression precipitates altered expression of specific P450 enzymes in the liver. The panel of P450 enzymes altered in expression included Cyp2b10 and Cyp2c54 both of which have been proposed to participate in arachidonic acid and xenobiotic metabolism, Cyp2r1 the vitamin D 25-hydroxylase, and Cyp1a1 involved in phase I xenobiotic and drug metabolism. This observation was confirmed and extended further by targeted RT-PCR analysis of a panel of 45 mouse P450s using gene specific primers. The RT-PCR analysis confirmed results from the affymetrix study, in addition to identifying alteration in the expression of additional P450s including Cyp3a11 that was not recognized by the microarray.

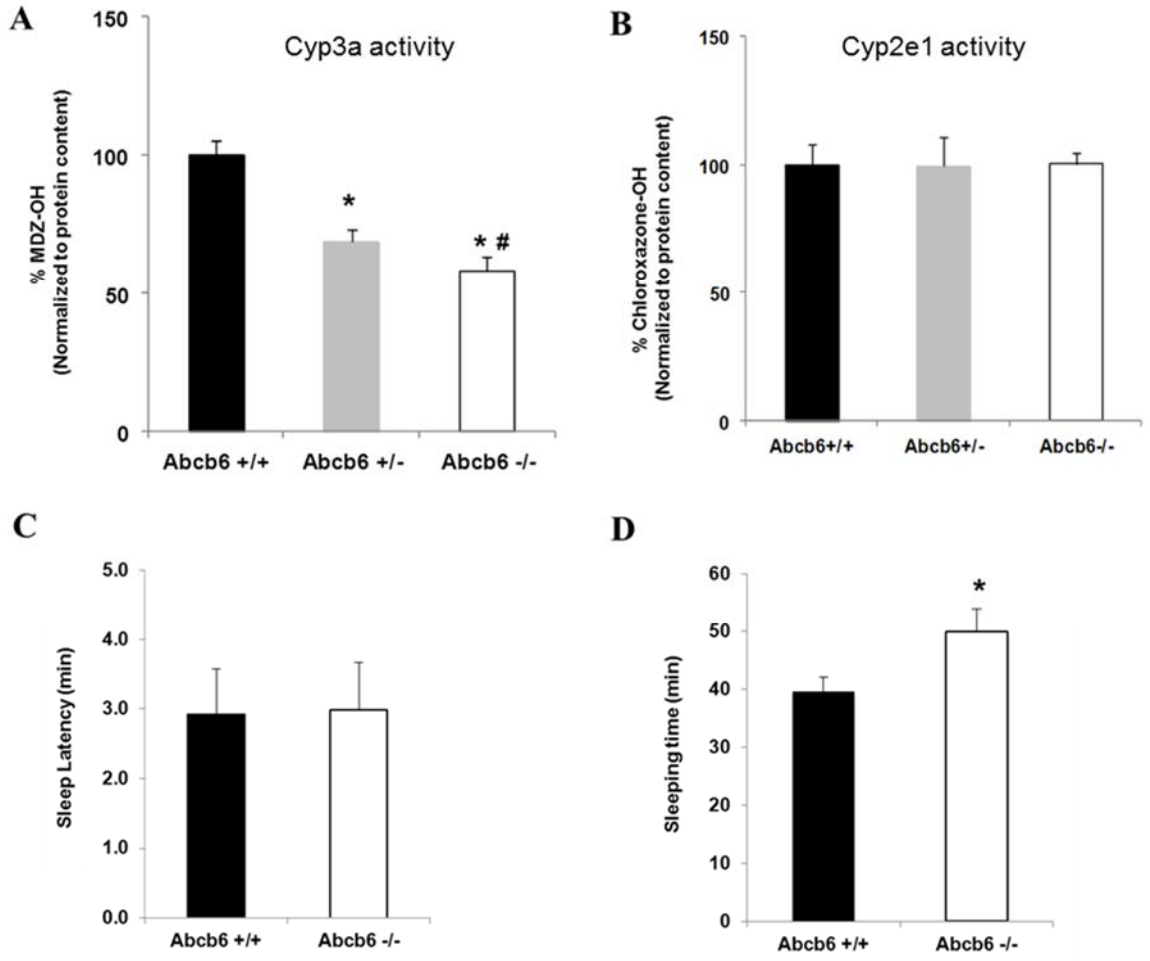
6.1.4. *Abcb6*$^{-/-}$ mice show decreased metabolism of the classic P450 substrate pentobarbital

Mammalian hepatic P450s involved in the metabolism of therapeutic agents include members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A subfamilies. Among these, quantitatively CYP3A and CYP2B forms account for nearly 30% and 19% of liver microsomal total CYP content respectively, with CYP3A forms involved in the metabolism of over 50% of available drugs (238). Thus, initial experiments were carried out to determine the effect of Abcb6 deficiency and the ensuing decrease in the Cyp3a and Cyp2b transcript to enzyme activity. For this purpose *in vitro* metabolism assays were performed using liver microsomes isolated from *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$ mice. Oxidation of midazolam to ketoconazole by Cyp3a11 and chloroxazone to pyridine by Cyp2e1 was used as specific markers for Cyp3a and Cyp2e1 activity respectively. Cyp2e1 activity was used as a negative control in these studies as Cyp2e1 expression was not altered in *Abcb6*$^{-/-}$ mice. Interestingly and consistent with the gene expression data we found that loss of Abcb6 expression compromised Cyp3a protein expression and basal Cyp3a activity but did not affect Cyp2e1 protein expression or activity (Figure 6.1.4, A and B and Figure 6.1.5, A and B).

Decreased Cyp3a activity in *Abcb6*$^{-/-}$ mice would be expected to alter the metabolism and hepatic clearance of Cyp3a substrates. Metabolism and hepatic clearance of classic xenobiotics pentobarbital have traditionally been used as an *in vivo* measure of altered pharmacokinetics/pharmacodynamics of Cyp3a substrate. Pentobarbital, an anesthetic, is inactivated by Cyp3a enzyme activity; therefore, the length of sedation following treatment with pentobarbital is indicative of Cyp3a activity. As shown in Figure 6.1.4D most wild type animals treated with pentobarbital woke up from sleep in approximately 40 minutes. In contrast essentially all the Abcb6-deficient mice required ~52 minutes to wake up. Thus, the knockout

animals have dramatically decreased basal Cyp3a activity, in agreement with the gene expression and *in vitro* activity results, and indicate that loss of Abcb6 function significantly increases pentobarbital sensitivity. Taken together these results suggest that the activity of a subset of hepatic P450s is altered in the absence of Abcb6.

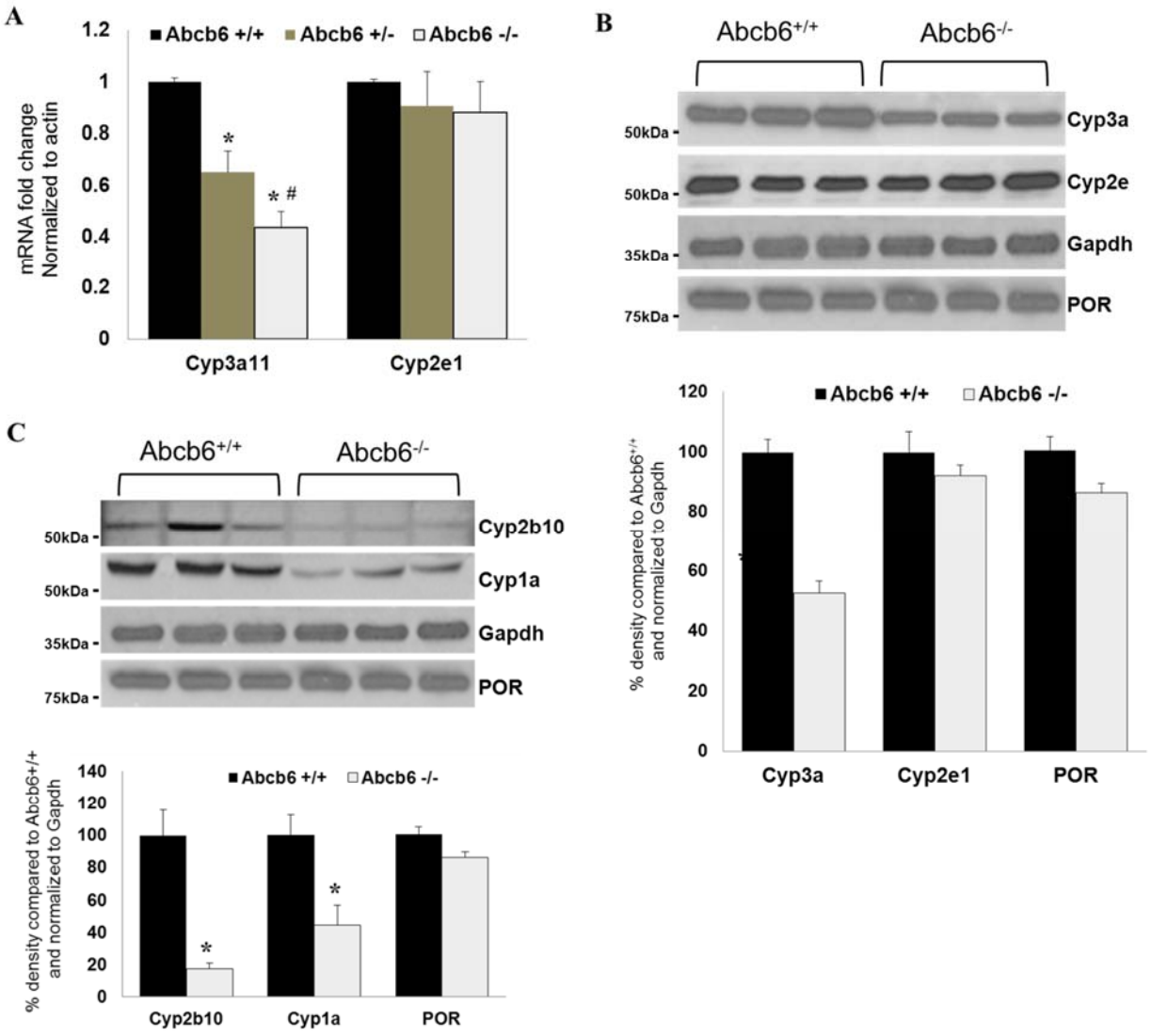
Figure 6.1.4.



Loss of Abcb6 expression compromises hepatic P450 activity.

Loss of Abcb6 expression results in decreased hepatic Cyp3a activity. (A & B), Oxidation of Midazolam (A) and Chloroxazone (B) is used to quantitate enzymatic activity of Cyp3a11 and Cyp2e1 levels in livers of *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice. Enzymatic activity was normalized to protein content. (C & D), The length of time between pentobarbital administration and the loss (Sleep Latency, C) and subsequent recovery of righting reflex (Sleeping time, D) were recorded as marker for pentobarbital clearance in *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$ mice. Values represent mean ± S.D. (error bars); n = 3 mice per experiment. Results shown are representative of two independent experiments. *, significantly different from *Abcb6*$^{+/+}$ control ($p < 0.01$). #, significantly different from Abcb6+/- ($p < 0.01$).

Figure 6.1.5.



Loss of Abcb6 expression compromises hepatic P450 expression.

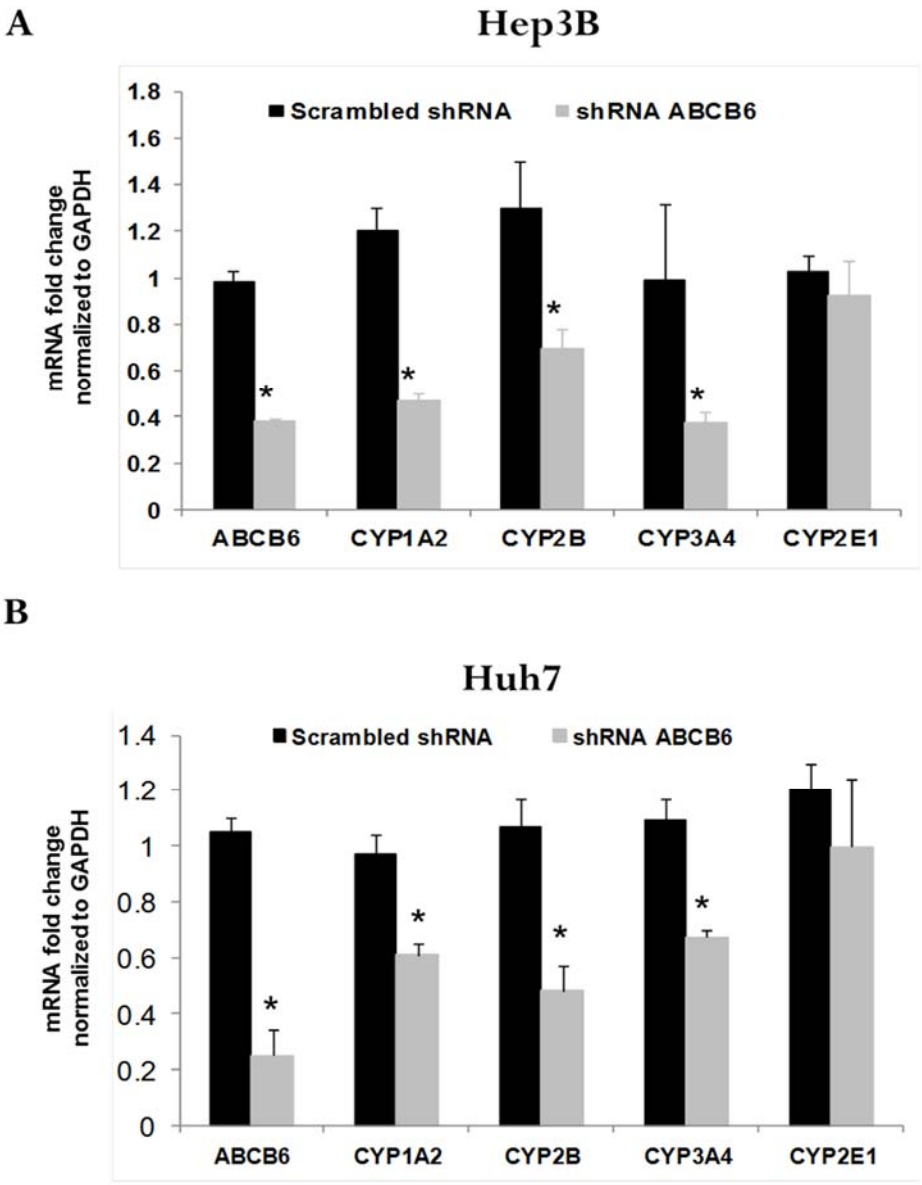
Loss of Abcb6 expression results in decreased hepatic P450s expression (A) Real-time PCR analysis of Cyp3a11 and Cyp2e1 mRNA levels in livers of *Abcb6*$^{+/+}$, *Abcb6*$^{+/-}$ and *Abcb6*$^{-/-}$ mice. P450s expression was measured using gene-specific primers and normalized to expression of Actin. (B) Immunoblot analysis (upper panel) of Cyp3a and Cyp2e expression in *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$ mice. ImageJ densitometry analysis (lower panel) of immunoblots panels from 2B. (C) Immunoblot analysis (upper panel) of Cyp2b10 and Cyp1a expression in *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$

