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PHYSIOLOGICAL AND TOXICOLOGICAL ROLES OF ABC TRANSPORTERS IN CELLULAR EFFLUX OF SUBSTRATES

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PHYSIOLOGICAL AND TOXICCOLOGICAL ROLES OF ABC TRANSPORTERS IN CELLULAR EFFLUX OF SUBSTRATES

DISSEETATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School at the University of Kentucky

By

Donna Jean Coy

Lexington, Kentucky

Director: Dr. Mary Vore, Professor of Toxicology

Lexington, Kentucky

2012

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ABSTRACT OF DISSERTATION

PHYSIOLOGICAL AND TOXICOLOGICAL ROLES OF ABC TRANSPORTERS IN CELLULAR EFFLUX OF SUBSTRATES

ATP-binding cassette (ABC) transporters are transmembrane proteins that transport a wide variety of substrates across intra and extra-cellular membranes. A few examples of endo and xenobiotic substrates are metabolic products, lipids, sterols, and drugs. An important function of ABC transporters involved in export is to prevent intracellular the buildup of toxic products. Several ABC transporters have also been associated with drug resistance upon treatment with chemotherapeutic agents. P-glycoprotein (P-GP) and the multidrug resistant (MRP) transporters of the ABC C family are examples of transporters that confer chemoresistance.

We have studied two unique roles of ABC transporters in the liver and the heart. In the liver, maintenance of bile secretion is important during lactation to ensure proper absorption of nutrients for the offspring. Three main ABC transporters are involved in this process: ABCB11 (transports bile acids), ABCB4 (transporters phospholipids), and ABCG5/ABCG8 (transports cholesterol). In the rat, expression of ABCB11 remains the same as the size of the bile acid pool increases. However, the expression of ABCG5/ABCG8 is abolished, preventing excessive export and loss of cholesterol from the liver. The regulation of these transporters during lactation maintains the production of bile acids from cholesterol by decreasing export while preventing toxicity from bile acids by maintaining bile flow.

Another protective role of ABC transporters is seen in oxidative stress-induced toxicity of cardiac tissue following treatment with Doxorubicin (DOX), a drug used in cancer treatment. Multidrug resistance protein 1 (Mrp1) can transport toxic products by conjugation with sulfate, glutathione (GSH) or glucuronide. In Mrp1−/− mice, DOX causes advanced cell damage through intracellular edema and increased apoptotic nuclei. However, Pgp expression increases upon DOX treatment, potentially compensating for the loss of Mrp1. Mrp1 can also transport GSH, GSH disulfide (GSSG), and products of oxidation, like GSH conjugates. In the absence of Mrp1, GSH levels are increased in the heart, providing protection against oxidative stress.
Both of these examples in liver and heart show the diversity of ABC transporters and the role they play in preventing cell toxicity. These studies also provide insight into ways to prevent cell toxicity through manipulation of ABC transport proteins.

KEYWORDS: ABC Transporter, ABCG5/ABCG8, cholesterol, Mrp1, glutathione
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...to my mom and dad
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Overview of ABC Transporters

Lipid membranes play an important role in cell survival and homeostasis by providing a barrier between the interior of the cell and its exterior environment. An essential function in every cell is the ability to import nutrients as well as export waste products and signaling molecules. Adenosine triphosphate (ATP)-binding cassette (ABC) transporters play significant physiological and toxicological roles in these export and import processes. ABC transporters utilize the hydrolysis of ATP to drive transport of substrates against their concentration gradient. Generally, in prokaryotic cells these transporters are importers while in eukaryotic cells they are exporters.

ABC transporters were first discovered in prokaryotic cells of *Escherichia coli* (*E. coli*) and *Salmonella typhimurium*. Import of nutrients in these bacterial cells was shown to be primarily dependent upon periplasmic substrate-binding proteins that utilized ATP hydrolysis for transport (16). Discovery of homology between gene sequences in the histidine transporter of *S. typhimurium* (92) and maltose transporter of *E. Coli* (75) led to the unearthing of the highly conserved super-family of ABC transporters. The first eukaryotic transporter to be discovered, by its involvement in multidrug resistance (MDR), was P-glycoprotein (*P-GP/MDR1/ ABCB1*) (33, 69, 82, 149). Today, 48 ABC transporters have been identified in humans. Mutations in these transporters can lead to a variety of genetic diseases, including bleeding, liver, and eye disorders (3, 105, 131). Examples of ABC transporters, their substrates, and corresponding disorders are listed in
Table 1.1. Below, we will be focusing on efflux transporters that play key roles in endo- and xenobiotic export from cells.

**Structure of ABC Transporters**

A typical ABC transporter consists of two membrane-spanning domains (MSDs), and two cytosolic nucleotide-binding domains (NBDs). ABC transporter domains can be encoded into separate polypeptides like the vitamin B12 importer BtuCD in *E. Coli* (122), they can be half transporters (one MSD and one NBD) that dimerize to form a functional transporter like *ABCG5* and *ABCG8*, or full-length and contain all four domains encoded into one peptide, as in P-glycoprotein. Some also have an extended transmembrane (TM) domain and consist of three MSDs such as MRP1 (Figure 1.1). In humans, the 48 genes have been divided into seven subfamilies A through G. Figure 1.1 shows examples of the structure of different family members.

Membrane-spanning domains consist of several TM helices (most commonly six) involved in substrate binding, specificity, and movement across the cell membrane. MSDs also have intracellular loops (ICLs) that interact with the NBDs and may assist in conformational alterations to facilitate substrate transport. Hydrolysis of ATP in NBDs confers a structural change in the MSD domain, allowing transport across the lipid bilayer. Nucleotide-binding domains consist of 200-300 amino acids and are highly conserved across genes (93). They contain a Walker A and B motif (93, 190) separated by 90-120 amino acids and a signature C-loop or LSGGQ motif upstream of the Walker B motif. The NBD also contains signature ABC motifs D-, H-, A-, and Q-Loops. Crystal
structures of three ABC transporters have been described: Vitamin B12 importer BtuCD from *E. Coli* (127), metal-chelate importer HI1470/1 from *Haemophilus influenza* (143), and the drug exporter Sav1866 from *Staphlococcus aureus* (49). In the two bacterial importers, the MSDs do not intertwine, whereas Sav1866 shows interaction of the two MSDs. One transmembrane domain (TMD) is formed by helices 4 and 5, and the other TMD is composed of helices 1, 2, 3, and 6 (49). This has also been proven through cross-linking studies of human P-gp (168) and by the crystal structure of mouse P-gp showing intertwining of the TMs (5).

**Mechanism of Action of an ABC Exporter**

Based on the structure of the above mentioned transporters and extensive studies of P-gp drug affinity, a model has been derived for the mechanism of transport, called the ATP-switch model (94). The model uses the switch between low and high affinity states of substrate binding, coupled with the ATP catalytic cycle, to explain the mechanism of transport. The whole process can be explained in four steps: substrate binding, ATP binding and substrate translocation, ATP hydrolysis, and return to the substrate binding conformation.

When ATP is not bound in the NBD, the transporter has an open conformation and a high affinity for substrate. In this configuration, the two MSDs are opened towards the cytosol with the NBD in an open conformation. Aller et al showed by crystal structure of the inward facing conformation that substrates could access the binding pocket from the inner leaflet of the plasma membrane or the cytosol (5). This
provides a hydrophilic pocket inside the transporter for substrate to bind. Substrate promiscuity results from the upper half of the binding pocket containing hydrophobic and aromatic residues while the lower half contains more polar residues (5).

Substrate binding induces a conformational change in the NBD which is propagated from the MSDs through interactions with the ICLs. At this point, the NBDs have a greater affinity for ATP. Binding of ATP causes the NBDs to form a closed dimer. This is sufficient to cause a large enough conformational change in the MSDs to put them in an outward-facing arrangement, causing translocation and export of the substrate from the cell due to decreased affinity for the substrate. The hydrolysis of two ATP molecules releases sufficient energy to be harnessed for conformational change in the MSDs (166), returning them to the open conformation. The closed formation of the NBD is a transient state. Hydrolysis of ATP is an unavoidable consequence of the closed conformation. The mechanism has been proposed to be through base catalysis (166) or substrate assisted catalysis (203). Either way, the hydrolysis leads to destabilization of the closed NBD conformation and the subsequent release of adenosine diphosphate (ADP) and a phosphate (Pi), returning the transporter to the open conformation, and once again making it accessible for substrate binding. Figure 1.2 summarizes the four steps involved in substrate transport.
Mammalian ABC Transporters: Multidrug resistance transporters

ABCB1/MDR1

ABCB1 (MDR1, P-glycoprotein, P-gp), referred to from here-on as P-gp, was first discovered in Chinese hamster ovary (CHO) cells that displayed drug resistance. It was found to be a 170 kDa glycoprotein thought to control drug permeation (107). Over-expression of P-gp in many tumor cell lines has conferred chemo-resistance. This resistance is attributed to decreased intracellular accumulation of the drug in question and increased energy-dependent drug efflux (47, 65, 180, 197). The gene was cloned in hamster (83), mouse (84), and human (151) and was found to have sequence homology with bacterial ATP transporters, becoming the first mammalian ABC transporter to be identified. P-gp contains two MSDs and two NBDs. In the mouse, two genes were identified, Abcb1a and Abcb1b (Mdr1a and Mdr1b), which share 80% homology with human P-gp and appear to play equal roles comparable to the human transporter.

P-gp is located in a variety of tissues including: hepatocytes, renal proximal tubule, enterocytes, the epithelium of the choroid plexus, the blood brain barrier (BBB), placenta, ovaries and testes. P-gp transports a variety of substrates including metabolic by products, xenobiotics, and numerous drugs. Most substrates are bulky amphipathic compounds that are hydrophobic electron donors (97). These include steroids, carcinogens, and drugs, to name a few. Many chemotherapeutic drugs are substrates for P-gp, including paclitaxel, vinca alkaloids, anthracyclines, camptothecins, and epipodophyllotoxins. Transcription of P-gp is constitutive, in that there are a variety of
factors that induce transcription. Transcription factors NF-Y, Sp1, and MDR promoter enhancing Factor 1 (MEF1) activate transcription, as well as induction of stress and activation of nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Mutations in p53 activate transcription and malignant transformation of a cell may induce transcription as well. Histone deacetylation, DNA methylation, as well as p53, can inhibit transcription of P-gp (158).

P-gp and other ABC transporters like Mrp1 (ABCC1) and BCRP (ABCG2) discussed below, play critical clinical roles in chemotherapeutic drug resistance. These ABC transporters have a wide-range of substrates and substrate specificity, complicating drug delivery to target treatment sites. Over expression of P-gp is linked to MDR in many drug-resistant cells lines as well as leukaemias and solid tumors (77, 177). P-gp in the gastrointestinal (GI) tract can prevent drug substrates from being absorbed and reaching target sites as well as preventing delivery to the brain across the BBB. Many drugs have been identified as chemosensitizers that inhibit P-gp, known as P-gp modulators, and sensitize cancer cells to chemotherapeutic drugs.

Examples of P-gp modulators that have been found are: calcium channel blockers, calmodulin antagonists, steroidal agents, protein kinase C inhibitors, immunosuppressant agents, antibiotics, and surfactants (63). Verapamil, an L-type calcium channel blocker, is P-gp modulator that causes hypotension and cardiovascular toxicity that is dose-limiting (63). Cyclosporine A is a cyclic undecapeptide that acts as an immunosuppressant. In children with sarcoma, it’s been shown to greatly increase
systemic toxicity resulting in sepsis (176). Although inhibitors like verapamil and cyclosporine A can be effective antagonists of P-gp, they result in high toxicity because of inhibition of transport in normal tissue (177).

**MRP1/ABCC1**

Multidrug resistance-associated protein 1 (MRP1, ABCC1) was first discovered in a small cell lung cancer cell line, H69AR. These cells had developed drug resistance to Doxorubicin (DOX) without the increase in expression of P-gp, leading to the discovery of another ABC transporter involved in drug resistance, MRP1 (39). In humans, there have been 12 ABC C family members discovered, ABCC1-ABCC12. Mrp1 is a 190 kDa heavily glycosylated ABC transporter that contains three MSDs and two NBDs. The additional TM domain MSD₀, and the ICL between MSD₀ and MSD₁, are important for trafficking and insertion in the plasma membrane (191, 192). Mrp1 is highly conserved between humans and rodents with 88% sequence homology. However, rodent Mrp1 cannot transport anthracyclines like Doxorubicin (171).

Mrp1 is ubiquitously expressed especially in heart, skin, lung, brain capillary endothelial cells, and the small intestine (64, 138). Substrates for Mrp1 are usually amphiphilic anionic compounds. Hydrophobic metabolic by products can also be substrates through conjugation with glutathione (GSH), sulfate, or glucuronic acid (41, 118). Mrp1 can also transport hydrophobic compounds in the presence of GSH. Transport specificity of Mrp1 overlaps with some MDR1 substrates, and includes epipodophyllotoxins, vinca alkaloids, anthracyclines (doxorubicin and danorubicin),
colchicine, and mitoxantrone (114). Transcriptional regulation of Mrp1 has been shown to involve many transcription factors, including Sp1, antioxidant response element (ARE) by binding to AP-1, and also through the JNK pathway upon treatment with doxorubicin. Oxidative stress has been shown to induce nuclear factor-like 2 (Nrf2) translocation from the cytosol to the nucleus and binding with ARE, inducing transcription of Mrp1 and antioxidative genes (90, 104, 136).