mice. ImageJ densitometry analysis (lower panel) of immunoblots panels from 2C. Gapdh and P450 oxidoreductase (POR) are used as loading control for the Immunoblot analysis. Gapdh used for the normalization of densitometric analysis. Values represent mean ± S.D. (error bars); n = 3 mice per experiment. Results shown are representative of three independent experiments. *, significantly different from *Abcb6*$^{+/+}$ control ($p < 0.01$). #, significantly different from *Abcb6*$^{+/-}$ ($p < 0.01$).

6.1.5. Loss of Abcb6 expression alters human P450 expression in hepatomas

Mechanisms that regulate human and mouse P450s are not always comparable. To test whether loss of Abcb6 expression has a similar phenotype on P450 expression in humans we measured P450 expression in human hepatoma cells where endogenous Abcb6 expression was knocked down using Abcb6 specific shRNA. Generation and characterization of Abcb6 knockdown hepatoma cells has been described previously. Consistent with P450 expression in Abcb6 knockout mouse, in Abcb6 knockdown Hep3B and Huh7 cells loss of Abcb6 resulted in decreased expression of a specific subset of P450s including CYP 2B, 3A4 and 1A2 gene transcripts (Figure 6.1.6A and B). Overall, these results suggest that Abcb6 mediated regulation of P450 is similar in mice and humans.

Figure 6.1.6.



Loss of Abcb6 expression compromises hepatic P450 expression in hepatoma cells.

Loss of Abcb6 expression results in decreased P450s expression. (A & B) Real-time PCR analysis of CYP1A2, CYP2B, CYP3A4 and CYP2E1 mRNA levels in hepatoma cells Hep3B (A) and Huh7 (B). P450s expression was measured using gene-specific primers and normalized to Gapdh expression. Values represent mean ± S.D. (error bars); n = 3 per experiment. Results shown are representative of three independent experiments. *, significantly different from Scrambled shRNA control ($p < 0.01$).

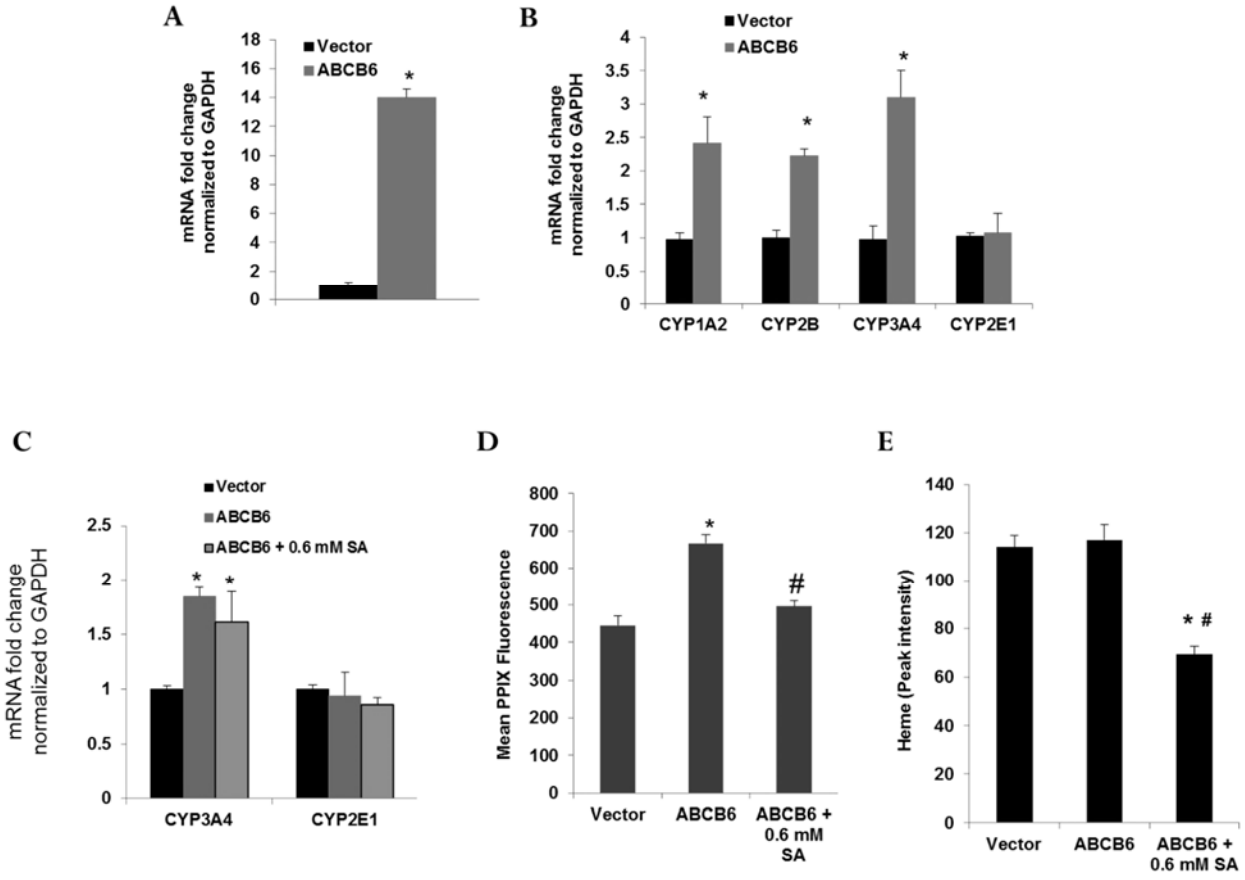
6.1.6. Abcb6 overexpression results in increased expression of a subset of P450 genes

Results from mouse and cell culture studies clearly demonstrate that loss of Abcb6 expression results in decreased expression of a specific subset of P450 genes. Based on these observations it is rationale to hypothesize that Abcb6 overexpression could lead to an increase in P450 expression and activity. To test this further, we measured P450 expression in cells engineered to overexpress Abcb6. Consistent with our hypothesis we found increased expression of a specific subset of P450s in Abcb6 overexpressing hepatomas including CYP1A2, CYP2B and CYP3A4 transcripts (Figure 6.1.7A). CYP2E1 expression, which we found to be unchanged in Abcb6 knockdown cells and knockout mouse models, was used as a control in these studies as well. Again consistent with previous observations Abcb6 overexpression did not affect CYP2E1 expression. Although we were able to demonstrate induced expression of P450s in Abcb6 overexpressing cells, we were unable to extend these studies to changes in P450 activity, because despite our extensive efforts the P450 activity assays used in these studies were not sensitive enough to detect P450 activity in these cell culture systems.

6.1.7. Heme independent P450 increase in Abcb6 overexpressing hepatoma

Heme is not only an important prosthetic group that modulates the structure and activity of hemoproteins such as P450s but also a regulatory molecule that controls the biosynthesis of various proteins. Evidence suggests that increased cellular heme levels are associated with increased expression of three CYPS; Cyp3a, Cyp2a5 and Cyp2b10 but not other members in this family. In our studies, Abcb6 overexpression induced not only Cyp3a and Cyp2b10 but also other members in this family, suggesting that Abcb6 mediated induction of CYP450 gene expression is independent of cellular heme levels. However, to confirm this further, we evaluated the expression of Cyp3a11, Cyp2a5, and Cyp2b10 expression in Abcb6 overexpressing cells in the absence of heme synthesis. Loss of heme synthesis in Abcb6 overexpressing cells was achieved by blocking heme synthesis using succinylacetone, a potent inhibitor of ALA-dehydratase the second enzyme in heme synthesis. We found that succinylacetone at a concentration of 0.6 mM resulted in 50% reduction in cellular heme levels (Figure 6.1.7 D and E), however this reduction in heme levels did not decrease CYP3A4 expression significantly (Figure 6.1.7C) suggesting that Abcb6 mediated induction of CYP3A4 is independent of cellular heme levels.

Figure 6.1.7.



Effect of heme biosynthesis Inhibition on P450 expression in Abcb6 overexpressor hepatoma

Inhibition of heme biosynthesis by succinyl acetone does not alter Abcb6 mediated induction of CYP3A4. (A & B) Real-time PCR analysis of Abcb6 (A) CYP1A2, CYP2B, CYP3A4 and CYP2E1 (B) levels in Hep3B cells overexpressing Abcb6. Real-time PCR analysis of CYP3A4 and CYP2E1 mRNA levels in Hep3B cells overexpressing Abcb6 treated with 0.6 mM SA (C) for 24 hr. Mean PPIX (D) and Heme (E) levels in Hep3B cells overexpressing Abcb6 treated with 0.6 mM Succinyl acetone (SA) for 24 hr. Values represent mean ± S.D. (error bars); n = 3 per experiment. Results shown are representative of three independent experiments. *, significantly different from vector control ($p < 0.01$).

6.1.8. Increased accumulation of androstanols in Abcb6 deficient mice

Direct activation by nuclear receptors represents one of the primary mechanisms by which P450 expression is regulated. The classical nuclear receptors are activated by their cognate ligands, which include endogenous metabolites and exogenous chemicals. Given that Abcb6 is an ABC transporter we hypothesized that loss of Abcb6 expression could in theory affect the levels of endogenous metabolites that could represent potential ligands for nuclear receptors. To test this hypothesis we performed a global metabolomics analysis using livers isolated from Abcb6 wildtype and Abcb6 knockout mice. To our surprise, we found that loss of Abcb6 expression lead to a significant increase in several androstane metabolites (Table 6.1.2), shown to be the naturally occurring inverse agonists that reverse transcriptional activation by nuclear receptor CAR. These results suggest that Abcb6 might regulate androstane homeostasis.

Table 6.1.2. List of androstanol and androrstenol derivatives

Androstane derivative	Formula	Mol. Wt.
2α, 3α-(Difluoromethylene)-5α-androstan-17β-ol acetate	$C_{22}H_{32}F_2O_2$	366.2370
3α-Hydroxy-2α-methyl-5α androstan-17-one	$C_{20}H_{32}O_2$	304.2402
17-Methyl-5α-androst-2-ene-1α, 17β-diol	$C_{20}H_{32}O_2$	304.2402
17β-Hydroxy-4α-methyl-5α androstan-3-one	$C_{20}H_{32}O_2$	304.2402
3β-Methoxyandrost-5-en-16β-ol	$C_{20}H_{32}O_2$	304.2402
3α-Hydroxy-2α-methyl-5α androstan-17-one	$C_{20}H_{32}O_2$	304.2402
17-Methyl-5α-androst-2-ene-1α, 17β-diol	$C_{20}H_{32}O_2$	304.2402
17β-Hydroxy-4α-methyl-5α androstan-3-one	$C_{20}H_{32}O_2$	304.2402
3β-Methoxyandrost-5-en-16β-ol	$C_{20}H_{32}O_2$	304.2402
17β-Hydroxy-6β-methyl-5αandrostan-3-one propionate	$C_{23}H_{36}O_3$	360.2664

Discussion

In our previous studies, loss of Abcb6 expression did not alter heme levels significantly at basal level; however, loss of Abcb6 expression significantly compromised PAH-mediated induction of hepatic porphyrin synthesis. These observations are in agreement with recent report by *Ulrich et al.,* demonstrating that in the absence of Abcb6, there is increase PPIX accumulation after phenylhydrazine treatment. These reports suggest, when stressed, loss of Abcb6 compromises cellular heme biosynthesis and function. In this context, it will be interesting to see if loss of Abcb6 expression *in vivo* (in the Abcb6 gene-deleted animals) compromises P450 activity and its effect on drug metabolism and disposition. Thus, in this study, we investigated the role of Abcb6 in expression and activity of P450s.

Cyp3a family is mainly responsible for drug metabolism function and is known to be regulated by cellular heme pool. In our initial analysis, we found Cyp3a11 expression and catalytic activity decreased and correlated with decrease in Abcb6 gene expression but there is no statistical decrease in heme pool of Abcb6 null mice, which suggest heme independent regulation of Cyp3a in Abcb6 null mice. Further analysis using succinylacetone, an inhibitor of heme biosynthesis, in Abcb6 overexpressor hepatoma supports heme independent regulation of Cyp3a by Abcb6 gene. *In vivo* this decrease in Cyp3a activity affects clearing of pentobarbital and affects sleep time in Abcb6 null mice. In addition some other known heme regulated cytochromes such as Cyp2a5 and Cyp1a along with several others P450 gene expression, which are not reported to be regulated by heme, was altered in Abcb6 null mice suggesting heme independent regulation of P450 genes in *Abcb6*$^{-/-}$ mice. Previously, increased Abcb6 expression has been demonstrated in several drug resistant cell lines and role of Abcb6 in drug resistance has been proposed but there is no direct evidence for Abcb6 mediated drug efflux. The observation from this study suggests that there could be altered drug metabolism through

altered P450 expression because of Abcb6 which is responsible for drug resistance. This hypothesis needs to be evaluated further.

Nuclear hormone receptor CAR and PXR are known to regulate P450 gene expression. CAR and PXR are ligand activated transcription factors which upon activation regulates downstream genes. CAR ligands such as androstanol and androrstenol which are endogenous steroids are known to function as inverse agonists which block transactivation of CAR. Our preliminary analysis suggests that there is an increase in androstane derivatives in Abcb6 null mice suggesting a possible role for Abcb6 in regulating the concentration of endogenous ligands of CAR. Future studies analyzing the steroid composition of Abcb6 null mice should provide answers that could help deduce the mechanism of P450 regulation by Abcb6.

In summary, the present study demonstrates that Abcb6 expression both *in vitro* and *in vivo* regulates P450 expression possibly through reduced nuclear activation of CAR. Future studies will focus on the mechanisms by which Abcb6 regulates P450 expression and the effect of decreased P450 expression on drug metabolism and toxicity. Overall, this suggests role of Abcb6 in the regulation of xenobiotic metabolism enzymes and drug metabolism.

Chapter 7: **DEVELOMENT OF *IN VITRO* ASSAY FOR SCREENING OF ABCB6 SUBSTRATES**

Abstract

In this study we developed an in vitro system with pure and active protein. Abcb6 overexpressed in HEK293 cells was solubilized from mitochondrial membranes and purified to homogeneity. Purified Abcb6 showed a high binding affinity for MgATP (Kd = 0.18 μM) and an ATPase activity with a Km of 0.99 mM. Reconstitution of Abcb6 into liposomes allowed biochemical characterization of the ATPase including (i) substrate stimulated ATPase activity (ii) transport kinetics of its proposed endogenous substrate coproporphyrinogen III and (iii) transport kinetics of substrates identified using a High-throughput screening (HTS) assay. Mutagenesis of the conserved lysine to alanine (K629A) in the Walker A motif abolished ATP hydrolysis and substrate transport. These results suggest a direct interaction between mitochondrial Abcb6 and its transport substrates that is critical for the activity of the transporter. Further, the simple immunoaffinity purification of Abcb6 to near homogeneity and efficient reconstitution of Abcb6 into liposomes might provide the basis for future studies on the structure, function of Abcb6.

Introduction

The ATP-binding cassette superfamily constitutes the largest and most broadly expressed class of proteins found in all kingdoms of life (239). They couple the hydrolysis of ATP to active transport of an array of biological compounds, including drugs, bile acids, peptides, steroids, ions, and phospholipids across membranes (240-243). Two highly conserved hydrophilic cytosolic NBDs, and at least two hydrophobic membrane-spanning domains (MSDs) characterize the eukaryotic ABC transporters (244,245). The MSDs serve as the substrate-binding sites and the transmembrane domains (located within the MSD) form the translocation pore. The two NBDs are responsible for the binding and hydrolysis of ATP, which provides energy for uphill movement of substrates across membranes. The TMDs and NBDs are found as homo- or heterodimers and can be arranged in any combination, e.g. as separate polypeptides, as single polypeptides or as half-size transporters with fused TMD and NBD. Each NBD contains the highly conserved Walker A and B motifs as well as the C-, H- and D-loops (246,247). The Walker A motif contains a lysine that coordinates with the gamma-phosphate of ATP, whereas the aspartate in Walker B interacts with Mg2+. The ABC signature or Walker C motif distinguishes ABC transporter proteins from other ATP-binding proteins. These domains are required to execute ABC transport activities.

The ATP binding cassette transporter subfamily member B6, Abcb6, gene encodes a membrane protein of 842 amino acids with a TMD followed by a NBD (248). Hydrophobicity and sequence homology analysis suggests that the TMD contains six transmembrane helices with the N and C termini located in the cytoplasm. The minimal functional unit has been suggested to be a homodimer residing in the outer mitochondrial membrane (28,249). Abcb6 has been characterized as a mitochondrial transporter involved in the translocation of COPIII from the cytoplasm into the mitochondria (28,29). COPIII a byproduct of heme synthesis in the cytoplasm requires active transport into the mitochondria to complete heme synthesis (41,250). Thus

Abcb6 has been characterized with a physiological role in heme synthesis. However, several other functions and localization for Abcb6 have been suggested, e.g. Abcb6 has been localized to the plasma membrane and specifies the new blood group system Langereis (Lan) (128). Loss of Abcb6 function has been associated with developmental defects including ocular coloboma (117). Further, exogenous expression of Abcb6 has been correlated with increased cell growth and proliferation while loss of Abcb6 expression results in delayed progression through the mitotic phase of the cell cycle (117,185). These observations along with the fact that Abcb6 unlike other mitochondria targeted ABC transporters lacks a classical mitochondrial targeting sequence has favored reports of the existence of differentially localized Abcb6 with potentially unique functional properties.

Here, we describe the development of a cell free system to understand the mechanistic relationship between ATP binding and hydrolysis and the coupling of these events to substrate transport mediated by the mitochondrial transporter Abcb6. Overexpression, purification and functional reconstitution of Abcb6 into liposomes permitted us to study the biochemical properties of Abcb6 for the first time in the absence of contaminating ATPases and carriers/ transporters. In addition, immunoaffinity purification of Abcb6 to homogeneity, allowed identification of two isoforms of Abcb6.

Results

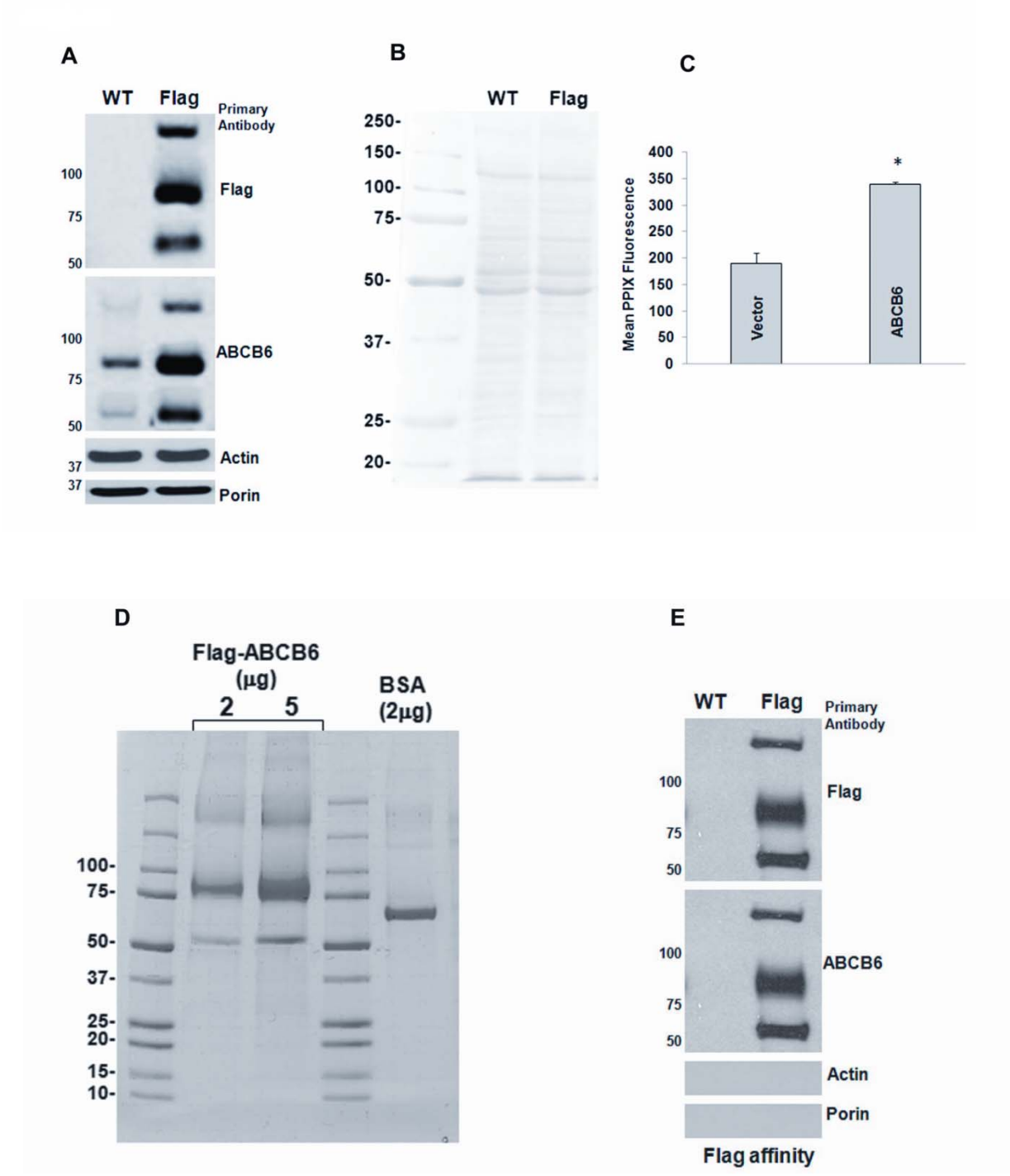
7.1. Overexpression and purification of Abcb6

To facilitate purification of Abcb6 for biochemical analysis, the protein was tagged at the C-terminus with the FLAG epitope and expressed from a lentiviral vector under the control of a strong constitutively active promoter. As shown in Figure 7.1A, immunoblot analysis of total cellular fraction, using antibody specific for the FLAG-epitope demonstrates induced expression of FLAG-tagged Abcb6 in HEK293 cells transduced with the Abcb6-FLAG lentiviral construct but not in vector only transduced cells. Ponceau staining of the western blot before immunoblot analysis demonstrates that the induced expression of Abcb6 in the overexpressing cell is not due to differences in loading (Figure 7.1B). To confirm that the addition of the C- terminal FLAG-tag to Abcb6 does not affect its function we measured heme synthesis in Abcb6-FLAG overexpressing cells. We have previously demonstrated that Abcb6 expression and function is directly related to cellular heme synthesis (28). As seen in Figure 7.1C cells overexpressing the FLAG tagged Abcb6 protein demonstrate increased heme synthesis relative to empty vector control cells, suggesting that the addition of the FLAG-tag to the C-terminus of Abcb6 does not alter its function. Interestingly, both the endogenous protein, and overexpressed protein resolve as three bands of about 180, 90 and 50 kDa, as evidenced by immunoblot analysis using antibodies specific for either Abcb6 or the FLAG-tag (Figure 7.1A).

For Abcb6 purification, in initial studies, total cellular fractions were employed as starting material. To solubilize the protein from the membranes, the non-ionic detergent Triton was effective in either the presence or absence of n-Octyl-β-D-glucopyranoside. Abcb6-FLAG in the detergent solubilized fractions was separated from other cellular constituents by anti-FLAG affinity chromatography. As seen in Figure 7.1D, under the conditions of loading and washing of FLAG-sepharose beads, the Abcb6-FLAG protein was quantitatively bound, and upon elution

with a buffer containing the FLAG peptide, was highly enriched and readily detectable by Coomassie blue staining. Bovine serum albumin (BSA) shown on the same gel was used as a loading control for the estimation of protein concentration (Figure 7.1D). The entire purification procedure produces approximately 40 μg of purified Abcb6 from one 150-cm^2 plate of confluent cells. Aside from the major Abcb6 monomer band of approximately 90 kDa, two additional staining bands, one with an apparent molecular weight approaching that of a homodimer (~180 kDa) and one with a lower molecular weight of approximately 50 kDa were also observed in the elution fractions (Figure 7.1D and E). All these three bands clearly contain Abcb6 as indicated by its detection with both Abcb6 specific monoclonal antibody (Figure 7.1E) as well as by MALDI-TOF peptide mass fingerprint analysis.

Figure 7.1.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Primary Antibody
Flag
ABCB6
Actin
Porin
100
75
50
37
B
WT
Flag
250-
150-
100-
75-
50-
37-
25-
20-
C
Mean PPIX Fluorescence
400
350
300
250
200
150
100
50
0
Vector
ABCB6
*
D
Flag-ABCB6 (µg)
2
5
BSA (2µg)
100-
75-
50-
37-
25-
20-
15-
10-
E
WT
Flag
Primary Antibody
Flag
ABCB6
Actin
Porin
Flag affinity

Figure 7.1. (continued)

Expression and purification of Abcb6 from total cell fraction.