Like P-gp, Mrp1 may play a protective role by efﬂuxing substrates from the cell. By efﬂuxing endo and xenobiotics, it may decrease cellular toxicity. However, Mrp1 null mice display no phenotype unless exposed to etoposide or inﬂammatory stress (194). An important role of Mrp1 is the transport of GSH and glutathione disulfide (GSSG). Transport of some Mrp1 substrates requires the presence of GSH or conjugation prior to efﬂux. Glutathione is an essential intracellular antioxidant and maintenance of GSH homeostasis plays a vital role in cell survival during oxidative stress. Mrp1 null mice have increased basal levels of GSH because of decreased export (147), while over expression of Mrp1 decreases cellular levels of GSH (40). Based on these ﬁndings, it appears that Mrp1 may play an important role in oxidative stress-induced tissue injury.

**BCRP/ABCG2**

In the late 1990’s, a breast cancer cell line highly resistant to Doxorubicin was found to lack expression of MDR1 and Mrp1. A new ABC transporter was found in the cells and named Breast Cancer Resistance Protein (BCRP), or ABCG2 (54). ABCG2 is a half transporter, where the gene encodes one MSD with six TM helices and one NBD.
Homodimerization of two ABCG2 half transporters creates a fully functional transporter. Over expression of ABCG2 alone in insect cells yields a functional transporter so it is not thought to heterodimerize with any other partners (156). 

ABCG2 is primarily expressed in the apical membrane of the small intestine, liver, mammary gland, testis, BBB, and placenta. It effluxes several chemotherapeutic drugs including mitoxantrone, doxorubicin, irinotecan, imatinib, and methotrexate (MTX), as well as some food carcinogens and vitamins such as riboflavin and folic acid (150). Like Mrp1, ABCG2 can also transport sulfate and glucuronide conjugates (182). ABCG2 transcriptional regulation is similar to MDR1 and Mrp1, with Sp1 and AP-1 binding sites (12). Expression can also be enhanced by estrogens by the estrogen response element (ERE), which has been shown in estrogen receptor (ER)-positive cells (55). The primary role of ABCG2 appears to overlap with the functions of MDR1 and Mrp1 as discussed above. The main role of Abcg2 is thought to involve prevention of drug accumulation and to increase clearance of endo and xenobiotics, which can be emphasized by its expression in tissues like the intestine, BBB, and placenta. On the other hand, ABCG2 may play a negative role in the mammary gland, where although it effluxes vitamins like riboflavin, other ABCG2 substrates like chemotherapeutic drugs and carcinogens can become concentrated in milk (106). Excretion of drugs into milk can be beneficial to the mother by preventing build-up of xenobiotics, however, it can result in a detrimental accumulation of xenobiotics in the suckling newborn.
Multidrug resistance ABC transporters and Doxorubicin

Doxorubicin

A chemotherapeutic drug that is transported by all three of the multidrug resistance ABC transporters discussed above is doxorubicin (DOX). Doxorubicin, also known by its trade name Adriamycin, is an anthracycline antibiotic chemotherapeutic drug derived from the bacterium *Streptomyces peucetius var. caesius*. It is used to treat a wide range of cancers; including leukemias, Hodgkin’s lymphoma, bladder, breast, stomach, lung, ovaries, thyroid, and soft tissue sarcoma. DNA intercalation and inhibition of topoisomerase II is its mode of action to kill cancer cells (48). However, DOX produces detrimental side effects in normal tissue through oxidative stress-induced injuries. Also, DOX has a high-affinity for cardiolipin, a negatively charged phospholipid located in the inner membrane of mitochondria, which results in DOX accumulation in mitochondria (157). Tissues that DOX negatively affects include heart (161), kidney (76), and brain (175).

Cardiac toxicity

Doxorubicin cardiotoxicity presents in a dose-dependent manner, with 5% of patients experiencing toxicity at cumulative doses above 500 mg/m$^2$. At doses above 600 mg/m$^2$, toxicity jumps to greater than 36%. Toxicity results in congestive heart failure, dilated cardiomyopathy, and sometimes death (132). In the heart, DOX is involved in multiple processes that produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in oxidative stress. One of the main paths to ROS
generation is redox cycling of the quinone moiety in DOX. In the presence of NADH in complex one of the electron transport chain (ETC) in mitochondria, the quinone ring undergoes one-electron reduction to a semiquinone. The semiquinone is quickly oxidized back to a quinone by oxygen, releasing a superoxide radical ($O_2^-$) (Fig. 1.3).

Production of ROS and RNS results in oxidation of membrane phospholipids. One common lipid peroxidation product from DOX-induced oxidative stress is (E)-4-Hydroxy-2-nonenal (HNE). HNE readily reacts with GSH to form the conjugate GS-HNE. Although conjugation is meant to detoxify HNE, it is still toxic to the cell and requires efflux and elimination from the body (95, 148). ABC transporters like Mrp1 are postulated to play a role in effluxing toxic products, such as GS-HNE. As discussed above, Mrp1 also transports GSH and GSSG; GSH is a non-toxic antioxidant that plays an essential role during oxidative stress. Therefore, Mrp1 may promote a balance between cellular concentrations of GS-conjugates, as well as GSH and GSSG, to maintain the redox state of the cell.

**Protective role of Mrp1**

In 1986, Ishikawa *et al* discovered that following perfusion of the isolated rat heart with HNE, intracellular levels of GSH are depleted while GS-HNE levels rise. Also, export of GS-HNE from the cell was a saturable process, indicating the involvement of a transporter (103), termed the GS-X pump. This transporter was later discovered to be Mrp1. Mice treated with DOX have increased Mrp1 expression in cardiac tissue as well as increased adduction of proteins to HNE (including Mrp1) (109). Although they do not
have a visible phenotype, Mrp1−/− mice do show sensitivity in highly proliferative cells, including in the testis, oropharyngeal mucosa, bone marrow and urinary collecting tubules in the kidney, where the chemotherapeutic drug etoposide has shown adverse effects (194, 195). In chapter 3 we will explore the protective role of Mrp1 in DOX-induced cardiac toxicity.

**Mammalian ABC Transporters: Physiological role in biliary excretion**

**BSEP/ABCB11**

The ABC transporter known as the bile salt export pump (BSEP/ABCB11) was first discovered in a screening of cDNAs in pig liver. Based on its homology to MDR1, it was first named sister of P-glycoprotein (spgp) (36). Its function was unknown except that its chromosomal location was associated with liver transport disorders (37). Gerloff et al. determined in 1998 that the homologous gene in rat liver was indeed an ATP-dependent transporter of bile salts (70). Human BSEP has 82% and 81% homology with rat and mouse, respectively, while both rodents share 90% homology (80, 169). Like MDR1, BSEP contains two MSDs and two NBDs.

Expression of BSEP has only been shown in liver (169). Its substrates include monovalent bile acids. Bile acids are detergents that help to emulsify lipid-soluble nutrients in the GI tract. BSEP in both rat and human transports the bile acid taurocholate at similar K_m values (roughly 4.2 µM) (28, 140). Mutations in BSEP that prevent normal function result in a severe cholestasis disorder called Progressive Familial Intrahepatic Cholestasis 2 (PFIC2). Cholestasis is defined as loss of bile flow,
which will be discussed in more detail below. For the above reasons, BSEP is concluded to be the main transporter responsible for bile salt secretion into bile. BSEP expression is regulated through the farnesoid X receptor (FXR). The promoter region of BSEP contains an inverted repeat that binds FXR:retinoid X Receptor (RXR) heterodimers (7). Hydrophobic bile salts are ligands for FXR, with chenodeoxycholic acid (CDCA) being the most effective agonist (119). A toxic bile salt, lithocholic acid, is a potent antagonist (200). Therefore, FXR acts as a sensor for intracellular bile acid levels in the liver. High concentrations of bile acids activate BSEP expression through FXR, promoting excretion into bile, and preventing the toxic build-up of bile acids in the liver.

**MDR2/ABCB4**

ABCB4/MDR2 or MDR3 in human (Mdr2 in rodents), is an ABC transporter that aids in the secretion of phospholipids into bile. It is a floppase that translocates or flops phosphatidylcholine (PC) from the inner leaflet to the outer leaflet of the cellular membrane, making it available for extrusion from the outer membrane into bile. In bile, PC decreases the detergent effect of bile acids by forming mixed micelles, discussed below. Mice lacking Abcb4 are unable to secrete PC into bile (164). Like BSEP and MDR1, ABCB4 has two MSDs and two NBDs, functioning as a full transporter. ABCB4 shares 90% homology with Abcb4 in mouse (85).

ABCB4 is primarily expressed in the liver canalicular membrane. Although mRNA has been detected at low levels in the adrenal gland, heart, striated muscle, spleen, and tonsils, no protein was detected (163). ABCB4 is regulated by FXR, with CDCA
increasing expression in both humans and rats (68, 108). Similar to BSEP, mutations in ABCB4 result in the cholestatic disease, PFIC3. The correlation of regulation of BSEP and ABCB4 through FXR likely serves to protect from cholestatic liver injury.

**ABCG5/ABCG8**

A set of genes that were found to be induced by the liver X receptor (LXR) are ABCG5 and ABCG8 (G5G8), or Sterolin 1 and Sterolin 2 (15). These ABC transporters are half transporters that heterodimerize to construct a fully functional transporter. Each half transporter consists of one MSD with 6 TM and one NBD and only share 28% amino acid homology. Neither half transporter has been found to homodimerize, as expression of only one results in retention of the protein in the endoplasmic reticulum (79). ABCG5 and ABCG8 are expressed in liver and small intestine to promote sterol secretion into bile and the intestinal lumen, respectively.

Substrates for G5G8 are neutral sterols, including cholesterol and the plant sterol sitostanol. Regulation of transcription of both genes is maintained by a small intergenic region of 374 base pairs and expression is bidirectional, so that both genes are equally expressed. In addition to positive regulation by LXR, hepatocyte nuclear factor 4α (HNF4α) binds GATA4 in the intergenic region to synergistically activate expression of G5G8 (172 2007 2007). Mice that lack an insulin receptor in the liver, and were given a high cholesterol diet, showed increased Abcg5/Abcg8 expression mediated by Foxo1 binding to the intergenic region (17). Thus, this intergenic region is essential for bidirectional stimulation of transcription.
The function of G5G8 as a transporter is still speculative and several theories are available. G5G8 may act as a cholesterol recipient from an intracellular donor. Alternatively, it may act as a floppase, similar to ABCB4, and flop cholesterol from the inner to outer leaflet, making it available for extrusion from the membrane. Lastly, it may function as an actual transporter. Mutations in G5 or G8 result in sitosterolemia, resulting in cholesterolemia and increased absorption and retention of plant sterols like sitostanol (15).

**Biliary transport, bile formation, and cholestasis**

**Bile acid synthesis**

The process of bile formation begins with the synthesis of bile acids in the hepatocyte from cholesterol catabolism (186). The primary bile acids cholic acid (CA) and CDCA, are formed by hydroxylation of cholesterol in the liver. Secondary bile acids are formed in the GI tract by bacteria which remove the hydroxyl group at position C-7. Bile acids become more toxic by removal of hydroxyl groups, increasing their hydrophobicity and thus detergent properties. In humans, secondary bile acids include deoxycholic acid (DCA) and lithocholic acid. Lithocholic acid is a highly toxic bile acid because it contains only one hydroxyl group. The bile acid pool of humans is more hydrophobic than the mouse. In mice, CDCA is converted into muricholic acids, which are much more hydrophilic and less toxic. In addition, bile acids are conjugated with taurine or glycine to form bile salts. This increases solubility in the GI tract and prevents permeability through the lumen of the GI tract. Bile acids are actively transported into
bile, reabsorbed in the terminal ileum of the GI tract via the apical sodium-dependent bile acid transporter (Asbt) and the organic solute and steroid transporter heterodimer alpha and beta (Ost\(\alpha/\beta\)). Bile acids are then reclaimed in the hepatocyte by the Na+-taurocholate cotransporting polypeptide (Ntcp). The recycling of bile acids is known as enterohepatic circulation.

**Mixed micelle formation and bile flow**

As discussed above, several ABC transporters are involved in transport of bile substrates at the apical or canalicular membrane in hepatocytes. BSEP, ABCB4, and G5G8 transport bile acids, phospholipids (PC), and cholesterol into bile, respectively. These three substrates come together to form mixed micelles that emulsify lipid soluble nutrients in the intestine to promote their absorption. Although the mechanism is not fully understood, based on our current knowledge of these transporters and bile flow, we can hypothesize how micelle formation takes place. First, bile acids are actively transported into bile by BSEP. ABCB4 flops PC to the outer leaflet of the cell membrane; once bile acids reach micellar concentrations, the available PC is taken up by bile acid micelles to form mixed micelles. Finally, the mixed micelles accept cholesterol from G5G8 (Figure 1.4) (145, 162).