Total cell fractions were analyzed by SDS-PAGE (4-15%) followed by (A) immunoblotting and (B) Ponceau staining. 50 µg of protein were applied per lane. Ponceau staining was used to confirm uniform loading of samples. Actin and Porin are cellular and mitochondrial proteins serving as controls. (C) Abcb6-FLAG overexpressing cells show increased heme synthesis compared to vector control cells. Values represent mean ± SD. '*' significantly different from vector control cells; $p < 0.01$. (D and E) Solubilization and purification of FLAG-tagged Abcb6 via FLAG-affinity chromatography. (D) 2 and 5 µg of protein eluted from the affinity column was analyzed by SDS-PAGAE (4-15%) followed by Coomassie staining. Bovine Serum Albumin (BSA; 2 µg) used as a loading control for the estimation of protein concentration is shown on the same gel. Affinity purified Abcb6-FLAG protein bands (at 180, 90 and 50 kDa) were identified as Abcb6 by (E) immunoblotting (500 ng-purified protein) using Abcb6-specific antibody and peptide mass fingerprinting. Results representative of three independent experiments.

7.2. Purified mitochondrial Abcb6 binds ATP and displays ATPase activity

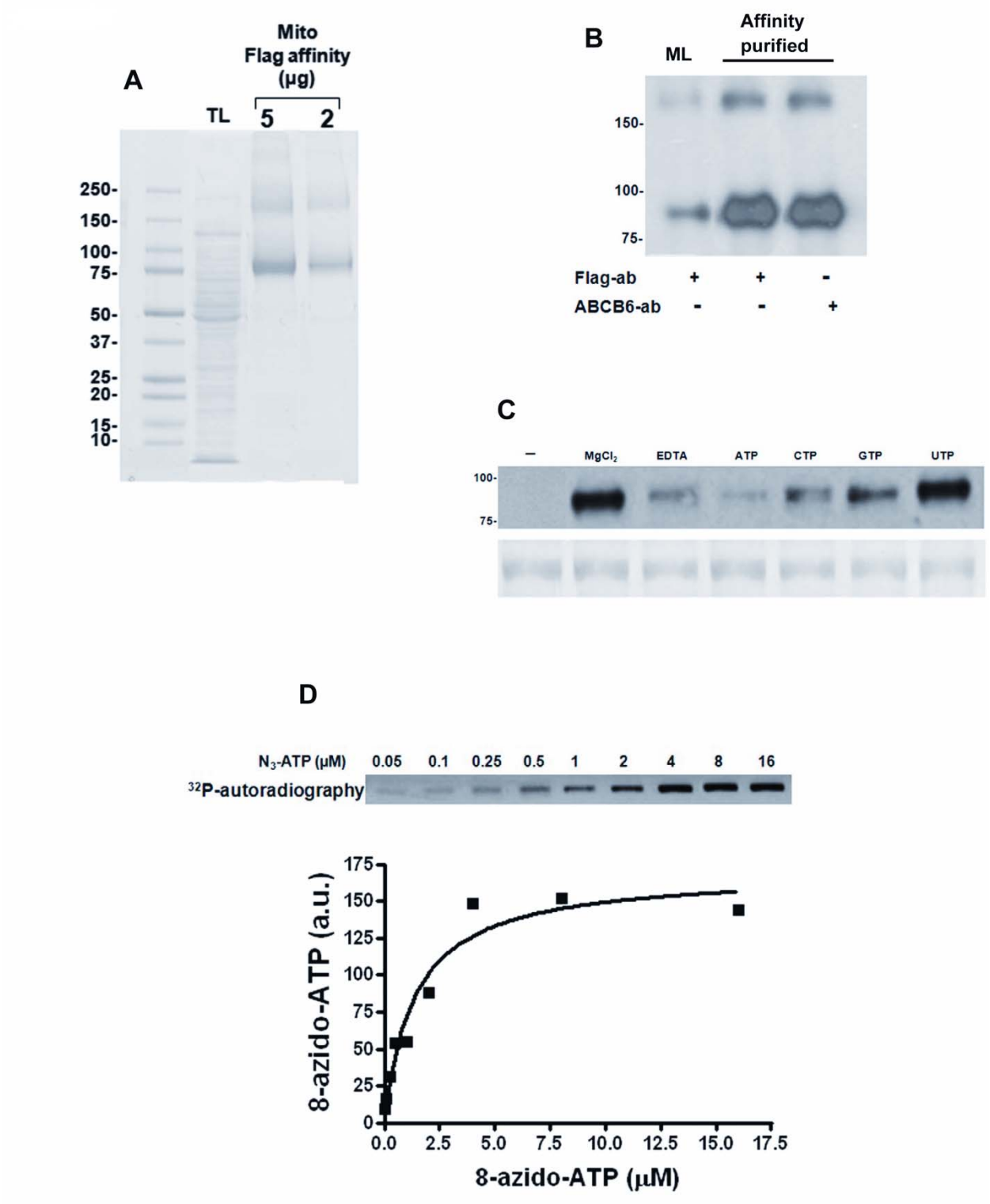
To identify the form of Abcb6 expressed in the mitochondria, differential density gradient centrifugation was used to isolate relatively pure mitochondrial fractions from Abcb6-FLAG overexpressing cells. Proteins in the mitochondrial membrane were solubilized with Triton in the presence or absence of n-Octyl-β-D-glucopyranoside. Detergent solubilized fractions were subjected to anti-FLAG affinity chromatography to separate Abcb6-FLAG from other mitochondrial membrane proteins. Proteins bound to the affinity column were subsequently eluted by treatment with a buffer containing the FLAG peptide. As seen in Figure 7.2A , Abcb6-FLAG protein was highly enriched in the eluted fraction and readily detectable by Coomassie blue staining. In contrast to Abcb6 purified from total cellular fraction, Abcb6 eluted from the mitochondrial preparations revealed only two bands; the Abcb6 monomer band of approximately 90 kDa, and the potential homodimer of approximately 180 kDa (Figure 7.2A). These results are consistent with our previous observations demonstrating a ~90 kDa Abcb6 monomer protein band and a ~180 kDa potential Abcb6 homodimer band seen in mitochondria isolated from Abcb6 overexpressing cells (28,69). Both the 90 and 180 kDa bands contain Abcb6 as indicated by its detection with both Abcb6 specific monoclonal antibody (Figure 7.2B) as well as by MALDI-TOF peptide mass fingerprint analysis.

Transport functions mediated by members of the ABC transporter family require ATP binding and hydrolysis. To examine the ATP binding property of Abcb6, the detergent-solubilized, purified mitochondrial Abcb6 protein was incubated with 8-azido-[α-32P]ATP and the azido photoprobe interacting with Abcb6 was irreversibly cross-liked by irradiation with ultraviolet light. The Abcb6-ATP interacting complex was separated from free probe by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and were subjected to autoradiography for detection of 8-azido-[α-32P]ATP labeling and to Coomassie blue staining

for determination of protein content (Figure 7.2C). A major band of about 90 kDa consistent with the molecular mass of mitochondrial Abcb6 was identified by Coomassie blue staining (Figure 7.2C: lower panel) and found to be photoaffinity labeled by 8-azido-[α-32P]ATP (Figure 7.2C: upper panel). Labeling of Abcb6 by 8-azido-[α-32P]ATP required magnesium and was strongly inhibited by the addition of EDTA (1 mM) or excess cold ATP (10 mM) (Figure 7.2C). Nucleotide specificity of Abcb6 appeared to be in the following order; ATP > CTP > GTP > UTP (inhibition from 85% to 10%; Figure 7.2C). The apparent affinity constant for 8-azido-ATP was determined to be 1.39 ± 0.37 μM (Figure 7.2D). Photoaffinity labeling of Abcb6 by 8-azido-[α-32P]ATP (5 μM) was inhibited by MgATP with an IC_{50} value of 1.03 μM and an estimated Hill coefficient approaching a value of 1 (0.87) (Figure 7.2E). Based on the apparent affinity of 8-azido ATP, a dissociation constant for ATP of 0.18 μM was estimated. Taken together these data indicate that Abcb6 had retained its ability to bind ATP through the purification process and like other ABC transporter proteins has relatively broad nucleotide specificity.

Purified recombinant ABC transporters have been reported to possess intrinsic ATPase activity. To determine the intrinsic ATPase activity of immune affinity purified Abcb6 we used a spectrophotometric assay to measure the amount of Pi released from ATP. The dependence of the rate of hydrolysis on ATP concentration shown in Figure 7.2F exhibited Michaelis-Menten behavior and, when expressed as a Lineweaver-Burk plot (Figure 7.2G), showed a linear relationship, yielding a Km for ATP of approximately 0.99 ± 0.11 mM and Vmax of 492.3 ± 17.25 nmol/mg/min. In parallel experiments the ATPase activity of the ATPase-inactive mutant of Abcb6 (Abcb6-MT) was also analyzed; it was solubilized and purified identically to the wild type protein in similar yields (Figure 7.3) The purified mutant protein showed very little ATPase activity suggesting that the observed Abcb6-WT-ATPase activity is Abcb6 specific (Figure 7.2F). In addition, we found that the purified Abcb6 protein was able to interact with and its ATPase activity was stimulated by its proposed substrate COPIII.

Figure 7.2.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Mito
Flag affinity
(μg)
TL
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2
250-
150-
100-
75-
50-
37-
25-
20-
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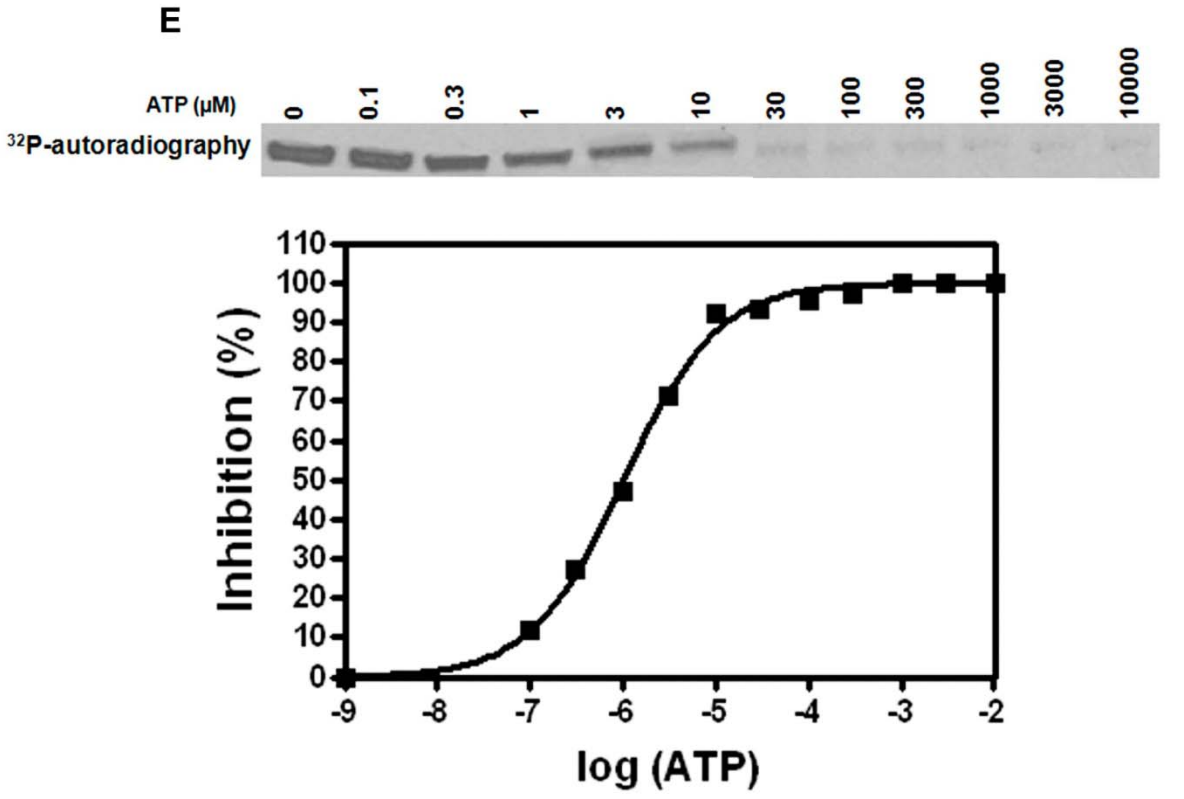
B
ML
Affinity
purified
150-
100-
75-
Flag-ab + + -
ABCB6-ab - - +
C
–
MgCl2
EDTA
ATP
CTP
GTP
UTP
100-
75-
D
N3-ATP (μM) 0.05 0.1 0.25 0.5 1 2 4 8 16
32P-autoradiography
8-azido-ATP (a.u.)
175
150
125
100
75
50
25
0
0.0 2.5 5.0 7.5 10.0 12.5 15.0 17.5
8-azido-ATP (μM)

Figure 7.2. (continued)



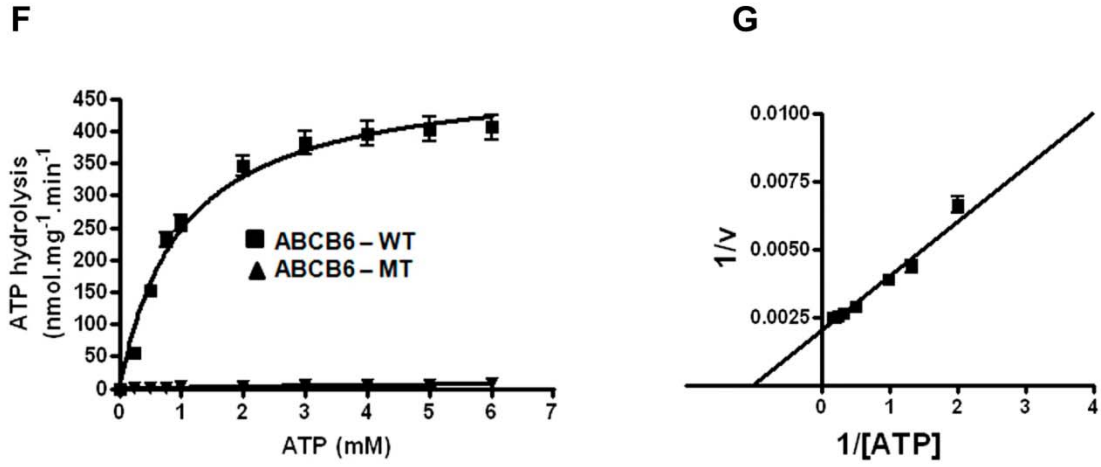


Figure 7.2. (continued)

Purified mitochondrial Abcb6 binds ATP and shows intrinsic ATPase activity.