Bile is both a digestive and excretory fluid. Mixed micelles play a digestive role by emulsifying lipid soluble nutrients. Bile’s excretory role involves elimination of substances that are not excreted in urine. These include insoluble conjugates or protein bound substances such as bile acids, cholesterol, bilirubin, heavy metals, and drugs. To
prevent accumulation of toxic substrates during cholestasis, upregulation of ABC transporters like Mrp3, can promote urinary excretion by effluxing substrates across the basolateral domain of the hepatocyte into the bloodstream for urinary excretion. Bile flow is an osmotic process with the rate-limiting step being bile acid transport across the canalicular domain (22). Concentrated solutes actively transported into bile result in an osmotic force of water from tight junctions and aquaporins, creating bile flow. In addition to mixed micelle components, other ABC transporters efflux substrates into bile. Elimination of drugs is performed by efflux into bile by MDR1, ABCG2, and Mrp2. Mrp2, like Mrp1, can also transport GSH, which contributes to bile flow.

Liver disease and cholestasis

Cholestasis is a common liver disease that is caused by impairment of bile flow, thus preventing excretion of constituents in bile. Cholestasis can develop from blockage in the bile duct or genetic disorders in bile transport. Symptoms include: pruritis, jaundice, dark or discolored urine, clay or white colored stool, inability to digest certain foods, and pain in the right upper part of the abdomen. In the absence of bile flow, bile salts and bilirubin levels in serum become elevated. Because bile acids act as detergents, accumulation can cause damage to cell membranes, and in the liver, can cause cirrhosis and necrosis. Here we will focus on genetic defects in the ABC transporters involved in efflux of bile substrates.

One hereditary disease that often results in liver failure and death without a liver transplant is PFIC. There are different forms of PFIC based on mutations in three
different transporters. PFIC1 is caused by mutations in the P-type ATPase ATP8b1, which is a flippase for phosphatidylyserine and phosphotidylethanolamine. Mutations in ATP8b1 result in defective bile acid transport (25, 26). Loss of function of the two ABC transporters BSEP and ABCB4 are responsible for PFIC2 and PFIC3, respectively. PFIC2 is characterized by low levels of bile acids in bile but normal bile acid synthesis. This in turn leads to high levels of bile acids in the blood and hyperbilirubinemia. Eighty-two mutations in the BSEP gene that cause severe PFIC2 cause the protein to not be expressed in the canalicular membrane or a significantly reduced expression (170). A majority of these are missense mutations (79%), with E297G and D482G appearing in 58% of the cases. In addition, PFIC3 results in an inability to secrete phospholipids into bile. Many patients present symptoms at a young age and half often require liver transplantation (59). Also, women with PFIC 3 are predisposed to intrahepatic cholestasis of pregnancy (ICP) (52), discussed below. In PFIC3 patients, the liver is unable to secrete PC into bile, which is not only a substrate of mixed micelles, but also chaperones bile acids to prevent damage to cell membranes. Therefore in the absence of PC secretion, bile acids cause injury to the bile duct epithelium resulting in cholangitis.

Mutations in G5G8 result in a recessive disorder called sistosterolemia, as discussed above. Sistosterolemia patients have a decreased ability to secrete sterols into bile, as well as to prevent their absorption in the GI tract. Symptoms of the disease include cholesterolemia, phytosterolemia, and premature coronary artery disease (15). Normally, only 5% of plant sterols are absorbed in the GI, however, in patients with
sistosteronemia, 30-40% of these sterols are absorbed. In addition, loss of G5G8 function in the liver results in decreased clearance of plant sterols into bile.

Besides inherited forms of cholestasis, there are also acquired forms. Some examples are blockage caused by gallstones, drug-induced, endotoxin or infection induced and ICP, which we will focus on now. ICP is reversible and usually presents in the third trimester of pregnancy, when hormone levels (estrogen and progestin) are highest. The most common symptom is itching (pruritis), usually in palms of hands and soles of feet, and in extreme cases, jaundice. Estrogen-treated rats are an animal model that has been used to study estrogen-induced cholestasis, which correlates with possible hormonal causes of ICP. Rats treated with ethinylestradiol show decreased taurocholate (TC) transport in hepatocyte canalicular membrane vesicles (21) as a result of decreased BSEP levels (117). Estradiol-17β-D-glucuronide (E₂17G) is an estrogen metabolite known to cause estrogen-induced cholestasis (188).
**Research Objectives**

As can be seen, ABC transporters play a variety of roles in normal cell function and maintenance of homeostasis. In the next two chapters we will examine both their physiological roles and protective roles in toxicity *in vivo* using rat and mouse models. Chapter 2 summarizes a project characterizing biliary excretion of micelle substrates and alterations during lactation to provide insight into transporter function using a model of normal cell function. In Chapter 3 we will use a disease model of DOX-induced cardiac toxicity to explore protective roles that ABC transporters play to prevent cell damage, particularly oxidative stress.

Our laboratory has shown significant changes in the metabolism and transport of bile acids and bile flow during lactation in the rat. In Chapter 2, we used rat and mouse models to characterize changes in ABC transporter expression and bile components during lactation. The aims of this study were as follows:

1. Determine if ABC transporters Bsep, Abcb4, and Abcg5/Abcg8 have altered expression during lactation, when the size of the bile acid pool is increased.
2. Characterize concentrations of bile acids, phospholipid, and cholesterol in bile in lactating and virgin rodents as well as maximal transport of these substrates upon stimulation with taurocholate.

We compared ABC transporter mRNA expression in lactating rodents at mid-lactation to non-lactating controls to identify possible differences in secretion of bile acids, phospholipids, and cholesterol. Subsequently, we determined the concentrations of
these substrates in bile collected by cannulation of the gallbladder or bile duct in mice and rats, respectively. To determine if maximal secretion of bile acids, phospholipid, and cholesterol could be reached, we performed isolated liver perfusions. Taurocholate infusion was used to maximize bile acid efflux and subsequently bile flow. Linear regression analysis was used to correlate bile acid-dependent phospholipid and cholesterol secretion to see if they are similarly coupled in lactation when compared to virgin control animals. We further examined Abcg8 protein expression by western blot and immunolocalization studies.

In Chapter 3 we aimed to characterize the role of ABC transporters using a drug-induced cell damage model. We utilized Mrp1<sup>−/−</sup> mice to examine the role of Mrp1 in Doxorubicin-induced cardiac toxicity. The aims of this study were as follows:

1. Determine if Mrp1 plays a protective role in DOX-induced cardiac toxicity by analyzing ultrastructural damage, expression of antioxidant proteins, and intracellular levels of GSH and GSSG.

2. Examine expression of ABC transporters known to cause MDR and common antioxidants to determine if their expression is altered to compensate for or alleviate potential negative side effects resulting from the loss of Mrp1 expression.

Wild type or Mrp1<sup>−/−</sup> mice were treated with DOX or saline and sacrificed at between 12 and 72 h to measure cardiac toxicity. Electron microscopy was used to examine ultrastructural damage in the mitochondria, cytosol, and the whole cell. Antioxidant
protein expression was determined for Sod1, Sod2, and catalase as indicators of oxidative stress. Cellular levels of GSH and GSSG were measured by high performance liquid chromatography (HPLC) to evaluate the redox state of the cell. Protein expression of Abcb1a, Abcb1b, and Abcg2 was measured, as well as accumulation of DOX in heart tissue, to determine if other ABC transporters were upregulated or downregulated in the absence of Mrp1 and in response to DOX treatment.
<table>
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<td>Sitosterolemia</td>
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**Figure 1.1** Structure of ABC transporters. Cartoon representation of (A) full length transporter, (B) extended full length transporters with an extra MSD domain at the N-terminus (MSD₀), and (C) half transporters that require dimerization for full function.
Figure 1.2. ABC transporter efflux of substrate: ATP-switch model. Cartoon representation of the 4 step ATP-switch model of an ABC transporter. (1) Transporter is in the open conformation to allow substrate binding. (2) ATP binds in the NBD domain, resulting in a closed conformation and substrate translocation. (3) Hydrolysis of ATP. (4) Return to the open conformation that is ready for substrate binding.
Figure 1.3. Redox cycling of Doxorubicin. The one-electron addition to the quinone moiety in ring C of doxorubicin results in the formation of a semiquinone that quickly regenerates its parent quinone by reducing oxygen to $O_2^-$, which generates additional ROS (i.e. $H_2O_2$). $O_2^-$ and other ROS are scavenged and detoxified by superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and GSH regenerated by GSH reductase (GR). However, in an oxidative stress environment, $O_2^-$ can initiate lipid peroxidation and generate the reactive end product, 4-hydroxy-2-nonenal (HNE), leading to toxicity and cell death.
Figure 1.4. Transport of mixed micelle substrates in bile. Cartoon representation of mixed micelle formation in bile. (1) bile acids are transported into bile via ABCB11/BSEP; (2) ABCB4/MDR2 flops phosphatidylycholine (PC) from the inner leaflet to the outer leaflet of the canalicular membrane, making PC available for uptake by bile acids. (3) ABCG5/ABCG8 makes cholesterol available to be incorporated into mixed micelles. ATP8B1/FIC1 flips aminophospholipids inward to allow PC and cholesterol to accumulate for extraction from the outer leaflet of the canalicular membrane. Figure from Elferink and Paulusma, Eur J Physiol. Vol. 453, 2007.
CHAPTER TWO

ABCG5/ABCG8-INDEPENDENT BILIARY CHOLESTEROL EXCRETION IN LACTATING RATS

Background

The liver plays a central role in lipid and cholesterol homeostasis. Synthesis of bile acids from cholesterol and secretion of bile acids is highly integrated and tightly regulated to maintain cholesterol homeostasis, optimize intestinal lipid absorption and minimize intracellular accumulation of cytotoxic bile acids (35). In Chapter one we discussed how bile acids, phospholipids, and cholesterol are secreted from hepatocytes and form mixed micelles that act in the small intestine to promote absorption of lipid-soluble nutrients, including dietary lipids, lipid-soluble vitamins, and cholesterol (98) and the role of ABC transporters. During lactation in rats, bile flow, bile acid synthesis and secretion are all increased (18, 112), as is the size and hydrophobicity of the bile acid pool (18, 198). We have shown that the expression of Ntcp in the basolateral domain of the hepatocyte, Bsep/Abcb11 expressed in the apical domain of the hepatocyte, and the ileal apical transporter Asbt are all increased in the rat dam during lactation (30, 124-126). We have further shown that the increase in the bile acid pool is mediated by increased expression of cholesterol 7α-hydroxylase (Cyp7a1), which catalyzes the rate-limiting step in the conversion of cholesterol to bile acids (198).

Lactation increases nutrient and energy demand in the rodent by 4-5 fold, resulting in a 2-3 fold increase in food intake (45, 88). Although the efficiency of nutrient absorption in the dam increases to meet both the needs of the dam and for incorporation of nutrients into milk, lactating rats are likely in negative energy balance
at all times of day (14, 185). We have speculated that the increased size and hydrophobicity of the bile acid pool might serve to increase the efficiency of absorption of cholesterol and lipid-soluble nutrients in lactation (30). An increase in the size and hydrophobicity of the bile acid pool would also likely impact the biliary excretion of lipids, i.e., phospholipids and cholesterol, into bile.

ATP-binding cassette transporters Mdr2, Bsep, and Abcg5/Abcg8 heterodimers in the canalicular membrane of hepatocytes mediate the biliary secretion of phospholipids, bile acids, and cholesterol, respectively. Mdr2 is a floppase that flops phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (58). Bsep is the primary bile acid transporter on the canalicular membrane and is responsible for biliary excretion of monovalent bile acids (6), while Abcg5 and Abcg8 form heterodimers that function to promote sterol excretion into bile (91).

Based on our data demonstrating increases in essential bile acid transporters in the liver and intestine during lactation (30, 124-126), we hypothesized that the expression of the key transporters of mixed micelle components, i.e., Mdr2, Bsep, and Abcg5/Abcg8 would also be regulated during lactation. We measured mRNA expression of these transporters and basal concentrations of bile acids, phospholipids and cholesterol in bile in rats and mice. We also infused taurocholate, a hydrophobic bile acid, to characterize the coupling of phospholipid and cholesterol biliary secretion to that of taurocholate. These studies demonstrated that despite a nearly complete absence of expression of Abcg5/Abcg8 mRNA and Abcg8 protein in lactating rats, basal
biliary concentrations of cholesterol were unchanged, consistent with an Abcg5/Abcg8-independent pathway of cholesterol secretion in lactating rats. However, maximal cholesterol secretion into bile was decreased in lactation following taurocholate infusion in the absence of Abcg5 and Abcg8. In order to determine if similar effects were present in other species, we characterized expression of Mdr2, Bsep and Abcg/Abcg8 and biliary excretion of their substrates in lactating mice. Whereas Mdr2 mRNA expression was decreased in both rats and mice, only mice exhibited a decrease in the maximal biliary secretion of phospholipids.
Materials and Methods

Chemicals and reagents

General reagents were purchased from Sigma Aldrich (St. Louis, MO). TRIzol Reagent and Superscript III First-Strand Synthesis System for RT-PCR were purchased from Invitrogen Life Technologies (Carlsbad, CA). Light Cycler DNA Master SYBR Green 1, Light Cycler 480 and master mix/universal probes were purchased from Roche Diagnostics (Indianapolis, IN), and RNeasy Mini kit from Qiagen Inc. (Valencia, CA). Secondary antibodies and chemiluminescence reagents were purchased from Pierce Chemicals (Rockford, IL). Calnexin antibody was purchased from Nventa (San Diego, CA). Rabbit anti-Abcg5 and mouse anti-Abcg8 antibodies have been previously described (79, 202). Antibodies for immunofluorescence including polyclonal antibody against ZO-1, Alexa Fluor-488 conjugated goat anti-mouse, Alexa Fluor-568 conjugated goat anti-rabbit; and ProLong gold antifade reagent with Dapi were purchased from Invitrogen Life Technologies.