(A and B) Solubilization and FLAG affinity purification of Abcb6-FLAG from mitochondria. 2 and 5 µg of protein eluted from the affinity column was analyzed by SDS-PAGAE (4-15%) followed by (A) Coomassie staining and (B) immunoblott (500 ng-purified protein) using Abcb6 specific and FLAG-specific antibody. (C – G) ATP binding and ATPase activity of detergent-solubilized FLAG-tagged Abcb6. (C-top panel) Phosphor image analysis of purified Abcb6 (2 µM) labeled with 8-azido-[α-32P]ATP followed by (C-bottom panel) Coomassie blue staining to verify equal protein loading. The effect of EDTA (1mM), ATP (10 mM) and various nucleotides (0.3 mM each) on Abcb6 labeling with 8-azido-[α-32P]ATP is also shown. (D) Binding assay with increasing concentrations of 8-azido-[α-32P]ATP. The photo cross-linking efficiencies were estimated from (D-top panel) phosphor imaging analysis and by (D-bottom panel) quantifying the spots and plotting the intensities against the concentration of 8-azido-[α-32P]ATP. The apparent Kd(azidoATP) value for Abcb6 (1.39 µM) was obtained from the best fit of the binding data to a hyperbolic curve. (E) Competition of 8-azido-[α-32P]ATP binding to Abcb6 (2 µM) by MgATP. (E-top panel) Photo cross-linking was performed with 5 µM of 8-azido-[α-32P]ATP with increasing concentrations of MgATP and (E-bottom panel) the IC_{50} for MgATP was derived by plotting labeling intensities corresponding to Abcb6 as a function of unlabeled MgATP concentrations. The sample without competitor was set to 100%. From the curve, an IC_{50} value of 0.82 µM for Abcb6 was estimated. a.u., arbitrary units. (F and G) ATPase activity of wild type and mutant Abcb6. The ATPase activity of Abcb6 (5 µM) was measured as a function of the ATP concentration at 37°C. Purified Abcb6 is active in ATP hydrolysis. The data were fitted to (F) Michaelis-Menten and (G) Lineweaver-Burk plot resulting in a Km of 0.99 mM and a Vmax of 492.3 nmol/mg/min. The Abcb6-nonfunctional mutant (Abcb6-MT) showed only background ATPase activity. Results representative of three independent experiments. TL: total lysate; ML: mitochondrial lysate; Flag-ab: Flag specific antibody; Abcb6-ab: Abcb6 specific antibody.

7.3. Reconstitution of Abcb6 into lipid membranes

To analyze Abcb6 in its membrane-embedded state, purified mitochondrial Abcb6 (both WT and MT forms) was reconstituted into preformed detergent-destabilized liposomes composed of 0.13 µmol PC; 0.25 µmol PS; 0.67 µmol PE and 0.25 µmol ES. Following reconstitution detergent was dialyzed from the proteoliposomes as described in methods. Efficiency of Abcb6 reconstitution into liposomes was analyzed by discontinuous Nycodenz density gradient centrifugation. As seen in Figure 7.3A reconstitution of Abcb6 into liposomes is evident from the co-migration of Abcb6-FLAG containing liposomes to the top of the Nycodenz gradient. A majority of reconstituted proteoliposomes float into the 5% fraction of the gradient. In contrast, a very small fraction of the purified non-reconstituted protein floats into the 40% fraction, which represents the bottom of the gradient (Figure 7.3A). These data suggest that both the Abcb6-WT-FLAG and Abcb6-MT-FLAG proteins were successfully reconstituted into liposomes with a reconstitution efficiency of ~80%. Integrity of liposome-reconstituted Abcb6 was analyzed by resolving the proteoliposomes obtained from the 5% fraction of the gradient by SDS-PAGE. As seen in Figure 7.3B and C analysis of Abcb6 reconstituted liposomes by Coomassie staining (Figure 7.3B) and immunoblot using Abcb6 specific antibody (Figure 7.3C) demonstrate that Abcb6 was not degraded during the reconstitution process.

The orientation of Abcb6 following insertion into liposomes was determined by the protease protection assay of the C-terminal FLAG-tag in the presence and absence of detergent. The C-terminal FLAG-tag in Abcb6 is accessible to protease-mediated degradation on the outside of the intact vesicles with an ‘NBD-out’ orientation but not with an ‘NBD-in’ orientation. In contrast, in the presence of detergent, the C-terminal FLAG-tag in Abcb6 is accessible to protease degradation irrespective of NBD orientation. Comparison of the protease assay data of intact and detergent-permeabilized vesicles revealed that about 70-80% of Abcb6-FLAG was inserted into liposomes in an “NBD-out” orientation (Figure 7.3D).

Figure 7.3.

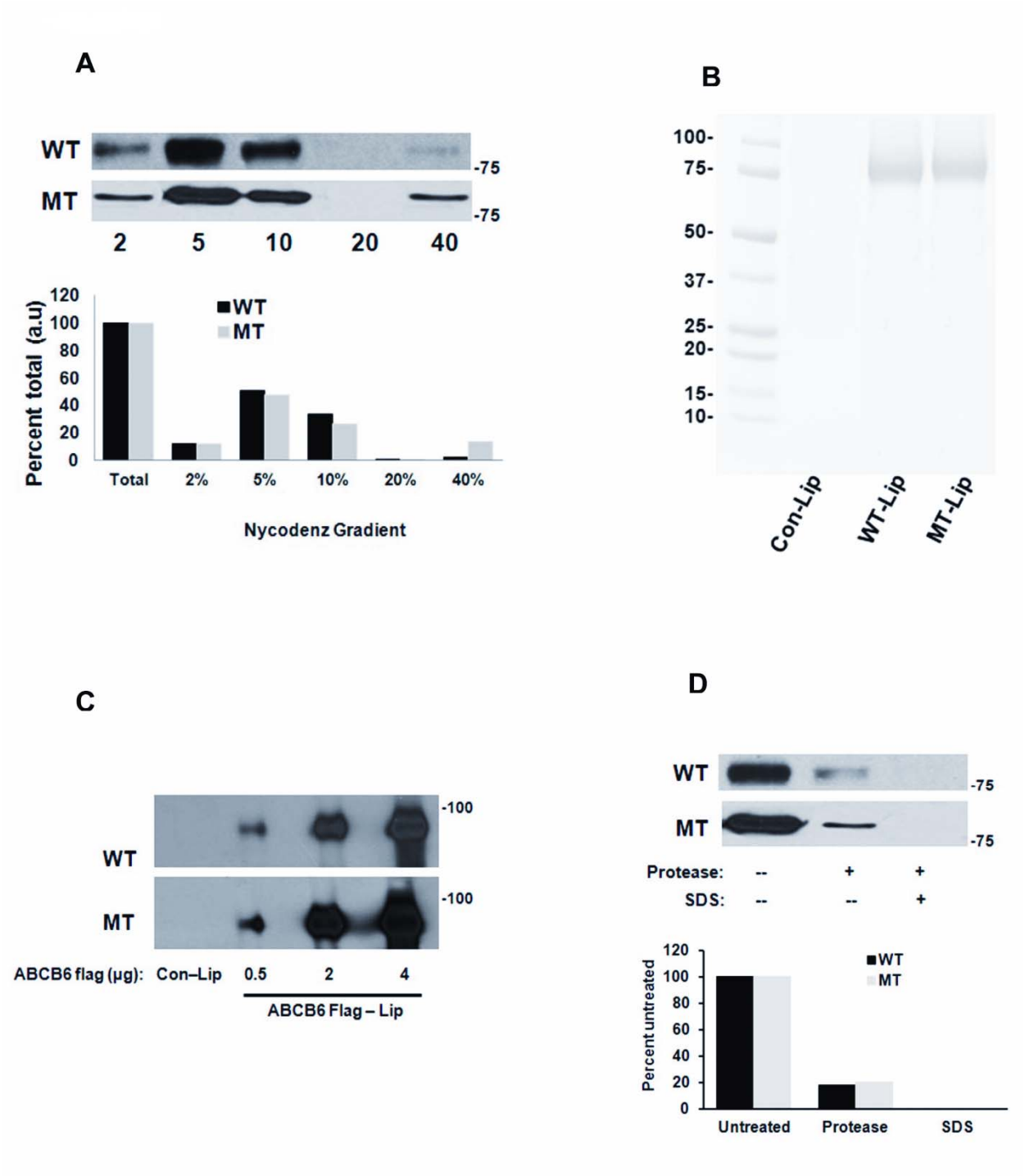

A
WT
MT
-75
-75
2
5
10
20
40
Percent total (a.u)
120
100
80
60
40
20
0
WT
MT
Total
2%
5%
10%
20%
40%
Nycodenz Gradient
B
100-
75-
50-
37-
25-
20-
15-
10-
Con-Lip
WT-Lip
MT-Lip
C
WT
MT
-100
-100
ABCB6 flag (μg):
Con–Lip
0.5
2
4
ABCB6 Flag – Lip
D
WT
MT
-75
-75
Protease:
--
+
+
SDS:
--
--
+
Percent untreated
120
100
80
60
40
20
0
WT
MT
Untreated
Protease
SDS

Figure 7.3. (continued)

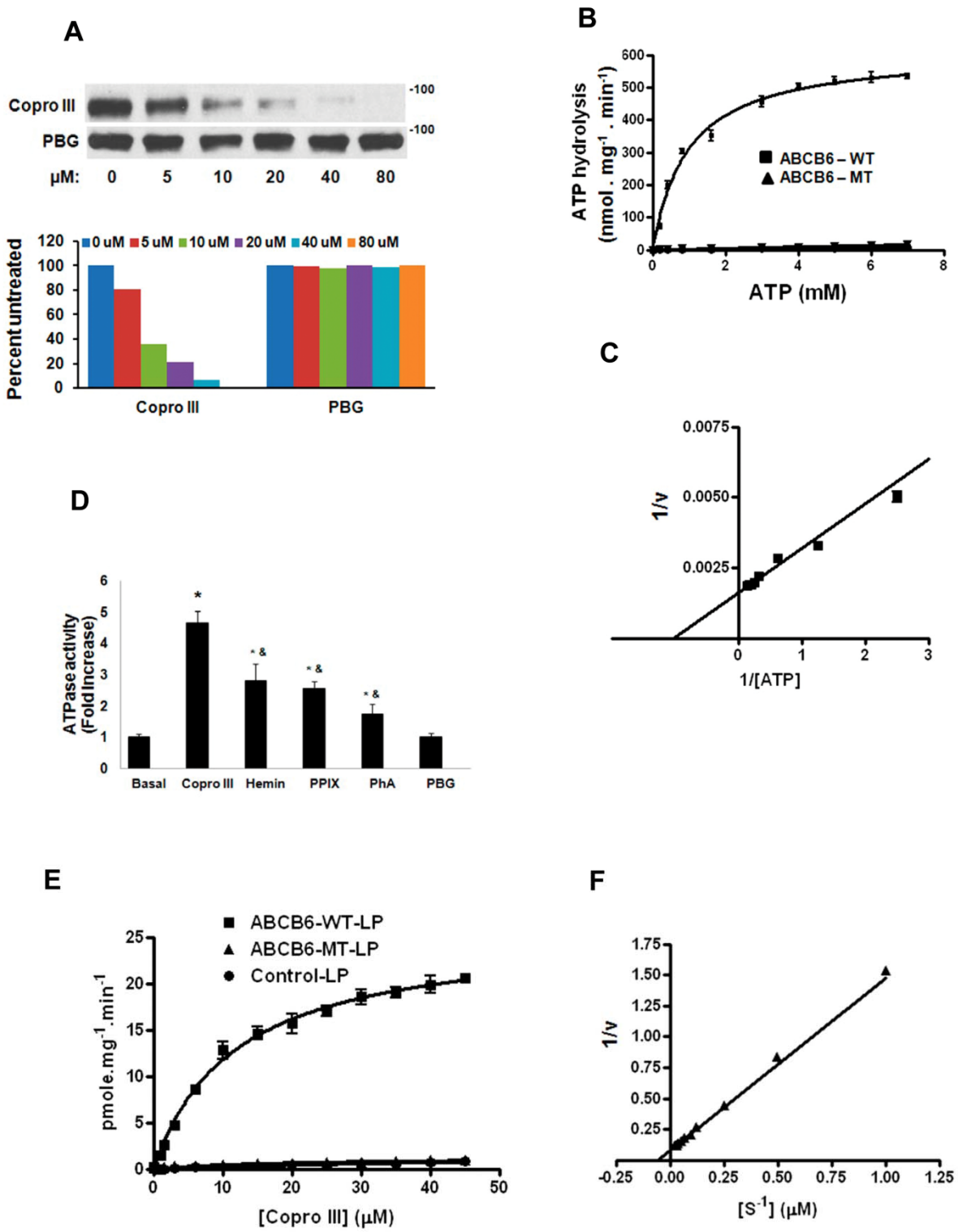
Purified Abcb6 was efficiently reconstituted into liposomes.