Animal care

Female, 11-12 week old Sprague-Dawley rats and C57BL/6 mice were purchased (Harlan Industries; Indianapolis, IN) as timed-pregnant or virgin controls and were maintained on a 12 hour light/dark cycle (6 am lights on/6pm lights off) in a temperature controlled environment. Animals had free access to Teklad Global Diet 2018 (Harlan Laboratories; Cincinnati, OH) and water. Litter size was culled within 24 h of birth to 9-11 or 7-9 pups for rats and mice, respectively. Animal protocols were conducted in accordance with the National Institutes of Health Guidelines for the Care
and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Female virgin rats or mice (control group, C), rats at late gestation (day 19 of gestation), and post-partum (PP) rats or mice during early lactation (day 3 and 5 PP), midlactation (day 10 and 12 PP), and late lactation (day 19 and 20 PP, just prior to weaning) were killed at 1600 hours. Post-weaning rats were killed 55 days following weaning of the pups.

**Real-time PCR analysis**

Total RNA from liver was isolated with TRIzol Reagent and purified with Qiagen RNeasy Mini kit, followed by cDNA synthesis with SuperScript III. To determine mRNA expression, real-time PCR was performed on a Roche LightCycler with a SYBR Green kit (rat Abcg5, Abcg8, Mdr2, 18S) and the Light Cycler480 with Light Cycler 480 master mix/universal probes (mouse Abcg5, Abcg8, Mdr2, Bsep, 18S). Amplification of diluted cDNA template was used to create a semiquantitative standard curve by plotting the cycle number vs. the log of the fluorescence measurement at the threshold. Conditions used for amplification using the LightCycler were as follows: denaturation for 30s at 95°C and 40 cycles of 95°C for 0 s; 60°C for 15 s (Abcg5), 65°C for 15 s (Abcg8), 57°C for 15 s (Mdr2), 56°C for 20 s (18S); and 72°C for 15 s (Abcg5, Abcg8, Mdr2), 30 s (18S). Primers were as follows: Abcg5 (forward, 5’-CGCAGGAACCCTTCAACCC-3; reverse, 5’-TGTCAAGTGGATGGAGAGCT-3); Abcg8 (forward, 5’-GATGCTGGCTATCATAGGGAGC-3; reverse, 5’-TCTCTGCTTGATAACGTCGA-3); Mdr2 (forward, 5’-CCCACAGGGGTACGATTAGCA-3; reverse, 5’-CGCCGATGAATTCCCTTAGAC-3); 18S (forward, 5’-GTAACCCGTTGAACCCCA-3; reverse, 5’-CCATCCAATCGGGTAGAGCG-3).
For the LightCycler 480, primers and probe sets were designed using the Roche Universal Probe Library (www.universalprobelibrary.com) to amplify intronic-spanning regions of the gene. Reactions were performed as follows: denaturation for 5 min at 95°C, and 45 cycles of 95°C for 15 s; 60°C for 30 s. The following probes were used: #81 (18s), #10 (Bsep and Abcg8), #31 (Abcg5), #4 (Mdr2)

**Preparation of membrane proteins**

Whole liver (mouse) or 100-200 mg liver (rat) was homogenized using a Polytron homogenizer in Buffer A (250 mM sucrose, 2 mM MgCl₂, 20 mM Tris-HCl, pH 7.5) and centrifuged at 2000x g for 10 min at 4°C. The supernatant was collected and centrifuged at 120,000x g for 45 min at 4°C. The membrane pellet was suspended in Buffer B (80 mM NaCl, 2 mM CaCl₂, 1% Triton x-100, 50 mM Tris-HCl, pH 8) and protein concentrations were determined by BCA assay (Pierce).

**Immunoblot analysis**

Protein samples were prepared in protein sample buffer containing β-mercaptoethanol (final concentration 1.2%) and were boiled at 95°C for 5 min. Size-fractionation was performed on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (5% (w/v)) dry milk in Tris buffered saline with 0.2% Tween 20, TTBS) for 1 h at 4°C. Primary antibodies were diluted 1:500 in blocking buffer and incubated overnight at 4°C. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were diluted (1:15,000) and incubated for 1 h at room temperature. Proteins were visualized
by chemiluminescence and protein loading was assessed by blotting with calnexin. Densitometry was used for semiquantitative analysis of expression levels.

**Confocal microscopy analysis of ABCG8 and ZO-1**

Confocal microscopy studies were done in collaboration with Dr. Greg Graf at the University of Kentucky. Livers from virgin and lactating Sprague Dawley rats (PPd21) were dissected, immediately imbedded in Tissue-Tek O.C.T. Compound 4583 (Sakura Finetek) and placed on dry ice, then stored at -80°C. Liver sections (14 µm) were prepared with a Microm HM 560 CryoStar microtome. Sections were fixed with methanol (for 5 min at -20°C), rehydrated in PBS (pH 7.4) and incubated in Buffer A (PBS + 1% BSA (w/v)) at 22°C for 1 h to block non-specific binding. For ABCG8 and ZO-1, sections were incubated with a monoclonal antibody against ABCG8 (16 µg /mL) and a polyclonal antibody against ZO-1 at a dilution of 1:100 in Buffer A at 4°C for 16 h. Sections were washed with Buffer A and incubated with Alexa Fluor-488 conjugated goat anti-mouse and Alexa Fluor-568 conjugated goat anti-rabbit (2 µg/ml) in Buffer A at 22°C for 1 hr. After washing 3 times with PBS and once with distilled water, slices were mounted in aqueous mounting media (ProLong gold antifade reagent with Dapi), and imaged on a Zeiss Axiovert 200M confocal microscope equipped with an Apotome.

**Basal bile collection**

Rats and mice were anesthetized with urethane (1 g/kg, ip) and the bile duct cannulated (rats), or ligated and the gall bladder cannulated (mice) with PE-10 tubing. In rats, basal bile was collected for approximately 10 minutes before single-pass liver perfusion following cannulation of the bile duct. In mice, bile was collected for 45 min.
For both rats and mice, bile volume was determined gravimetrically assuming a density of 1.0. Samples were frozen at -20°C until analysis of bile components.

**Single-pass liver perfusion**

Single-pass rat liver perfusion was performed as previously described (125). The liver was perfused at a flow rate of 3.5 to 4.0 mL/min/g liver via the portal vein with Krebs-Henseleit buffer [NaCl (118.5 mM), NaHCO₃ (24.9 mM), KH₂PO₄ (1.2 mM), MgSO₄ (1.19 mM), KCl (4.74 mM), CaCl₂ (1.27 mM), and glucose (5 mM), pH 7.4]. Perfusate was oxygenated with 95% O₂/5% CO₂, and the liver was maintained at 36 ± 1°C. Bile was collected every 5 min and volume determined gravimetrically assuming a density of 1.0. Taurocholate was infused into the portal vein cannula at increasing concentrations ranging from 4 to 250 nmol/mL for 15 min each. In mice, the liver was perfused through the portal vein with Krebs-Henseleit buffer at a flow rate of 5 mL/min/g liver and bile collected every 10 min. Taurocholate was infused into the portal vein cannula at increasing concentrations ranging from 5 to 120 nmol/mL for 10 min each.

Total phospholipids and cholesterol were measured in bile using enzymatic colorimetric kits from Wako Pure Chemical Industries (Richmond, VA). Quantitation of total bile acids in bile was performed enzymatically by measuring 3α-hydroxy bile acids as described previously (174) for rats, or using a colorimetric kit from Wako for mice.

**Statistical analysis**

All data are expressed as the mean ± SEM for n = 3 to 8 animals per group. Statistical analysis was performed with Student’s t-test, one way-ANOVA followed by
Tukey’s multiple comparison test, or linear regression analysis with GraphPad 4.0 software (San Diego, CA) as indicated in figure legends.
Results

Changes in expression of transporters of mixed micelle components in rats

Expression of bile acid transporters in the basolateral domain (Ntcp) and the canalicular domain (Bsep) of the hepatocyte, and the apical domain of the enterocyte (Asbt) are all increased in early (Ntcp, Bsep) and mid-late (Asbt) lactation in the rat (30, 124-126). Further, the bile acid pool size is increased by mid-lactation (PPd10) in rats, and remains elevated until weaning (30, 198). We therefore extended these studies to determine if there were changes in the expression of Mdr2 and Abcg5/Abcg8 that mediate biliary secretion of phospholipids and cholesterol, respectively. Mdr2 mRNA expression was significantly decreased by 45% at PPd10 (Fig 2.1D), while expression of Abcg5 and Abcg8 mRNA was profoundly decreased throughout lactation (Fig 2.1A-C). Thus, Abcg5 mRNA expression was decreased 80% at PPd3, 97% at PPd10, and 93% at PPd21. Similarly, Abcg8 mRNA expression was decreased 94% at PPd3, 98% at PPd10, and 97% at PPd21 (Fig 2.1A, B). Pregnant or post-weaning rats (day 55) showed no significant difference in Abcg5 or Abcg8 mRNA expression compared to controls (Fig 2.1A, B). Immunoblot analysis confirmed the absence of Abcg8 expression in PPd10 rats (Fig 2.1C). Confocal immunofluorescence analysis showed that while ZO-1 localization in the apical membrane and architecture were maintained, Abcg8 expression could not be detected in hepatocytes from lactating rats at PPd21 (Fig 2.2). However, there were no differences in bile acid, phospholipids and cholesterol concentrations in bile under basal conditions (Table 2.1).
Effects of taurocholate infusion on secretion of mixed micelle components in rats

The biliary excretion of phospholipids and cholesterol are known to be coupled to that of bile acids (91), such that taurocholate infusion increases secretion of bile acids (30), which in turn increases incorporation of phospholipids and cholesterol into bile acid micelles, thereby increasing their secretion into bile (58). Since there were no major changes in biliary bile acid, phospholipid or cholesterol concentrations under basal conditions, we measured the coupling of cholesterol and phospholipids to the increased bile acid secretion following infusion of taurocholate. Secretion rates were normalized to liver weight, and regression analysis was used to model secretion of phospholipids and cholesterol as a function of bile acid secretion (Fig 2.3, A, B), or cholesterol secretion as a function of phospholipid secretion (Fig 2.3C). The slopes, which denote the molecular coupling of the two substrates, were all significantly different from zero. As shown in Fig 2.3 and Table 2.2, the coupling of phospholipids to bile acids secretion was only modestly impaired, (p<0.005) despite the decrease in Mdr2 mRNA expression seen at this time. In contrast, cholesterol secretion was markedly decreased in response to taurocholate infusion, as shown by the decreased slope of the regression line, consistent with a decreased coupling of cholesterol to bile acid secretion into bile (Fig. 2.3B, Table 2.2, p<0.0001). Although the slopes were unchanged between control and post partum animals for phospholipid dependent cholesterol secretion, post partum animals had visibly lower secretion rates of cholesterol (Fig. 2.3C) with a significantly lower positive intercept (0.3741, control; 0.03284, PPd10; p < 0.0001).
Changes in expression of transporters of mixed micelle components and their substrates in mice.

In order to determine if the markedly decreased expression of Abcg5/Abcg8 seen in the lactating rat was also present in lactating mice, we examined expression of Bsep, Mdr2 and Abcg5/Abcg8 expression in lactating mice and female controls. Bsep mRNA expression was significantly elevated during early lactation (2.5–fold increase, PPd5; Fig 2.4A) but there were no differences when compared to controls at later stages of lactation. The mouse also showed a significant 58% decrease in Mdr2 mRNA expression at mid-lactation (PPd12) (Fig 2.4B), but only a moderately decreased mRNA expression of Abcg5 and Abcg8 (Fig 2.5A,B). Thus, Abcg5 mRNA expression was decreased 40% at PP day 5, 65% at PP day 12, and 53% at PPd20 (Fig 2.5A), while Abcg8 mRNA expression was not significantly different at PP day 5, but was decreased 63% at PP day 12 and 53% at PPd20 (Fig 2.5B). Although protein expression of Abcg5 and Abcg8 was somewhat decreased (Abcg5 by 28% and Abcg8 by 21%) at PPd12, these decreases were not significantly different when compared to controls (Fig 2.5C).

Next, we measured the basal concentrations of cholesterol, phospholipid, and bile acids in bile to determine if altered transporter expression correlated with transport activity. In mice, the bile acid concentration was significantly elevated 1.5 fold in PP day 11 mice (Table 2.1), consistent with increased expression of Bsep mRNA. In agreement with no significant changes in mouse Abcg5 and Abcg8 protein levels, there were no changes in biliary cholesterol concentrations in the postpartum mouse. Phospholipid
concentrations were also not changed in post partum mice when compared to controls (Table 2.1), despite decreases in Mdr2 mRNA expression (Fig 2.4B).

**Effects of taurocholate infusion on secretion of mixed micelle components in mice.**

There was significant positive coupling between bile acids and phospholipid secretion in both control and postpartum mice, however lactating mice showed a decreased slope (Fig. 2.6A, Table 2.2, p< 0.005), consistent with the decreased Mdr2 mRNA expression in mice at this time (Fig 2.4B). Bile acid dependent cholesterol secretion remained positively coupled in control and lactating mice to essentially the same extent (Fig. 2.6B). Phospholipid-dependent cholesterol secretion was also positively coupled (Fig. 2.6C) and postpartum animals had an increased slope (Table 2.2, p<0.0001), consistent with the decreased coupling of taurocholate and phospholipid biliary secretion.
Discussion

The most striking and unexpected findings in the present studies were the profoundly decreased expression of Abcg5/Abcg8 in lactating rats, particularly in the face of unchanged basal cholesterol concentrations in bile, compared to control rats. Earlier studies (101) had shown an increased cholesterol concentration in bile of lactating rats. Significant evidence indicates that the half-transporters Abcg5 and Abcg8 are required for cholesterol secretion into bile, in that deletion of both half-transporters in mice leads to a marked decrease (90%) in biliary cholesterol (202). Further, cholesterol secretion is linearly correlated with the gene copy number of Abcg5/g8 in mice (201). In humans, mutations in either ABCG5 or ABCG8 gene cause an autosomal recessive disorder known as sitosterolemia, characterized by decreased biliary excretion of cholesterol, and increased intestinal absorption of cholesterol and phytosterols, such as sitosterol (15). However, there is also evidence of Abcg5/Abcg8-independent pathways of cholesterol secretion in mice. Diosgenin is a plant sterol that stimulates cholesterol excretion (137). This induction of cholesterol secretion is independent of Abcg5/Abcg8 induction (113), even though biliary cholesterol secretion is not induced in Abcg8−/− mice fed diosgenin, suggesting that expression of the co-transporters is still required.

The present study offers further evidence in a physiological model of an Abcg5/Abcg8-independent pathway of cholesterol secretion in lactating rats. However, consistent with the requirement for Abcg5/g8, there was a significantly
decreased coupling of cholesterol secretion to bile acid secretion into bile in lactating vs control rats upon infusion of the relatively hydrophobic bile salt, taurocholate. Although the basis for the normal basal cholesterol secretion in lactating rats is not clear, it is likely due to the increased size and hydrophobicity of the bile acid pool (198). These changes, coupled with the increased flux of these hydrophobic bile acids across the canalicular membrane in lactating rats, is sufficient to extract adequate amounts of cholesterol from the membrane to maintain basal concentrations without the need for a transporter.

Transport of cholesterol by ABC transporters besides Abcg5/Abcg8 has been demonstrated in the presence of an extracellular acceptor(133, 135). Thus, ABCA1 transports cholesterol when either apolipoprotein A-I (apoA-I) or taurocholate are present to serve as acceptor molecules (135). Similarly, Mdr2 expressed in HEK cells effluxed cholesterol together with phospholipid when 0.5 - 1 mM taurocholate was present in the media as an acceptor molecule (133). These data clearly demonstrate that Mdr2 is able to directly efflux cholesterol together with phospholipid from cells as long as there is an acceptor molecule, in this case, taurocholate (133). Our data indicate, however, that in the presence of a marked increase in the flux of infused taurocholate across the canalicular membrane, there is apparently insufficient cholesterol in the outer hemi-leaflet of the membrane bilayer to mediate sustained high levels of biliary cholesterol in the absence of transporters to flop additional cholesterol from the inner to the outer leaflet of the membrane. Evidence for such a mechanism is also seen in Atp8b1 deficient mice. Mutations in mice deficient in both Atp8b1 and
Abcg8 demonstrate increased cholesterol secretion when compared to wild-type controls; this has been attributed to decreased detergent resistance of the canalicular membrane due to the accumulation of phosphotidylserine in the outer leaflet of the membrane (81). However we found no changes in mRNA expression of Atp8b1 in control vs lactating rats (data not shown). Decreased detergent resistance may also play a part in lactation, when bile acids, which act as detergents, are increased. Finally, there may be an alternative transporter for cholesterol when Abcg5 and Abcg8 are not present. Further studies would be needed to identify the mediators of such a putative pathway of cholesterol secretion. While scavenger receptor class B type I (SR-BI) has been suggested to mediate cholesterol flux across the membrane (193), we found no changes in its mRNA expression in lactating vs control rats (data not shown).

The physiologic or teleological bases for loss of expression of Abcg5/Abcg8 expression in lactation are not known. We postulate that the increased size and hydrophobicity of the bile acid pool in lactating rats relative to control rats would markedly increase the basal levels of cholesterol in bile were normal expression of Abcg5/Abcg8 expression retained. Thus, the relatively hydrophilic muricholic acids that are the major bile acids in control rats (75 mole% of the bile acid pool) are decreased to 62 mole% by 14 d of lactation, while the relatively hydrophobic taurocholic acid is increased from 20 mole% in control rats to 31 mole% in lactating rats (198). Loss of biliary cholesterol could be detrimental for several reasons. Most importantly, cholesterol and lipids are essential components in milk that support membrane synthesis and brain development in the pups. Cholesterol is secreted into milk (~16
mg/day in the rat (38)), with 32-40% synthesized *de novo* in the mammary gland, and 11% from dietary sources, so that cholesterol synthesized in the liver makes up the majority of cholesterol secreted in milk (73). Also, biliary excretion of cholesterol serves as a route of its elimination from the body, both directly and following its catabolism to bile acids. The liver obtains cholesterol from peripheral tissues, intestinal absorption, and *de novo* synthesis. Fifty percent of cholesterol catabolized in the liver is used for the production of bile acids and 40% is secreted directly into bile (34). Hepatic cholesterol synthesis increases about 50% and 300% per total organ at 14 and 21 days of lactation, respectively (61). Since the bile acid pool size is increased almost 3-fold in lactation (198), this implies that greater than 50% of cholesterol is catabolized to bile acids in lactation, mediated by the increased expression of Cyp7a1, the rate limiting enzyme in this process (198). Finally, cholesterol is a component of mixed micelles in bile (98). These mixed micelles are essential for emulsifying lipid soluble nutrients for absorption from the small intestine. Increased cholesterol secretion into bile decreases cholesterol absorption in the small intestine (116). Taken together, these data suggest that the lactating dam needs to conserve cholesterol; the decreased expression of Abcg5 and Abcg8 in the liver during lactation may thus serve to minimize cholesterol elimination into bile.

Hormonal changes that occur during lactation may play a role in Abcg5 and Abcg8 regulation. We have shown that prolactin, one of the essential hormones involved in maintaining milk production, is involved in regulation of biliary secretory function. Prolactin increases the capacity of the liver to secrete taurocholate into bile.
(125) by increasing the expression of Ntcp and Bsep in the rat during lactation (30). Leptin is another hormone that regulates biliary secretory function. Leptin is secreted from adipocytes and increases energy expenditure while reducing energy intake by decreasing appetite (2). Lactation is characterized by hypoleptinemia, likely reflecting the required increased energy intake (142, 165). Leptin also promotes hepatic cholesterol clearance (183), and mice lacking leptin or the leptin receptor (ob/ob and db/db, respectively) have reduced protein expression of Abcg5 and Abcg8 (154). In addition, leptin administration to ob/ob mice decreases the size and hydrophobicity of the bile acid pool (101). Decreased leptin during lactation may thereby contribute to decrease the maximal cholesterol excretion and permit expansion of the bile acid pool. Prolactin has been reported to inhibit leptin release from adipocytes (23, 121) and may thereby play a role in leptin regulation during lactation.

Mdr2 is required for Abcg5 and Abcg8-dependent biliary cholesterol secretion, where transgenic mice that overexpress ABCG5/ABCG8, but lack Mdr2, also lack increased biliary cholesterol secretion (116). Phospholipid secretion into bile is abolished in Mdr2−/− mice and cholesterol secretion is significantly inhibited, however, Mdr2+/− animals had biliary cholesterol concentrations similar to controls (56, 57, 164). While we found Mdr2 mRNA expression to be decreased in both rats and mice at mid-lactation, mRNA may not reflect protein levels since Mdr2 protein has been reported to increase in the rat during lactation (165).
Clear species differences were seen in Abcg5/Abcg8 expression and cholesterol secretion into bile upon infusion of taurocholate. While Abcg5/Abcg8 expression in the rat was essentially completely abolished during lactation at the mRNA and protein level (Fig 2.1), mice showed a modest decrease in mRNA expression, and protein levels were not affected (Fig 2.5). Species and gender differences in Abcg5/Abcg8 expression and regulation have been shown between rats and mice, where male rats tend to have higher expression in the liver than females and upon cholesterol feeding, Abcg5/Abcg8 mRNA expression decreases in rats but increases in mice (51). In the present studies, lactating rats were able to maintain maximal phospholipid secretion (Fig 2.3A) whereas lactating mice had significantly reduced phospholipid secretion in response to infusion of increasing concentrations of taurocholate (Fig 2.6A). Although mRNA expression may not reflect protein levels, there was a positive correlation between Mdr2 mRNA expression and maximal phospholipid secretion in mice.

In summary, the present studies demonstrate an Abcg5 and Abcg8-independent pathway of sustained basal cholesterol biliary concentration and secretion in the lactating rat. These findings represent a novel yet physiologic model in which the lactating dam is able to down-regulate expression of these important transporters in the face of an increased bile acid pool size that is more hydrophobic, apparently to minimize loss of biliary cholesterol.
Table 2.1. Basal bile concentrations of bile acids, phospholipids, and cholesterol in control vs lactating rats and mice.

<table>
<thead>
<tr>
<th></th>
<th>Bile Acids (mmol/L)</th>
<th>Phospholipid (mmol/L)</th>
<th>Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=7)</td>
<td>43.6 ± 5.3</td>
<td>4.3 ± 0.50</td>
<td>0.45 ± 0.10</td>
</tr>
<tr>
<td>PPd12 (n=10)</td>
<td>47.2 ± 4.7</td>
<td>5.0 ± 0.40</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>27.4 ± 2.7</td>
<td>2.98 ± 0.27</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>PPd12 (n=4)</td>
<td>40.9 ± 2.7*</td>
<td>3.03 ± 0.21</td>
<td>0.63 ± 0.04</td>
</tr>
</tbody>
</table>

Basal bile was collected in control and postpartum (PPd12) rats and mice. *, p<0.05 vs. control by Student’s unpaired t-test.
Table 2.2. Influence of taurocholate on bile secretion in control vs lactating rats and mice.

<table>
<thead>
<tr>
<th></th>
<th>ΔPL/ΔBA</th>
<th>$r^2$</th>
<th>ΔCH/ΔBA</th>
<th>$r^2$</th>
<th>ΔCH/ΔPL</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=7)</td>
<td>0.057 ± 0.003</td>
<td>0.8229</td>
<td>0.0059 ± 0.0004</td>
<td>0.6858</td>
<td>0.099 ± 0.005</td>
<td>0.7652</td>
</tr>
<tr>
<td>PPd12 (n=10)</td>
<td>0.046 ± 0.003**</td>
<td>0.6401</td>
<td>0.0032 ± 0.0005***</td>
<td>0.2446</td>
<td>0.084 ± 0.006</td>
<td>0.5442</td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>0.094 ± 0.006</td>
<td>0.8700</td>
<td>0.0095 ± 0.0009</td>
<td>0.7270</td>
<td>0.098 ± 0.008</td>
<td>0.7896</td>
</tr>
<tr>
<td>PPd12 (n=4)</td>
<td>0.068 ± 0.004**</td>
<td>0.9199</td>
<td>0.013 ± 0.0008*</td>
<td>0.8835</td>
<td>0.17 ± 0.014***</td>
<td>0.8275</td>
</tr>
</tbody>
</table>

Values determined from linear regression. The slope of the line represents bile acid (BA) dependent phospholipid (PL) secretion (ΔPL/ΔBA), BA dependent CH secretion (ΔCH/ΔBA), or PL dependent CH secretion (ΔCH/ΔPL).* , p<0.02; **, p<0.004; ***, p<.0001 vs. control. All regression lines were significantly different from zero, p<0.0001.
**Figure 2.1.** Expression of Abcg5 and Abcg8 and Mdr2 in Control (C), late pregnancy (G19), postpartum (PP days 3, 10, 21), and 55 days post-lactating (PL d55) rats. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA (n=3-6) to amplify **(A)** Abcg5 **(B)** Abcg8 and **(D)** Mdr2 mRNA expression. Each bar represents the mean ± S.E.M. **(C)** Immunoblot analysis was performed to determine protein expression of Abcg8 in control and PPd10 rats (ns, non-specific). *, P<0.05; **, p<0.01; ***, p<0.003 vs control as indicated by Student’s unpaired t-test or Mann Whitney test (Abcg5, C vs PPd10).
Figure 2.2. Immunolocalization of ABCG8 in rat liver sections from lactating and virgin rats. Cryosections (14 µm) from liver of lactating (A-C) and virgin (D-F) rats were probed with antibodies directed against Abcg8 (A, D) and the tight junction marker, ZO-1 (B, E). Nuclei were labeled with DAPI (blue). Merged images are shown in panels C and F. Scale bar is equal to 20 µm.
Figure 2.3. Effect of taurocholate (TC) infusion (4-200 nmol/mL) on biliary cholesterol (CH) and phospholipid (PL) secretion in control and PPd11-12 rats. Each regression line represents bile acid (BA) dependent PL secretion (A), BA dependent CH secretion (B), and PL dependent CH secretion (C) in control (solid line, n=7) and PPd12 (dashed line, n=10) rats. Data points represent individual samples of control (squares) and PPd12 (triangles) rats. Regression analysis results are shown in Table 2.2.
Figure 2.4. Expression of Bsep and Mdr2 in control (C) and postpartum (PP days 5, 12, 20) mice. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA of control and post-partum mice, n=4-8 per group for (A) Bsep in mice or (B) Mdr2. Each bar represents the mean ± S.E.M. *, p<0.05; **, p<0.01 vs. control as indicated by One-way ANOVA.
**Figure 2.5.** Expression of Abcg5 and Abcg8 in control (C) and lactating (PP days 5, 12, 20) mice. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA in control and post-partum animals (n=4-7) to amplify (A) Abcg5, (B) Abcg8 mRNA expression. Each bar represents the mean ± S.E.M. Immunoblot analysis was performed to determine protein expression of (C) Abcg5 and Abcg8. *, p<0.05; **, p<0.01; ***, p<0.001 vs. control mice as indicated by One-way ANOVA. Immunoblot analysis of Abcg5 and Abcg8 showed no significant difference in protein expression between C and PP mice despite changes in mRNA expression.
Figure 2.6. Influence of taurocholate (TC) infusion (5-120 nmol/mL) on biliary cholesterol (CH) and phospholipid (PL) secretion in control and lactating (PPd12) mice. Each regression line represents bile acid (BA) dependent PL secretion (A), BA dependent CH secretion (B), and PL dependent CH secretion (C) in control (solid line, n=6) and PPd12 (dashed line, n=4) mice. Data points represent individual samples of control (squares) and PPd12 (triangles) mice. Results of the regression analysis are shown in Table 2.2.
CHAPTER THREE