Efficiency of Abcb6 reconstitution in liposomes was analyzed by (A) Western blot analysis of a flotation assay of Abcb6-FLAG proteoliposomes (2 µg of each fraction) in a Nycodenz gradient, (B) Coomassie blue staining of equal amounts of proteoliposomes (25 µg of protein) obtained from the 5% fraction of the gradient and (C) Immunoblotting of equal amounts of proteoliposomes obtained from the 5% fraction with Abcb6 specific antibody. Flotation assay shows co-migration of majority of Abcb6 proteoliposomes into the 5% fraction of the gradient while unincorporated protein is found at the bottom of the gradient (40%). Coomassie staining and immunoblotting show comparable efficiency of reconstitution of both Abcb6-wildtype and Abcb6-mutant protein. (D) Membrane orientation of reconstituted Abcb6-FLAG (4 µg protein) was determined by protease protection assay utilizing the FLAG-tag at the C-terminus and orientation of Abcb6 was followed by a FLAG-antibody. SDS was used to permeabilize the proteoliposomes (almost 100% cleavage). Data in all panels are derived from three independent measurements. WT: Abcb6 wildtype (functional) protein; MT: Abcb6 mutant (non-functional) protein; Con-Lip: control liposomes; WT-Lip: Abcb6 wildtype liposomes; MT-Lip: Abcb6 mutant liposomes.

7.4. Liposome reconstituted Abcb6 interacts with its proposed substrate coproporphyrinogen III.

ABC transporters associate with their transport substrates to facilitate its movement across the membrane. Using hemin-agarose affinity chromatography, we have previously demonstrated that Abcb6 associates with COPIII, a potential endogenous transport substrate of Abcb6 (28). To determine if liposome reconstituted Abcb6 retained its ability to associate with COPIII we performed hemin-agarose affinity chromatography as described (28). We found that COPIII was able to displace liposome reconstituted Abcb6 from hemin-agarose in a dose dependent manner (Figure 7.4A). In contrast, porphobilinogen, a molecule that has been previously demonstrated not to interact with Abcb6, does not compete with hemin-agarose for Abcb6 (Figure 7.4A). These results suggest that liposome reconstituted Abcb6 retains its ability to interact with its transport substrates.

Figure 7.4.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Percent untreated
0 uM 5 uM 10 uM 20 uM 40 uM 80 uM
Copro III
PBG
B
ATP hydrolysis (nmol . mg-1 . min-1)
ABCB6 – WT
ABCB6 – MT
ATP (mM)
C
1/v
1/[ATP]
D
ATPase activity (Fold Increase)
Basal
Copro III
Hemin
PPIX
PhA
PBG
E
ABCB6-WT-LP
ABCB6-MT-LP
Control-LP
pmole.mg-1.min-1
[Copro III] (μM)
F
1/v
[S-1] (μM)

Figure 7.4. (continued)

Liposome reconstituted Abcb6 shows substrate stimulated ATPase activity and ATP dependent substrate transport.

(A) Liposome reconstituted Abcb6 interacts with its substrates. (A – top panel) Abcb6 liposomes (4 µg protein) were subjected to hemin affinity chromatography in the presence or absence of either coproporphyrinogen III or porphobilinogen and bound protein was subjected to 4-15% SDS-PAGE followed by western blot using FLAG M2 antibody. (A – bottom panel) Percent binding was estimated by plotting the band intensities corresponding to untreated Abcb6, which was set to 100%. (B - D) Liposome reconstituted Abcb6 shows both basal and substrate stimulated ATPase activity. ATPase activity of Abcb6-WT and Abcb6-MT proteoliposomes (25 - 50 µg protein) was measured as a function of the ATP concentration at 37°C. The data were fitted to (B) Michaelis-Menten and (C) ineweaverBurk plot resulting in a Km of 0.97 ± 0.070 mM and a Vmax of 614.4 ± 11.53 mol/mg/min. The Abcb6-nonfunctional mutant (Abcb6-MT) showed only background ATPase activity. (D) Liposome reconstituted Abcb6 shows substrate stimulated ATPase activity. Fold change was calculated relative to basal ATPase activity. Values represent mean ± SD. '*' significantly different from basal ATPase activity; $P < 0.01$. '&' significantly different from COPIII ATPase activity; $p < 0.01$. (E and F) Effect of substrate concentration on ATP dependent COPIII uptake by Abcb6 liposomes (25 – 50 µg protein). Kinetic parameters were determined by (E) fitting the data to Michaelis-Menten and (F) linear regression analysis of the Lineweaver-Burk transformation of the data points. Transport kinetics of liposome reconstituted Abcb6 showed a Km of 11.97 µM and Vmax of 29.6 pmol/mg/min. Copro III: Coproporphyrinogen III; PBG: Porphobilinogen; PPIX: Protoporphyrin IX; PhA: Pheophorbide A.

7.5. ATPase activity of reconstituted Abcb6 is stimulated by coproporphyrinogen III (COPIII)

To determine the ATPase activity of liposome reconstituted Abcb6 the release of inorganic phosphate from ATP was assayed as described for the purified protein. As shown in Figure 4B lipid reconstituted Abcb6 demonstrated basal ATPase activity that was comparable to the ATPase activity of the purified detergent solubilized protein (Figure 7.2F). The basal ATPase activity was specific to Abcb6 because no such activity was observed either in the presence of control liposomes (data not shown) or in the presence of liposomes carrying an ATPase inactive mutant of Abcb6 (Abcb6-MT) (Figure 7.4B). Further, the basal activity was reduced to 98% by ortho-vanadate that inhibits ABC transporters and P-type ATPases, indicating that no additional ATPases are present in the Abcb6 proteoliposome preparations (data not shown). The dependence of the rate of hydrolysis on ATP concentration shown in Figure 7.4B exhibited Michaelis-Menten behavior and, when expressed as a Lineweaver-Burk plot (Figure 7.4C), showed a linear relationship, yielding a Km for ATP of approximately 0.97 ± 0.07 mM and Vmax of 614 ± 11.53 nmol/mg/min.

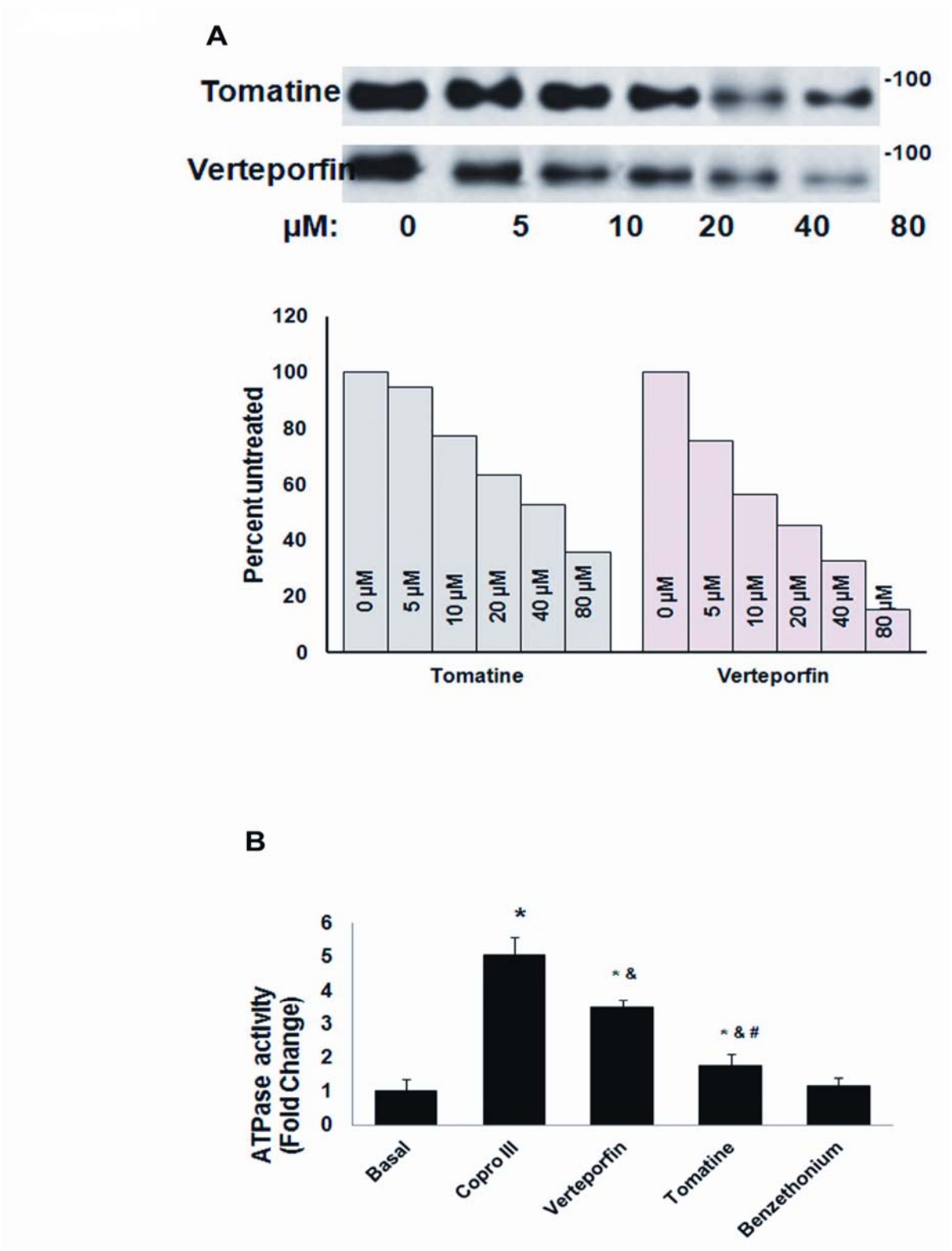
Stimulation of ATPase activity by transport substrates is a feature that is common to most ABC transporters. Consequently, it was of interest to determine whether known Abcb6 substrates affected its ATPase activity in a similar manner. As shown in Figure 7.4D ATPase activity of liposome reconstituted Abcb6 was strongly stimulated in the presence of COPIII, hemin and protoporphyrin IX (COPIII > Hemin > PPIX) but not in the presence of PBG. This multifold increase in Abcb6 ATPase activity by COPIII, hemin and PPIX suggests that these compounds are potential transport substrates of Abcb6. These results are in agreement with our earlier observations demonstrating that porphyrins such as COPIII and hemin are potential substrates of Abcb6 (28).

7.6. Coproporphyrinogen III transport kinetics of reconstituted Abcb6

Abcb6 is proposed to be involved in the transport of COPIII from the cytoplasm into the mitochondria. However, a direct role for Abcb6 in COPIII transport has not been demonstrated in the absence of other potential interacting components (mitochondrial preparations). Thus, kinetic parameters of COPIII transport mediated by Abcb6 were carried out with the Abcb6 proteoliposomes. According to the Lineweaver-Burk analysis, the apparent Km for COPIII was 11.97 ± 1.10 μM with a Vmax of 25.79 ± 0.81 pmole/mg/min (Figure 7.4E and F). To confirm these observations further COPIII uptake studies were performed in control liposomes and in liposomes reconstituted with either a transport competent Abcb6 (Abcb6-WT) or the transport incompetent Abcb6 (Abcb6-MT) protein. As seen in Figure 7.4E COPIII uptake in Abcb6-MT proteoliposomes were very low and reflected transport rates similar to what was seen in control liposomes (Figure 7.4E).

We recently developed a HTS assay to identify potential substrates and inhibitors of Abcb6 (69). Two of the compounds identified in the HTS assay, tomatine and verteporfin were transported into mitochondria in an Abcb6 dependent manner but a third compound benzethonium was not transported by Abcb6 (69). We used the transport features of these three molecules to test and validate the robustness of liposome reconstituted Abcb6 as a potential model to identify Abcb6 transport substrates. As with COPIII, both tomatine and verteporfin competed with hemin binding to Abcb6 (Figure 7.5A), stimulated Abcb6 ATPase activity (Figure 7.5B) and were transported into liposomes by Abcb6 (Figure 7.5C and D) with varying transport kinetics (verteporfin Vmax 5.13 ± 0.23 pmol/mg/min; tomatine Vmax 2.7 ± 0.19 pmol/mg/min). However, unlike verteporfin and tomatine, benzethonium did not stimulate Abcb6 ATPase activity (Figure 7.5B) nor was it transported by Abcb6 proteoliposomes (data not shown). These results are consistent with the transport properties of Abcb6 observed using mitochondrial fractions isolated from Abcb6 overexpressing cells (69).