PROTECTIVE ROLE OF MRP1 IN DOXORUBICIN-INDUCED CARDIAC TOXICITY

Background

As discussed in Chapter one, multidrug resistance (MDR) is a major challenge in the treatment of cancers with chemotherapeutic drugs (173), with Pgp and Mrp1 being two of the main transporters involved in chemo resistance. ABC transporters can efflux many types of chemotherapeutic drugs, including vinca alkaloids, anthracyclines, actinomycin-D, and paclitaxel (78). In addition to MDR, which is the leading cause of cancer-related death, chemotherapeutic drugs can have toxic side effects that contribute to their limited efficacy. Some milder side effects include fatigue, bleeding, nausea and vomiting, hair loss, and a depressed immune system. More serious side effects include cardiotoxicity, hepatotoxicity, nephrotoxicity and encephalopathy. Here we will focus on doxorubicin-induced cardiotoxicity.

The anthracycline doxorubicin (DOX) is a commonly used chemotherapeutic in the United States for a variety of malignant tumors, including breast cancer, leukemia, and sarcomas. However, DOX has severe side effects in the heart, resulting in cardiac toxicity. About 5% of patients display some form of cardiac toxicity (160). In the United States, this results in more than 9,000 cases and 2,000 deaths annually. One major side effect of cancer patients treated with DOX is cardiomyopathy (67, 152), with an incidence 2.5 times higher than in untreated patients (53). Although the mechanism of cardiac toxicity from DOX has not been completely elucidated, oxidative stress has been shown to play a pivotal role in toxicity.
One of the major toxic products of oxidative stress from DOX is the lipid peroxidation product, 4-hydroxy-2-nonenal (HNE). HNE is an α,β-unsaturated aldehyde derived from ω-6 polyunsaturated fatty acids (27, 60). As a potent electrophile, HNE reacts with cysteine, histidine, and lysine residues of proteins, possibly resulting in functional impairment (60). Production of HNE can be seen in as little as 3 hours after DOX treatment (31, 123, 129). HNE can be partially detoxified by conjugation with GSH, producing GS-HNE (187). Metabolism of HNE and efflux of GS-HNE are thought to play a protective role in alleviating intracellular toxicity.

In Chapter one, we discussed Mrp1 and its capacity to transport GSH conjugates, including GS-HNE. Our laboratory has previously shown that upon treatment of mice with DOX, Mrp1 expression in sarcolemma of cardiomyocytes is increased by six hours and remains elevated after 24 hours (109). In the same study, protein adduction by HNE was increased after DOX treatment and maximal after 72 h. Mrp1 was also shown to be adducted by HNE, resulting in its decreased transport capacity (109). In addition, increased Mrp1 expression and adduction with HNE was found in mitochondria (110). Sarcolemma membrane vesicles from FVB mice lacking Mrp1 are unable to transport GS-HNE, indicating that Mrp1 is the sole transporter of GS-HNE in the mouse heart (111). Based on these findings, we further investigated the protective role Mrp1 may play in exporting toxic products of oxidative stress from cardiomyocytes after DOX treatment in a C57Bl/6 Mrp1 null mouse model.
Materials and methods

Chemicals and reagents

General reagents were purchased from Sigma Aldrich (St. Louis, MO). TRIzol Reagent and Superscript III First-Strand Synthesis System for RT-PCR were purchased from Invitrogen Life Technologies (Carlsbad, CA). Light Cycler 480 and master mix/universal probes were purchased from Roche Diagnostics (Indianapolis, IN). Secondary antibodies and chemiluminescence reagents were purchased from Pierce Chemicals (Rockford, IL). P-glycoprotein (C219) antibody was purchased from Calbiochem (EMD Millipore, Billerica, MA). Breast cancer resistance protein antibody (BXP-53) was purchased from Enzo Life Sciences (Farmingdale, NY). Catalase and SOD1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SOD2 antibody was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY).

Animal care

Mrp1−/− mice were a gift from Gary Kruh, Department of Medicine and Cancer Center, University of Illinois, Chicago, IL. Mrp1−/− mice were originally established by Lorico et al in 1997 (128). The Mrp1 gene was inactivated using homologous recombination by replacing a fragment from the second NBD with a neomycin-resistance gene cassette in C57/BL6 mice. We received an Mrp1−/− breeding pair that was F7. Wild type C57/BL6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred with Mrp1−/− mice to produce heterozygous breeding pairs. From there, C57/BL6 and Mrp1−/− littermates were bred in-house and maintained in the Division of Laboratory Animal Research facility. Food and water was provided ad
libitum. Animal protocols were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

**Animal treatment and tissue collection**

Wild type C57Bl/6 (WT) and Mrp1<sup>−/−</sup> mice were treated with 20 mg/kg Doxorubicin-HCl (Bedford Laboratories, Inc., Bedford, OH) or an equivalent volume of 0.9% sterile saline intraperitoneally (ip). For electron microscopy studies, generation F8 Mrp1<sup>−/−</sup> mice were bred at the University of Kentucky, while C57Bl/6 mice were purchased from Jackson. All other studies used the F4-F7 generation of in-house bred littermates of C57Bl/6 and Mrp1<sup>−/−</sup> mice. Mice were anesthetized with Isoflurane followed by decapitation and hearts were removed at various times ranging from 12 h to 3 days (72 h) post treatment and processed immediately for ultrastructural studies or frozen for molecular and biochemical studies.

**Real-time PCR analysis**

Total RNA from heart was isolated with TRIzol Reagent, followed by cDNA synthesis with Superscript III. To determine mRNA expression, real-time PCR was performed on a Roche Light Cycler 480 with Light Cycler 480 master mix/universal probes (mouse Abcb1a, Abcb1b, Abcg2, 18s). Amplification of diluted cDNA template was used to create a semiquantitative standard curve by plotting the cycle number vs. the log of fluorescence measurement at the threshold. Probe sets were designed using the Roche Universal Probe Library (www.universalprobe-library.com) to amplify intronic-spanning regions for the gene. Reactions were performed as follows:
denaturation for 5 min at 95°C, and 45 cycles of 95°C for 15 s; 60°C for 30 s. The following probes were used: no. 18 (Abcb1a), no. 62 (Abcb1b, Abcg2), no. 48 (18S). Primers were as follows: Abcb1a (forward, 5’-GGGCATTACTTCAAACTTATCA-3’; reverse, 5’-TTTACAAGCTTCATTCCAATTCAA-3’); Abcb1b (forward, 5’-CGAAGATGGGCAAAAAGAGT-3’; reverse, 5’-AGCGAAACATCCCAAATACG-3’); Abcg2 (forward, 5’-GCACAGAAGGCCTTGAGTA-3’; reverse, 5’-AGGGTTGTGGTAGGGCTCAC-3’).

**Immunoblot analysis**

Whole heart tissue was homogenized using a Polytron homogenizer in a buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and the following protease inhibitors: 10 mg/L aprotinin, leupeptin, pepstatin, and 100 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by Bradford assay (Pierce). Protein samples were prepared in protein sample loading buffer containing β-mercaptoethanol (final concentration 5%) and all samples were boiled at 95°C for 5 min except for Mrp1 Pgp, and Abcg2 protein preparations. Size fractionation was performed on 4-12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer [5% (wt/vol) dry milk in Tris-buffered saline with 0.2% Tween 20] for 1 h at 4°C. Primary antibodies were diluted as follows: Pgp, 1:5,000; Abcg2, 1:500; catalase, SOD1, and SOD2, 1:1000; in blocking buffer and incubated overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and anti-rat IgG were diluted (1:10,000) and incubated for 2 h at room temperature. Proteins were visualized by chemiluminescence, and protein loading was
assessed by blotting with GAPDH or β-Actin. Densitometry was used for semi-quantitative analysis of expression levels.

**Ultrastructural examination by electron microscopy analysis**

Heart left ventricle tissue taken from the tip of the heart was fixed, embedded, and processed for electron microscopy and quantification of mitochondrial, cytosolic, and total cell damage in collaboration with Dr. Terry Oberley (University of Wisconsin-Madison, Wisconsin) as previously described (32). Briefly, embedded blocks from each heart for an individual mouse were sectioned and transferred to copper grids. Imaging was performed with an electron microscope (Hitachi H-600) at 75 kV. Random sampling was achieved by scanning at low magnification so that damage wasn’t apparent. Sample were scanned top to bottom and right to left, with photographs being taken of whole cardiomyocytes every 10-15 grids at 10,000x magnification.

The following characteristics were classified as mitochondrial damage: mitochondrial swelling, presence of myelin figures, loss of cristae in mitochondria, degeneration of mitochondria with disorganized cristae, lysosomal degradation, vacuolization of mitochondria, and mitochondrial membrane disruption. Cytoplasmic damage was characterized as follows: myofibrillar disorganization, intracytoplasmic vacuolization, intracellular edema, presence of myelin figures, and disruption of cell membranes. Damaged area was measured in μm² as the average of damaged area (either mitochondrial or cytoplasmic) divided by the total mitochondrial or cytoplasmic area, respectively. Image analysis was performed using Scion Image Beta 4.02 (Scion Corporation, Frederick, MD).
GSH and GSSG HPLC analysis

Whole heart homogenate was used for glutathione derivatization and quantification of GSH and GSSG as previously described (159) with minor modifications. Redox-quenching buffer (20 mmol/L of HCl, 5 mmol/L of diethylenetriaminepenta-acetic acid, and 10 mmol/L of ascorbic acid) was prepared and used to prepare 5% tricholoroacetic acid, 10 mM N-ethylmaleimide (NEM), and 100 mM dithiothreitol. Fifty milli-molar monobromobimane (MBB) was dissolved in HPLC-grade acetonitrile. Glutathione disulfide (GSSG) samples were incubated with NEM to trap thiols followed by incubation with dithiothreitol (DTT) to reduce GSSG to GSH, resulting in 2 GSH molecules per GSSG molecule. Monobromobimane-derivatized samples were centrifuged at 5,000xg for 5 min and the supernatant was assayed by reverse-phase HPLC for thiol-bimane fluorescence. A linear gradient was used from 0 to 100% solvent B (50% methanol, 0.25% acetic acid in water) in solvent A (10% methanol, 0.25% acetic acid in water) over 40 min with a flow rate of 0.8 mL/min. Fluorescence detection at Ex$_{370}$/Em$_{485}$ was performed with Waters 2475 Multi λ fluorescence detector as described (159). Quantitation using fluorescence intensities versus time of elution were done using Waters Breeze chromatography software v. 3.2 (Waters Corporation Milford). Integration of peak areas was converted to nmol equivalents from the area under the GSH or GSSG standard curve.

Doxorubicin concentration

DOX concentration was measured by DOX fluorescence as previously described (115). Briefly, 200 µL of whole heart homogenate was added to 1.5 mL of acidified
isopropanol, 100 µL of 10% Triton X-100, and 200 µL of water. Samples were centrifuged and extracted overnight at -20°C. The next day, samples were warmed to room temperature, vortexed for 5 min, and centrifuged at 15,000×g for 10 min. DOX was added to whole heart homogenate extracted from saline-treated mice to obtain a standard curve. Quantification of DOX was performed fluorometrically at λ<sub>ex</sub>470 and λ<sub>em</sub>590. Concentrations obtained include DOX fluorescent metabolites.

**Statistical analysis**

All data are expressed as mean ± SE for n = 3-7 animals/group. Statistical analysis was performed using Student’s t-test or two way-ANOVA followed by a multiple comparisons test using GraphPad 4.0 software (San Diego, CA) as indicated in figure legends.
**Results**

Mrp1⁻/⁻ mice displayed the same physical characteristics as WT mice after DOX treatment, including lethargy and weight loss (Table 3.1). Body weight was decreased in both groups by almost 10% 24 h after DOX treatment and was decreased 16% by 72h (Table 3.1). Hearts in both WT and Mrp1⁻/⁻ mice appeared visibly smaller in size after DOX treatment when compared to saline treated mice. However, due to the decreased body weight after DOX treatment, heart weights were similar in all treatment groups (Table 3.1). In this regard, both WT and Mrp1⁻/⁻ mice respond similarly to DOX treatment.

**Ultrastructural changes in left ventricle after DOX treatment**

DOX has been shown to cause cardiac injury that can be measured through morphological changes in the C57Bl/6 mouse as assessed by EM of left ventricle tissue slices. Velez et al showed that C57/BL6 mice had increased damage to mitochondria and total cell damage 72 h after DOX treatment when compared to saline treated mice (184). To determine if Mrp1⁻/⁻ mice would have different ultrastructural damage compared to WT mice, we examined mitochondrial, cytosolic, and total cell damage in both WT and Mrp1⁻/⁻ mice treated with saline or DOX for 24 and 72 h. Ultrastructural damage was measured based on the criteria explained in the above methods for mitochondrial and cytosol. Cardiomyocytes that displayed at least one characteristic of injury were considered damaged.

After 24 or 72 h DOX treatment, both WT (Fig. 3.1A) and Mrp1⁻/⁻ (Fig. 3.1B) mice developed measurable morphological changes. After 24 h treatment, WT animals
showed mitochondria vacuolization which was enhanced at 72 h with intracellular vacuolization of mitochondria and abnormal cristae (Fig. 3.1A). In Mrp1\textsuperscript{-/-} mice, intracellular vacuolization was seen at 24 h with advanced toxicity at 72 h, including abnormal cristae, degeneration of mitochondria, and markers of autophagy (Fig. 3.1B). Mitochondrial damage was significantly increased in both WT and Mrp1\textsuperscript{-/-} mice at 24 h (6 fold and 5.7 fold, respectively) and 72 h (11.6 fold and 18 fold, respectively) when compared with saline controls, with damage maximal at 72 h in both groups (Fig. 3.1C). However, there were no significant differences between DOX treated WT and Mrp1\textsuperscript{-/-} mice. The percentage of cytosolic damage, although increased, was less than that of mitochondria at both 24 and 72 h after DOX treatment. Cytosolic damage was significantly increased 34 fold in WT mice after 24 h treatment when compared to WT saline controls, but not in Mrp1\textsuperscript{-/-} mice because of high variability, although the percent damage was greater when compared to WT (9.9% vs. 6.2%, respectively) (Fig. 3.1C). Differences between WT and Mrp1\textsuperscript{-/-} mice were not seen until 72 h after treatment, where cytosolic damage was significantly increased in Mrp1\textsuperscript{-/-} mice (18 fold) when compared to WT mice (2.2 fold) (Fig. 3.1C). Total cellular damage was increased after DOX treatment in both groups but there was no difference between WT and Mrp1\textsuperscript{-/-} mice (data not shown).

**Cellular edema and apoptotic nuclei after DOX treatment**

Mitochondrial and cytosolic damage was increased in both WT and Mrp1\textsuperscript{-/-} mice after DOX treatment (Fig. 3.1). However, Mrp1\textsuperscript{-/-} mice only showed increased cytosolic damage when compared to WT in cytosol at 72 h. In addition to advanced toxicity
markers (Fig. 3.1B), edema was prevalent in Mrp1\(^{-/}\) mice compared to controls. Types of edema seen in Mrp1\(^{-/}\) mice included intracellular edema, intercellular edema at tight junctions, and edema around the nucleus (Fig. 3.2A). In addition to edema, Mrp1\(^{-/}\) mice showed evidence of apoptotic nuclei 72 h after DOX treatment (Fig. 3.2A), with an increase of 13\% when compared to saline treated Mrp1\(^{-/}\) mice (Fig. 3.2B). Figure 2C shows a quantitative analysis of apoptotic nuclei in WT and Mrp1\(^{-/}\) mice after DOX or saline treatment.

**Doxorubicin concentration**

Since Mrp1\(^{-/}\) mice showed increased cytosolic damage after 72 h DOX treatment, we determined the concentration of DOX in whole heart homogenate to identify possible differences in DOX retention. It has been previously shown in mdr1a\(^{-/}\) mice that DOX accumulates in heart tissue upon intravenous (i.v.) DOX treatment (10). We measured the DOX concentration 24 and 72 h after treatment but found no differences between WT and Mrp1\(^{-/}\) mice at either time point (Fig. 3.3). Because we used fluorescence as the method of detection, fluorescent DOX metabolites may be contributing to the total DOX concentration.

**Expression of antioxidant proteins after DOX treatment**

Because DOX-induced cardiac toxicity is mainly thought to be induced by oxidative stress, we examined protein expression of key antioxidants involved in detoxifying ROS. Copper Zinc Superoxide dismutase (SOD, SOD1), Manganese SOD (SOD2), and catalase protein expression were assayed by immunoblot 24 or 72 h after treatment with saline or DOX. Saline treated Mrp1\(^{-/}\) mice displayed increased Sod1
protein expression, however, this was not significant upon post hoc analysis (Fig. 3.4A p=0.022, row factor). These results postulate that basal levels of Sod1 may be elevated in Mrp1−/− when compared to saline treated WT mice. There were no differences in protein expression of Sod2 and catalase (Fig. 3.4B,C). Sod1 is a cytosolic antioxidant and potential increased protein levels in Mrp1−/− mice may be a mechanism for protection of the cell against oxidative stress in the absence of Mrp1. Basal protein expression of these antioxidants as well as at earlier time-points after DOX treatment may clarify the potential increases in Sod1 we see in saline-treated Mrp1−/− mice.

**Changes in GSH and GSSG levels after DOX treatment**

Glutathione (GSH) is the most important antioxidant in the cell. It scavenges intracellular hydrogen peroxide (H₂O₂) in reactions catalyzed by the GSH-dependent enzymes glutathione peroxidase (GPx) (50). Enzymatic oxidation of GSH results in GSH disulfide (GSSG), which is then converted back to GSH by glutathione reductase (GR). Glutathione is also used to detoxify endo- and xenobiotics through conjugation, catalyzed by glutathione-S-transferase (GST). Changes in the ratio of GSH to GSSG can indicate the oxidative state of the cell, with decreases in the GSH:GSSG ratio confirming oxidative stress. Since examination of other antioxidants showed that these were unchanged (Fig. 3.4), we examined cellular concentrations of GSH and GSSG at various time points as another measure of oxidative stress. Mrp1−/− mice have increased intracellular basal levels of GSH (147), while overexpression of Mrp1 lowers intracellular levels of GSH and increases its efflux (40). To compare basal concentrations of GSH in WT and Mrp1−/− mice, we measured GSH in whole heart homogenate of untreated mice.
Mrp1<sup>−/−</sup> mice had 75% higher GSH concentrations when compared to WT mice (Fig. 3.5). To determine if GSH and GSSG levels changed after DOX treatment, we examined GSH and GSSG levels at 12, 24, and 72 h after DOX or saline treatment in WT and Mrp1<sup>−/−</sup> mice. GSH levels were unchanged in WT mice at all three time points (Fig. 3.6A,D,G). Mrp1<sup>−/−</sup> mice had increased GSH following both saline and DOX treatment at all time points, with mice at 12 h having significantly increased GSH in Mrp1<sup>−/−</sup> saline and DOX treated mice (Fig. 3.6A) and 72 h Mrp1<sup>−/−</sup> mice having significantly increased GSH in DOX treated animals when compared to WT DOX treated (Fig. 3.6G). Despite Mrp1<sup>−/−</sup> mice showing a trend for increased GSSG levels when compared to WT mice at all time points, GSSG concentrations were only significantly increased in Mrp1<sup>−/−</sup> mice after 72 h DOX treatment (Fig. 3.6H). The ratio of GSH/GSSG was unchanged in all groups at all time points (Fig. 3.6C,F,I). Normally, elevated GSSG levels will decrease the GSH:GSSG ratio, indicative of oxidative stress. Although Mrp1<sup>−/−</sup> mice had increased GSSG 72 h after treatment with DOX, elevated GSH levels kept the GSH:GSSG ratio from significantly changing (Fig. 3.6).

**Altered expression of ABC transporters**

Since Mrp1<sup>−/−</sup> mice had increased cytosolic damage after 72 h DOX treatment but no change in antioxidant levels or the GSH/GSSG ratio, we measured expression of other ABC transporters that could be compensating for the loss of Mrp1. As seen in Chapter 1, many ABC transporters have overlapping substrate specificities. Pgp, MRP1 (except in mice), and ABCG2 have all been shown to transport DOX. Up-regulation of Pgp or Abcg2 could play a compensatory role in Mrp1<sup>−/−</sup> mice to provide further protection from DOX.
toxicity. Although we saw no differences in DOX concentrations between WT and Mrp1−/− mice, these transporters could potentially prevent DOX accumulation at earlier time points. We measured mRNA expression of Abcb1a and Abcb1b in Mrp1−/− and WT mice at 24 and 72 h after DOX or saline treatment. At 24 h, expression of Abcb1a and Abcb1b mRNA were both increased in WT and Mrp1−/− mice treated with DOX, with Abcb1b mRNA being significantly increased (p<0.05) (Fig. 3.7A). At 72 h, only Abcb1b mRNA was significantly increased in DOX treated Mrp1−/− mice (p<0.05; Fig. 3.7B). To further examine the expression of Pgp, we measured protein levels at both time points. Protein expression of Pgp was significantly increased in Mrp1−/− mice 72 h after treatment with DOX (Fig. 3. 7D), confirming a possible compensatory mechanism of Pgp-mediated protection. Expression of Abcg2 protein was also measure at 24 and 72 h time points for all treatment groups but there were no changes in expression among any of the groups (data not shown).
**Discussion**

Doxorubicin is a chemotherapeutic antibiotic drug that causes dose-dependent cardiac toxicity (53). As shown in Figure 1.3, a quinone moiety in DOX undergoes redox cycling, resulting in lipid peroxidation and production of the toxic product HNE (4). HNE is partially detoxified by GSH through formation of the conjugate GS-HNE, which is a substrate for the ABC transporter, Mrp1. Our group has previously shown that mice treated with DOX have increased expression of Mrp1 in the heart, as well as increased HNE protein-adduction (109). In addition, we have also shown that Mrp1 expression increases in heart sarcolemma as well as mitochondria 24 h after DOX treatment (110). Because of increased expression and the ability to transport oxidation products such as GS-HNE, we postulated that Mrp1 plays a protective role in DOX-induced cardiac toxicity. We therefore used a Mrp1−/− mouse model to study cardiac toxicity and oxidative damage *in vivo*. We hypothesized that mice lacking Mrp1 would have increased toxicity upon DOX treatment that would be measurable by cardiac morphology and oxidative damage.

Velez et al. previously showed by ultrastructural electron microscopy analysis, that 72 h after DOX treatment, there was increased mitochondrial, cytosolic, and total damage in cardiac tissue of C57Bl/6 mice (184). Here, we have confirmed increases in mitochondrial and cytosolic damage for both WT and Mrp1−/− mice (Fig. 3.1). In the absence of Mrp1 expression, we expected the Mrp1−/− mice to have increased damage. Interestingly we discovered that Mrp1−/− mice have increased cytoplasmic damage after 72 h DOX treatment when compared to WT DOX treated mice. This increase in
cytoplasmic damage at 72 h most likely reflects increased edema in the Mrp1−/− mice (Fig. 3.2). DOX is commonly known to cause edema in rat and mouse heart tissue (13) due to an increased inflammatory response (1, 86). In our study, some edema was seen in myofibrils of WT mice treated with DOX (data not shown). In contrast, Mrp1−/− mice exhibited more severe edema intracellularly as well as extracellularly. One possible explanation for this is an increased inflammatory response in Mrp1−/− mice. Gibson et al. showed that lipopolysaccharide (LPS) treatment decreased Mrp1 mRNA and protein in microglia (74). Decreased expression of Mrp1 was partially thought to be a result of NF-κB signaling, resulting in retention of toxic chemicals in the cell, as well as decreased cell-cell communication. Inflammation through LPS has also been shown to decrease Pgp expression in the liver through production of pro-inflammatory cytokines (8, 89). DOX treatment is known to cause increases in the pro-inflammatory response by increasing cytokines, including TNFα, IL-1β, and IL-6 in myocardial tissue (139). Therefore, Mrp1−/− mice may be more susceptible to inflammation resulting in increased edema.

DOX-induced cellular apoptosis has been attributed to increased production of ROS (32). In this study, we found a 13% increase in apoptotic nuclei in Mrp1−/− mice treated with DOX (Fig.3.2), with virtually no apoptotic nuclei found in WT DOX treated mice. Increased apoptosis in the Mrp1−/− mice is likely a result of increased cellular damage from a combination of retention of toxic products in the cell and increased edema.
We examined antioxidant protein expression to determine whether Mrp1\(^{-/-}\) mice were possibly protected by altered levels of antioxidants. Increased expression of antioxidant proteins would relieve some of the oxidative stress resulting from DOX treatment by scavenging ROS and RNS. For example, DOX has been shown to decrease mRNA expression of catalase (9). We found that Sod1 protein expression is potentially increased in Mrp1\(^{-/-}\) mice treated with saline at 24 h (Fig. 3.4A), indicating a possible protective role of Sod1 in Mrp1\(^{-/-}\) mice. However, we found no changes in expression of Sod2 or catalase after DOX treatment in both WT and Mrp1\(^{-/-}\) mice (Fig. 3.4B,C). Although the protein levels of Sod2 and catalase did not change, it is possible that antioxidant activity was altered or expression was different at earlier time points. Assays of basal protein expression and enzymatic activity assays would further elucidate the role of key antioxidants in the protection of DOX-induced cardiac oxidative stress.