Figure 7.5.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Verteporfin
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Percent untreated
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100
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0 μM
5 μM
10 μM
20 μM
40 μM
80 μM
Tomatine
Verteporfin
B
ATPase activity (Fold Change)
6
5
4
3
2
1
0
*
* &
* & #
Basal
Copro III
Verteporfin
Tomatine
Benzethonium

Figure 7.5. (Continued)

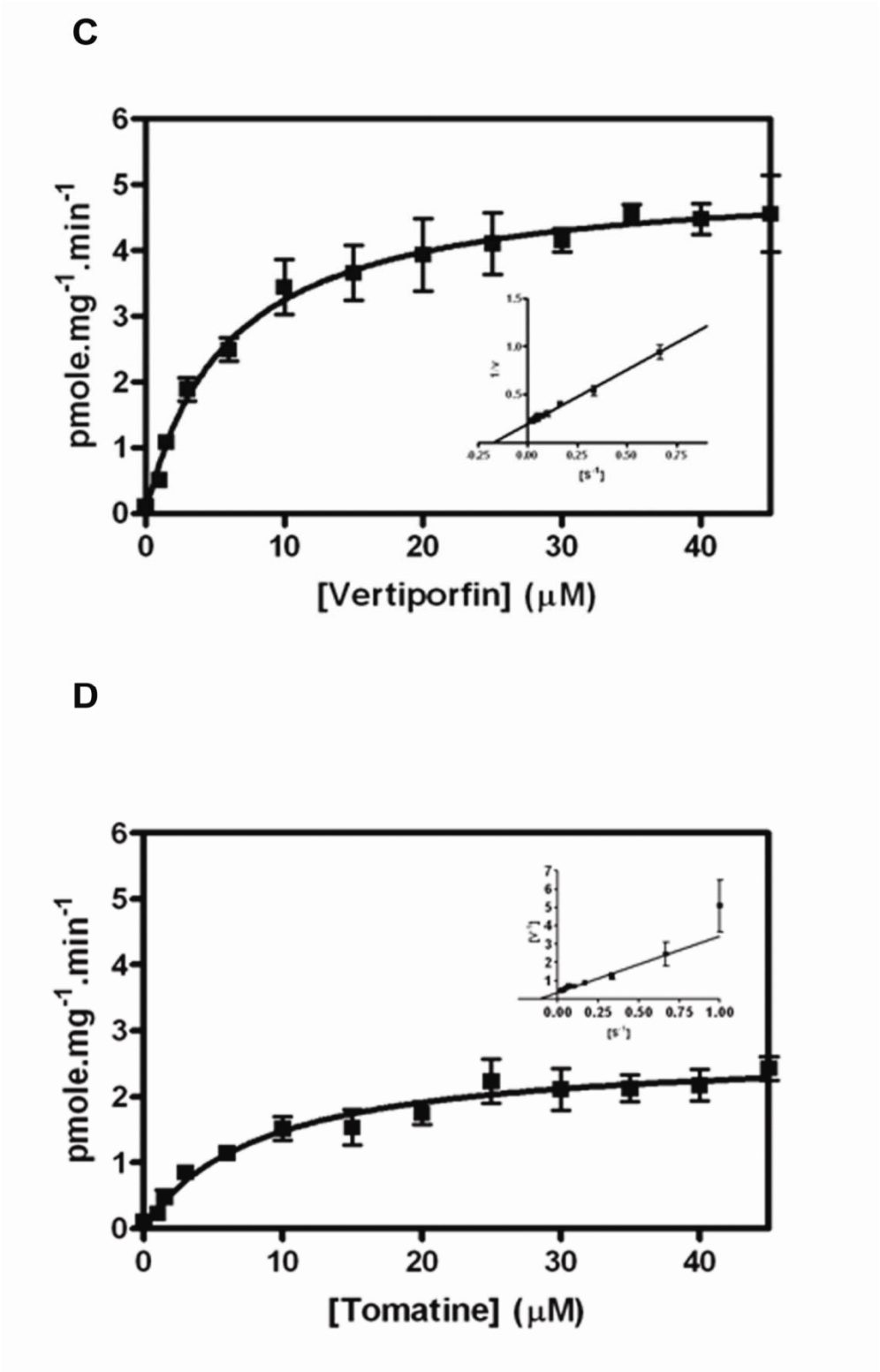

Figure 7.5. (Continued)

Liposome reconstituted Abcb6 shows ATP dependent transport of molecules identified in a HTS assay.

(A) Liposome reconstituted Abcb6 interacts with both tomatine and verteporfin. (A – Top panel) Abcb6 liposomes (4 µg protein) were subjected to hemin affinity chromatography in the presence or absence of molecules identified in the HTS assay and bound protein was subjected to 4-15% SDS-PAGE followed by western blot using FLAG M2 antibody. (A – Bottom panel) percent binding was estimated by plotting the band intensities corresponding to untreated Abcb6 which was set to 100%. (B) Liposome reconstituted Abcb6 shows increased ATPase activity in the presence of molecules identified in the HTS assay. Values represent mean ± SD. '*' significantly different from basal ATPase activity; $P < 0.01$. '&' significantly different from Copro III ATPase activity; $p < 0.05$. '#' significantly different from vertiporfin ATPase activity; $p < 0.01$. (C and D) Liposome reconstituted Abcb6 shows ATP dependent transport of (C) verteporfin and (D) tomatine. Kinetic parameters were determined by linear regression analysis of the Lineweaver-Burk transformation of the data points.

Discussion

Mitochondrial ABC transporters are difficult to study because of the two-membrane architecture of mitochondria, problems associated with analyzing transport process, and the high abundance of other ATPases and carriers/transporters. Thus the development of an in vitro system with pure and active protein, is a necessary prerequisite toward understanding the mechanistic relationships between ATP binding and hydrolysis and coupling of these events to translocation of substrates across the lipid membranes. In this study, we describe for the first time a successful purification protocol and biochemical characterization of the putative mitochondrial porphyrin transporter Abcb6. The purification procedure comprised of lentiviral-mediated overexpression of Abcb6 carrying a C-terminal FLAG tag followed by a single step solubilization of Abcb6 from mitochondrial membranes and immunoaffinity purification using the FLAG antibody. Purified Abcb6 was found to be mostly monodisperse by PAGE electrophoresis and was efficiently reconstituted into liposomes allowing its biochemical characterization with respect to ATP binding, ATP hydrolysis and transport kinetics.

A prerequisite for the purification of integral membrane proteins is the dissolution of the biological membrane in which the protein resides so that the membrane protein exists in solution in a monodisperse state while maintaining its structure in a physiological relevant condition. The success of a purification strategy is thus largely dependent on the type of detergent used, for both initial solubilization and the subsequent steps of column chromatography. The choice of detergent (Triton and n-Octyl-β-D-glucopyranoside) used for solubilization and purification of Abcb6 appeared acceptable as reasonable amounts of Abcb6 were extracted from the membrane, with preserved oligomeric state and activity of the proteins. After purification by immunoaffinity chromatography, Abcb6 bound MgATP with a relatively high affinity (Kd = 0.18 μM), and showed an ATPase activity of 0.493 μmol/mg/min. The observed

activity is similar to that reported for ABC transporters such as MRP1 (0.46 μmol/min/mg; (251) but is relatively high compared to ABC transporters e.g. MRP3 (0.075 μmol/mg/min; (252) and MsbA (0.15 μmol/mg/min; (253). Interestingly Abcb6 ATPase activity, is lower than activities seen for other mitochondrial ABC proteins such as Mdl1 [2.3 μmol/mg/min; (254)] and Atm1 [1.9 μmol/min/mg; (255)]. The relatively high ATPase activity indicates that immunoaffinity purified Abcb6 is in an active state. This conclusion is further supported by the observation that solubilized and reconstituted Abcb6 have comparable ATPase activities. Further, given that the apparent affinity for MgATP is 700 times higher than the Michaelis-Menten constant for ATP hydrolysis (Km = 0.99 mM) this implies that ATP binding and ATP hydrolysis are potentially distinct steps.

There is increasing evidence that Abcb6 localizes to multiple cellular compartments including the plasma membrane (204,256-258). However, the precise identity, substrate specificity (if any), and functional significance of the differentially localized Abcb6 is not well defined. In our studies, although the principal form of Abcb6 purified from total cell fractions was a ~90 kDa protein, a second band of ~50 kDa was also purified following affinity chromatography. Both the purified bands contain Abcb6 as confirmed by western blot and MALDI-TOF analysis. The ~90 kDa band represents the mature form of the protein, which has been previously described as localizing to the mitochondria (28,249). Interestingly however, the 50 kDa band was not seen in the Abcb6 purified from mitochondrial fractions suggesting that the ~50 kDa form of Abcb6 might localize to cellular compartments other than the mitochondria. The ability of our experimental protocol to purify sufficient quantities of these two forms of Abcb6 should in the future enable us to characterize their localization, identity and potential functional significance.

It is well established that the lipid environment in which a membrane protein is reconstituted can affect its reconstitution and interaction with its substrates thus modulating its

activity (259). For example, it has been shown that MRP3, which is expressed in both the liver and the brain, exhibits efficient reconstitution and highest ATPase activity when reconstituted in liver lipids but not in brain lipids (252). Similarly, certain lipids significantly influence the characteristics of purified Pgp ATPase activity and the apparent coupling between its drug-binding and catalytic sites (260,261). In contrast, the ATPase activity of human ABCA4 could not be stimulated by its substrate, all-trans-retinal, when the protein was reconstituted in vesicles composed of single synthetic lipids (262). Thus, overall, there appears to be considerable variability among the ABC transporters with respect to their response to their lipid environment.

Results from the present study suggest that for Abcb6 a combination of various phospholipids constitutes an optimal environment for substrate binding and stimulation of basal ATPase activity. However, whether these values are true representations of the ATPase activity of native Abcb6 requires further investigation. The fact that the basal ATPase activity of lipid reconstituted Abcb6 was relatively higher than that observed for the purified protein suggests that the lipid environment may play a role in influencing the ATPase activity of native Abcb6. In this context, and given that Abcb6 is ubiquitously expressed, native Abcb6 in different organ systems or in different cellular compartments might exhibit varied ATPase activity and potentially different substrate specificities. We speculate that these suppositions could provide the basis to explain some of the Abcb6 loss of function phenotypes described in the literature (115-117,132,263,264).

Many ABC transporters have been shown to possess intrinsic ATPase activity that is stimulated in the presence of transported substrates. The best-investigated example is the mammalian Mdr1, which possesses an ATPase activity stimulated by various drugs that are known to be transported (265,266). The ATPase activity of lipid reconstituted Abcb6 was stimulated by porphyrins known from previous studies to be potential substrates for this

transport protein (28,29). COPIII has the highest affinity for Abcb6 and this compound stimulated ATPase activity by approximately five fold above basal activity. The lack of substrate stimulated ATPase activity in control liposomes and in liposomes reconstituted with a non-functional mutant of Abcb6 indicates that the observed ATPase activity is specific to Abcb6. In addition to COPIII significant induction in ATPase activity was also observed with verteporfin a compound which we recently identified as a potential substrate of Abcb6, by a HTS assay that was developed to identify functional modulators of Abcb6 (69). In contrast, tomatine, which was also identified as a potential transport substrate of Abcb6, was not as efficient as verteporfin in stimulating Abcb6 ATPase activity. The observation that porphobilinogen, which lacks a completed ring structure is unable to stimulate ATPase activity, leads to the speculation that transport substrates of mitochondrial Abcb6 might require a ring structure.

The results presented here demonstrate for the first time transport of COPIII by purified Abcb6 in an artificial lipid bilayer system. The relatively high hydrophilicity and the inherent endogenous fluorescence capacity of COPIII facilitated analysis of its transport kinetics in this system. The lack of transport in control liposomes and in liposomes reconstituted with a non-functional mutant of Abcb6 confirmed that the ATP-dependent uptake of this substrate by Abcb6 proteoliposomes involved transport into the vesicle lumen. This conclusion is further supported by our findings that the Km values for COPIII were comparable with those reported previously in mitochondrial transport studies (29). The specific stimulation of Abcb6 ATPase activity by COPIII and the efficient transport of this molecule into proteoliposomes suggest that this molecule might be the primary substrate for mitochondrial Abcb6, and transporting across the mitochondrial membrane could be the physiological function of Abcb6. Collectively these data provide strong support for the conclusion that mitochondrial Abcb6 acts as an energy-driven pump that appears not to require additional components for its basal and substrate stimulated ATPase activity and substrate transport.

In summary, we have purified Abcb6 to homogeneity and reconstituted it into proteoliposomes. The reconstituted Abcb6 demonstrates both substrate stimulated ATPase activity and ATP dependent translocation of candidate transport substrates across the lipid membrane. These studies represent the first characterization of the ATPase activity and ATP dependent transport kinetics of purified Abcb6 in the absence of other potential interacting components. Abcb6 is increasingly being recognized as a relevant physiological and therapeutic target (115-117,128,186,263,264). Abcb6 expression is upregulated in many tumor cells with acquired chemotherapeutic resistance (132,187,234,235,267-269). However, the mechanism by which Abcb6 provides therapy resistance is not known. In this regard, reconstitution of purified Abcb6 into proteoliposomes in the absence of contaminating factors might facilitate mechanistic studies of ATP dependent Abcb6 mediated transport of therapy drugs. In addition, the simple immunoaffinity purification of Abcb6 to near homogeneity might provide the basis for future studies on the structure, function of Abcb6, and potentially help characterize the identity, localization and functional significance of the smaller isoform of Abcb6.

Chapter 8: **CONCLUSIONS AND FUTURE DIRECTIONS**

8.1. Summary and conclusions

The present dissertation provides a comprehensive analysis of the physiological function, regulation of expression and clinical significance of the mitochondrial ABC transporter Abcb6. Initial characterization of Abcb6 proposed a role for this protein in heme synthesis. Consistent with this observation Abcb6 expression is induced in response to signals that promote heme synthesis. Under conditions of induced heme synthesis Abcb6 expression is regulated by the nuclear hormone receptors AhR, PXR and CAR which mediate transcriptional induction of Abcb6. Studies using loss of function and gain of function mutations in Abcb6 helped define the clinical role of this transporter in porphyria and toxicities associated with oxidative stress. These same studies also provided the first examination of a heretofore-unknown role for Abcb6 in drug metabolism, and cholesterol and steroid homeostasis. Finally the dissertation describes the simple immunoaffinity purification of Abcb6 to near homogeneity and its reconstitution into liposomes, which should provide the basis for future studies on the structure, function of Abcb6, and potentially help characterize the substrates for this mitochondrial protein.

Chapter 4 comprehensively presents the regulation of Abcb6 by xenosensors (nuclear receptors AhR, CAR and PXR) and arsenic. Using specific ligands for nuclear receptor AhR (3-MC, B[a]P and TCDD), CAR (TCPOBOP) and PXR (PCN) we demonstrated that Abcb6 expression is up-regulated in mice after ligand exposure. Moreover*, in vivo* studies in AhR-null, CAR-null and PXR-null mice demonstrated that lignad mediated induction of Abcb6 is regulated by AhR, CAR and PXR, respectively. We also show that sodium arsenite induces Abcb6 expression in a dose-dependent and time-dependent manner both in mice and in hepatoma cells. Using Nrf2 null mice we were able to show that arsenite-induced Abcb6 expression is not mediated by the redox-sensitive transcription factor Nrf2. Further, using promoter activation studies in association with EMSA and ChIP assay, we demonstrated direct interactions of AhR

protein with the AhR binding sites in the Abcb6 promoter. These studies are the first to describe direct transcriptional activation of Abcb6 by xenobiotics and also implicated drug activation mechanisms for Abcb6 similar to those found in inducible P450s.

Chapter 5 demonstrates the role of Abcb6 in PAH mediated porphyria and also provides evidence of a protective role for Abcb6 in arsenic toxicity. Using Abcb6 knockdown hepatoma cells (Huh7 and HepG2) and primary hepatocytes from Abcb6 knock-out mice, we demonstrate that loss of Abcb6 expression does not alter porphyrin levels in the absence of B[a]P; however, loss of Abcb6 expression significantly compromised B[a]P-mediated induction of hepatic porphyrin levels. These results suggest that under normal physiological conditions, the absence of Abcb6 expression and the ensuing decrease in porphyrin biosynthesis is compensated by a complementary increase in mechanisms that regulate porphyrin synthesis. But when stressed with increased functional demand for heme, the compensatory mechanisms might not be sufficient to complement the loss of Abcb6 expression. These observations support recent findings by *Ulrich et al.* (29) demonstrating that in the absence of Abcb6, compensatory mechanisms are activated to compensate for the loss of Abcb6. However, when stressed, loss of Abcb6 compromises cellular heme biosynthesis and function.

Direct correlation between arsenic resistance and Abcb6 expression was suggested in various human and mouse cell lines (203,204). In *Chapter 5* we demonstrated that, in Hepatoma cells (HepG2 and Hep3B), knockdown of Abcb6 expression using Abcb6-specific siRNA sensitized the cells to arsenite toxicity. In contrast, stable overexpression of Abcb6 conferred a strong survival advantage by decreasing arsenite-induced oxidative stress. The protection against arsenic toxicity in Abcb6 overexpressors is in part mediated by its ability to potentiate catalase activity as treatment of cells with the catalase inhibitor 3-AT blocked Abcb6-mediated activation of catalase but had only a modest effect on the survival of cells exposed to sodium arsenite.

Collectively, results from *Chapter 5* suggest a role for Abcb6 in PAH mediated porphyria and also suggests that Abcb6 expression might be an endogenous protective mechanism activated to protect cells against arsenite-induced oxidative stress.

To investigate the role of Abcb6 *in vivo* we generated Abcb6 knockout mice on C57BL6/N background. *Chapter 6* describes generation of Abcb6 knockout mice and its initial characterization. The whole body knockout is viable and has sporadic stunted growth with the Mendelian inheritance showing 11% decrease in knockout number (observed 11% compared to 25%) than expected. Hepatic heme levels show a decreasing trend that correlates well with the number of Abcb6 alleles in the mouse genome. A global gene expression analysis using Affymetrix microarray demonstrates significant changes in gene expression in the liver of Abcb6 null mice compared to wild type controls. Mapping the gene expression changes in the knockout mice using the Ingenuity pathway analysis demonstrate that the highest number of genes altered are related to one of the following pathways; a) drug metabolism; b) growth and survival; c) cholesterol and lipid homeostasis; and d) development. Consistent with this observation Abcb6 knockout mice show decreased metabolism of the classic Cyp substrate pentobarbital, sporadic growth defects, decreased serum cholesterol after fasting and increase in androgens. These results suggest a heretofore-unknown role for Abcb6 in growth and development and in lipid and cholesterol homeostasis.

Chapter 7 of this dissertation describes establishment of a liposomal vesicular transport assay for purified Abcb6 protein. This assay provides an advantage over cell based assay in identifying and characterizing Abcb6 transport substrates. In this study, FLAG tagged Abcb6 was overexpressed in HEK293 cells and purified to homogeneity from solubilized mitochondrial membranes by FLAG-immunoaffinity purification. Purified Abcb6 protein was then reconstituted into liposomes and the liposome reconstituted Abcb6 was characterized for the ATPase activity including (i) substrate stimulated ATPase activity (ii) transport kinetics of its proposed

endogenous substrate coproporphyrinogen III and (iii) transport kinetics of substrates identified using a High-throughput screening (HTS) assay. Mutagenesis of the conserved lysine to alanine (K629A) in the Walker A motif of Abcb6 abolished ATP hydrolysis and substrate transport. In conclusion, results from *Chapter 7* suggest a direct interaction between mitochondrial Abcb6 and its transport substrates that is critical for the activity of the transporter. Efficient reconstitution of Abcb6 into liposomes might provide the basis for future studies on the structure, function of Abcb6.

Collectively, using a combination of tissue culture, mouse models and cell free systems, this dissertation explored the role of Abcb6 protein in arsenic toxicity, porphyria and drug metabolism. It also examined regulation of Abcb6 by xenobiotics and environmental toxicants. The findings from this dissertation suggest protective role of Abcb6 in arsenic toxicity and porphyria. It also provides valuable information on the role of Abcb6 in drug and xenobiotic metabolism and the effect of xenobiotics on regulation of Abcb6 expression. Finally the reagents developed during the course of these studies could continue to provide powerful tools in the future studies evaluating Abcb6's physiological and pathological significance.

8.2. Future directions

The results presented in this dissertation provide a foundation upon which the role of Abcb6 in normal physiology and disease pathology could be explored further. The results support the public health relevance of studying Abcb6 and in combination with the recent clinical findings suggests that Abcb6 might be a potential therapeutic target during development, oxidative stress response and metabolic disorders.

Potential future studies from results presented in Chapter 4:

Our future studies will focus on further understanding the regulation of Abcb6. Our results demonstrated transcriptional activation of Abcb6 by sodium arsenite so future studies will focus on understanding the mechanism of sodium arsenite–mediated activation of Abcb6. In addition to Nrf2, arsenic exposure has been shown to activate several transcription factors including p53, Sp1, AP-1, and NF-kβ (215-220). Preliminary analysis of the human and mouse Abcb6/Abcb6 promoter using a pattern search program (nubiscan) reveals several putative *cis*-elements that may be capable of binding AP-1, NF-kβ, and p53. Future studies will address the role of these transcription factors in the activation of Abcb6.

In silico analysis of Abcb6 promoter also identified potential *cis*-elements in the Abcb6 promoter region that may be capable of binding nuclear receptor PXR and CAR. Involvement of these elements will be investigated for Abcb6's induction through PXR and CAR. Current studies for PXR and CAR mediated activation of Abcb6 do not address the question of species differences so further investigation will be done using human primary hepatocytes and human hepatoma cells.

PAH mediated activation of Abcb6 has shown through binding of AhR to AhR response element in Abcb6 promoter but it is interesting to note that the AhRE sequence 5-GCGTG-3 is

similar to the HIF-1 response element sequence (consensus 5-RCGTG-3), suggesting a potential role of HIF and hypoxia in regulating Abcb6 expression. So future experiments will investigate role of HIF and hypoxia in regulating Abcb6 expression and significance of Abcb6 expression in hypoxic induction, if any. Another, interesting aspect of AhR mediated regulation of Abcb6 is mutation of the AhRE element at 115 bp (Abcb6-M2) showed an increase in promoter activation in response to B[a]P which was significantly higher than the unmutated native sequence. This observation suggests the potential existence of repressor motifs in close proximity to the AhRE motif at115 bp which needs to be further investigated. From the EMSA results (Figure 4.1.6C) it is interesting to note that treatment with B[a]P led to additional DNA-protein complex formation in the Abcb6 promoter. However, this interaction appeared to be independent of AhR interaction with the Abcb6 promoter. At present, it is not clear what these interacting proteins are and what if any is the significance of these interactions to Abcb6 expression and function. Future studies will try to identify these interaction and effect of those proteins to Abcb6 expression and function.

Potential future studies from results presented in Chapter 5:

The results from our arsenic exposure studies suggest the possibility that Abcb6 expression is induced in response to short-term low-dose exposure to arsenic and that this increased expression could protect against short-term acute arsenic toxicity. However, human diseases are associated with chronic arsenic exposure at moderate dose of arsenic. So, future studies need to address the effect of long-term moderate level arsenic exposure on Abcb6 expression and effect of Abcb6 expression to prolonged protection against arsenic toxicity using Abcb6-null mice. Another interesting observation from Arsenic protection study is that Abcb6 overexpressor has decreased oxidative stress at basal level and Abcb6 protection against sodium arsenite is mediated partially by its ability to potentiate catalase activity. Thus, future

studies will focus on mechanism(s) by which Abcb6 protects against arsenite toxicity and role of Abcb6 in maintenance of cellular oxidative stress.

Abcb6 was speculated as a multidrug resistance gene because of its elevated mRNA expression in drug-resistant cell lines (adriamycin, camptothecin, paclitaxel, and 5-fluorouracil–resistant tumor cell lines) (203,233,234). Based on these observations, it was suggested that the multidrug resistance phenotype could be attributed to Abcb6's ability to function as a transporter to reduce intracellular concentrations of the respective drug compounds. It is quite possible that a similar transport mechanism mediated by Abcb6 for inorganic arsenic or its metabolite could reduce cellular arsenic concentrations. Supporting the hypothesis of Abcb6 as a efflux transporter, cells engineered to overexpress Abcb6 demonstrate plasma membrane localization of Abcb6 (204). However, in our studies reported here, we were not able to detect endogenous Abcb6 expression at the plasma membrane. It is quite possible that the analytical techniques used were not sensitive enough to detect endogenous Abcb6 expression at the plasma membrane. Thus, future studies will evaluate endogenous expression of Abcb6 at the plasma membrane and its potential contribution to drug resistance and arsenic toxicity.

Potential future studies from results presented in Chapter 6:

Availability of Abcb6 knockout mice will increase our understanding of physiological function of Abcb6. Role of Abcb6 in transport of COPIII from *in vitro* studies was confirmed using Abcb6 knockout mice but its proposed effect on heme biosynthesis was not observed. In our preliminary observation there is no significant change in heme levels at basal in Abcb6 knockout mice suggesting activation of compensatory mechanism in Abcb6 knockout mice. Therefore, our future studies will try to understand the compensatory mechanism activated using gene array for mitochondrial transporters and carrier proteins. In addition, there is possibility of alternate pathway for heme biosynthesis bypassing need for COPIII transport by

Abcb6. Indeed, evidence of an alternative pathway via harderoporphyrinogen to protoporphyrinogen-IX and heme has been reported previously. Our future studies will explore potential possibility of increased alternate heme biosynthesis pathway in *Abcb6*$^{-/-}$ mice.

Our microarray data provided insight into biological and canonical pathways which are altered in Abcb6 knockout mice. Interestingly, organismal death pathway was increased the most suggesting susceptibility of Abcb6 knockout mice. In this context, it will be interesting to see the effect of various toxicants in Abcb6 knockout mice, starting with Arsenic against which Abcb6 is shown to protect hepatoma cells *in vitro*. Another interesting finding from the array is alteration in genes related to cholesterol homeostasis. So our future studies will focus on understanding role of Abcb6 in cholesterol homeostasis and also focus on disease related to cholesterol homeostasis such as heart disease, obesity and liver diseases. It will be interesting to examine the differences in steroids if any in Abcb6 knockout mice. Our future studies will focus on elucidating Abcb6's effect on steroids using metabolomics approach, we expect this will provide novel role of Abcb6 in gene expression regulation as several of steroid molecules function as endogenous ligands and signal for gene expression regulation (e.g. P450s).

Our preliminary data for drug metabolism enzymes P450s suggests a role for Abcb6 in regulation of P450s metabolism. So our future studies will illustrate effect of Abcb6 on P450 function and will also focus on regulation of P450 isomers. Our current results suggest decreased pentobarbital metabolism, we will further examine effect of Abcb6 on metabolism of some over the counter drugs and its adverse effect, if any.

Potential future studies from results presented in Chapter 7:

We have purified Abcb6 protein to homogeneity and the protein is active in the solubilized state. Our future studies will outsource this protein for structural analysis which will

provide insight into mechanism by which Abcb6 interacts with its substrates and the dynamics of transport. This will provide a unique opportunity for drug discovery to predict Abcb6 interacting molecules *in silico*. An *in vitro* liposomal assay developed in Chapter 7 will be used in future for confirming substrates of Abcb6 screened from HTS assay or *in silico* predictions.

In summary, future studies will explore the role of Abcb6 protein in arsenic resistance, porphyria and drug metabolism *in vivo* using the Abcb6 null mouse that we have developed. We will also explore the mechanism of Abcb6 regulation mediated by xenobiotics and environmental toxicants. Collectively these studies should help define the physiological and pathological significance of Abcb6 and provide and opportunity to explore its therapeutic relevance.

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