Glutathione is major antioxidant in the cell and its concentration is tightly controlled to maintain the redox status of the cell. Under oxidative stress conditions, changes in the GSH:GSSG ratio can be used as a measure of alterations in the redox state of the cell. When GSSG levels rise because of oxidative stress, the GSH:GSSG ratio decreases. Importantly in this study, Mrp1 is an essential transporter for efflux of GSH and GSSG. We confirmed previous results from Rappa et al of increased GSH levels in Mrp1\(^{-/-}\) mice (147), with Mrp1\(^{-/-}\) mice in this study having a higher basal level of GSH than WT mice (Fig. 3.5). Mrp1\(^{-/-}\) mice maintained higher concentrations of GSH after both saline and DOX treatment when compared to WT mice (Fig. 3.6). Elevated levels of GSSG, indicative of oxidative stress, were not established until 72 h after treatment with
DOX in Mrp1\textsuperscript{\textminus} mice, the same time increased cytoplasmic damage in Mrp1\textsuperscript{\textminus} mice was observed. Although GSSG levels were increased, there was no change in the GSH:GSSG ratio. This is most likely due to the already elevated levels of GSH in Mrp1\textsuperscript{\textminus} mice. Elevated levels of GSH that are maintained after DOX treatment is one way that Mrp1\textsuperscript{\textminus} mice are more protected against DOX-induced oxidative stress in the heart.

Although we did not measure the intracellular levels of conjugated HNE (GS-HNE) in these studies, increased basal levels of GSH would be expected to have an effect on the levels of free and conjugated HNE in the heart. Mrp1 is the sole transporter of GS-HNE in the heart so that the absence of Mrp1 abolishes GS-HNE transport (111). While conjugation of HNE is a mechanism of detoxification, GS-HNE still retains some toxicity (148) through feedback inhibition of GSTs (99). Therefore, elevated levels of GS-HNE, resulting from decreased efflux in the absence of Mrp1, can lead to increased intracellular levels of free HNE and subsequent toxicity. Our laboratory currently has on-going studies to measure levels of GS-HNE and GST expression in cardiac tissue in Mrp1\textsuperscript{\textminus} and C57Bl/6 mice.

We hypothesized that Mrp1\textsuperscript{\textminus} mice would display increased cardiac toxicity upon DOX treatment because of cellular retention of toxic products such as HNE. We found accordingly that Mrp1\textsuperscript{\textminus} mice have increased cytoplasmic damage including extensive edema and apoptotic nuclei at 72 h after DOX treatment when compared to Mrp1\textsuperscript{\textminus} saline treated mice and WT mice treated with saline or DOX. Upon discovering that Mrp1\textsuperscript{\textminus} mice have increased Sod1 protein levels and higher intracellular GSH than WT mice, we hypothesized the Mrp1\textsuperscript{\textminus} mice may be further protected by increased
expression of other ABC transporters. Since murine Mrp1 cannot transport DOX, we examined expression of other ABC transporters that could efflux DOX, Pgp and Abcg2. Budde et al. showed that mRNA expression of Pgp was increased in cardiac tissue upon treatment with DOX after 3 days (24). We found that Abcb1a and Abcb1b mRNA expression were both increased upon DOX treatment in both WT and Mrp1\(^{-/-}\) mice, with Abcb1b showing the most significant increases in Mrp1\(^{-/-}\) mice treated with DOX (Fig. 3.7). Upon examination of protein expression we found that increased mRNA expression did not correlate with protein levels. Mrp1\(^{-/-}\) mice had significantly higher Pgp expression 72 h after DOX treatment with no changes in protein expression seen in WT mice at either time point. These results indicate that both WT and Mrp1\(^{-/-}\) mice have increased transcription of Pgp, but only Mrp1\(^{-/-}\) mice have alter transcription resulting in increased expression of Pgp protein.

Although Pgp expression is increased, GSH conjugates like GS-HNE are not substrates for this transporter. Pgp may be playing a protective role by increasing efflux of other toxic chemicals, including DOX itself. Although we found no significant differences in DOX concentration between WT and Mrp1\(^{-/-}\) mice at 72 h, it is possible that Pgp plays a role in efflux at an earlier time point that we have yet to examine. Other Mrp transporters are unlikely to play a role in efflux of GS-HNE. Mrp1 has been identified as the primary transporter of oxidized GSH from the heart (134). Mrp2 has overlapping substrate specificity with Mrp1 but very low to no expression in the heart (46). Mrp3 is a low-affinity GSH transporter but also has little to no expression in heart tissue (96), and no protein expression of Mrp4 has been characterized in the heart (44).
In addition, mice that do not express Mrp1 have no active transport of GS-HNE from sarcolemma (111).

Conrad et al. identified several MRP1 single nucleotide polymorphisms (SNP) in humans (42). One SNP in particular, G671V, showed significantly increased DOX-induced toxicity that accounted for 6.4% of incidence of acute cardiac toxicity in a case-controlled cohort clinical study (115). Our group has shown in HEK293 cells, that the SNP G671V has 85% decreased transport of GS-HNE as well as 20% greater intracellular retention of DOX (111). The G671V SNP is located near the Walker A motif in the first NBD of MRP1. Although GS-HNE transport is blunted, transport of other well known substrates like LTC₄ and E₂₁₇G is maintained. These studies indicate that altered MRP1 transport, in particularly decreased GS-HNE transport, may contribute to increased cardiac toxicity upon DOX treatment. However, in our mouse model, complete absence of Mrp1 appears to induce other mechanisms of protection from cardiac injury, including increased intracellular GSH concentrations and increased Pgp expression.

Taken together, Mrp1⁻/⁻ mice are able to partially compensate for DOX-induced cardiotoxicity through increased expression of Abcb1a/b, Sod1, and increased levels of GSH. However, in the absence of Mrp1, these mice show greater cardiotoxicity indicated by greater cytoplasmic and extracellular edema, as well as increased apoptotic nuclei. More studies are needed to elucidate the pathways causing inflammation and edema, as well as advanced apoptosis in Mrp1⁻/⁻ mice. In these studies, using an acute dose equivalent to human toxicity levels limited maximal treatment time to 72 h, because the mice were very ill by day 3. Chronic studies using a lower dose and longer
periods of treatment could further aid clarification of the oxidative stress response in Mrp1−/− mice.
Table 3.1. Decrease in body weight and heart weight in DOX treated mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight (% decrease)</th>
<th>Heart weight (mg/g BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td><em>C57Bl/6 (WT)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n=4)</td>
<td>2.4 ± 1.5</td>
<td>-0.5 ± 1.9</td>
</tr>
<tr>
<td>DOX (n=5)</td>
<td>9.2 ± 2.3*</td>
<td>16.1 ± 2.2*</td>
</tr>
<tr>
<td><em>Mrp1-/-</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n=4)</td>
<td>1.6 ± 2.1</td>
<td>-1.3 ± 1.1</td>
</tr>
<tr>
<td>DOX (n=5)</td>
<td>9.8 ± 0.9*</td>
<td>16.1 ± 2.3*</td>
</tr>
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</table>

Representative body weight (BW) loss (% decrease) and heart weight (mg/g BW) in *C57Bl/6* mice or *Mrp1-/-* mice treated with doxorubicin (DOX) or saline for 24 and 72 h.

All mice treated with DOX had a significantly greater BW loss than those treated with saline. *, p < 0.001 by two-way ANOVA.
Figure 3.1. Ultrastructural damage induced by DOX treatment. Representative high magnification electron micrographs (x10,000) demonstrating ultrastructural lesions identified in C57BL/6J (WT; A) and Mrp1-/- (B) mice treated with Saline or DOX. Ultrastructural examination of mouse hearts at 24 and 72 h after DOX showed significant pathologic changes including: vacuolization within mitochondria (V), mitochondria with abnormal cristae (*) in WT and Mrp1-/- mice. Mrp1-/- mice show (*) organelles associated with autophagy, including lysosomes and lipofuscin (L), and degeneration of mitochondria (arrow). M; mitochondria. Myo; myofibril. (C) Quantitative analysis of damaged areas in C57BL/6J (WT) or Mrp1-/- mice treated with DOX or saline at 24 and 72h. Mitochondrial damage and cytoplasmic damage were analyzed. Mitochondrial damage was significantly increased at 24 and 72 h after treatment with DOX in both strains of mice. In contrast, cytoplasmic damage was significantly increased at 24 h in WT mice and 72 h in Mrp1-/- mice. In all cases, mitochondrial damage was greater than cytoplasmic damage. *p, ≤ 0.05 when compared with saline treatment.
**Figure. 3.2.** Cellular edema and apoptotic nuclei in Mrp1^−/− mice. (A) Representative high magnification electron micrographs (x8,000) demonstrating intracellular edema and apoptotic nuclei in Mrp1^−/− mice. (B) Quantitative analysis of apoptotic nuclei in C57BL/6J (WT) mice or Mrp1^−/− mice 24 and 72 h after treatment with DOX or saline. (C) Table representing the percent of apoptotic nuclei (average ± SE). Apoptotic nuclei were significantly increased in Mrp1^−/− DOX vs. saline (**p=0.002) and WT DOX vs. Mrp1^−/− DOX (*p=0.044). Statistical analysis assumed a binomial distribution.
Figure 3.3. Concentration of DOX in whole heart homogenate. Doxorubicin concentration was measured fluorometrically in WT (white bars) and Mrp1<sup>−/−</sup> (gray bars) mice treated with saline or DOX after 24 and 72 h (n=5). DOX concentration was decreased after 72 h with no significant differences between WT and MRP1<sup>−/−</sup> mice.
**Figure. 3.4.** Protein expression of antioxidant enzymes. Antioxidant protein expression was measured by immunoblot in WT and Mrp1\(^{-/-}\) mice 24 h (white bars) and 72 h (gray bars) after treatment with saline or DOX treatment (\(n=3\)). Quantitative analysis of protein expression of (A) Sod1, (B) Sod2, and (C) catalase, with representative western blots (D). Two-way ANOVA analysis of Sod2 expression determined the column factor (saline vs. DOX) and row factor (C57Bl/6 vs. Mrp1\(^{-/-}\)) to be significant (\(p=0.026\) and \(p=0.022\), respectively), however, post hoc analysis showed no significant differences in expression of Sod1, Sod2, or catalase between treatment groups. Antioxidants Sod2 and catalase were unchanged at both time points in all treatment groups.
Figure. 3.5. Basal Concentration of GSH in whole heart. Concentration of GSH in WT and Mrp1⁻/⁻ mice was measured in whole heart homogenate by HPLC in untreated mice (n=3). GSH was significantly increased in Mrp1⁻/⁻ mice. *, p<0.03 by student’s t-test.
Figure. 3.6. Concentrations of GSH, GSSG, and the GSH:GSSG ratio at 12h (A,B,C), 24h (D,E,F), and 72h (G,H,I) in WT and Mrp1⁻/⁻ mice after saline (white bars) or DOX treatment (gray bars; n=3-5). GSH was significantly increased in Mrp1⁻/⁻ mice 12h and 72h after treatment with saline and DOX relative to WT mice. GSSG was significantly increased in Mrp1⁻/⁻ mice 72 h after DOX, indicative of increased oxidative stress. *, p<0.05; **, p<0.01 by two-way ANOVA. However, GSH:GSSG ratios were unchanged at anytime in WT or Mrp1⁻/⁻ mice treated with DOX or saline.
Figure 3.7. Expression of Pgp after DOX treatment. Quantitative analysis of mRNA expression of Abcb1a (white bars) and Abcb1b (gray bars) in WT and Mrp1⁻/⁻ mice 24 (A) and 72h (B) after saline or DOX treatment (n=5). Each bar represents the mean ± SE. Representative western blot analysis at 24 (C) and 72h (D) after treatment (n=3). Protein expression was significantly increased at 72h in Mrp1⁻/⁻ mice treated with DOX. *, p<0.05 by two-way ANOVA.
CHAPTER 4

DISCUSSION

The objective of the projects summarized in Chapter 2 and 3 were to study protective mechanisms of ABC transporters using biologically diverse, yet relevant models. As reviewed in Chapter 1, ABC transporters play important roles in protecting organisms from potential toxicity by preventing toxic substrates from entering and/or accumulating in the cell. Examples include transporters like Pgp and Mrp1, which are located in the BBB, GI tract, and placenta. In addition, ABC transporters are involved in normal cell function, including secretion of steroids, GSH, and bile substrates. In Chapter 2, we used lactating rat and mouse models to investigate the role of ABC transporters in biliary secretion under conditions of altered metabolic demands. Chapter 3 utilized a drug-induced tissue damage model to examine protective effects of ABC transporters and their role in cellular toxicity. Together, these models characterize unique roles of ABC transporters in toxicity utilizing states of normal cell function and in cellular disease.

Altered biliary secretion during lactation: Role of ABC transporters

Our group has shown that the expression of bile acid transporters Ntcp and Asbt as well as the ABC transporter Bsep/Abcb11, is increased during lactation, increasing with uptake and efflux of bile acids across the hepatocyte (30, 124-126). Cholesterol 7α-hydroxylase (Cyp7a1), which catalyzes the rate-limiting step of bile acid synthesis, along with the total bile acid pool size, are increased as well (198). Others have shown peak lactation to increase rodent food intake two- to threefold (45, 88), as well as
increase day time food intake by 30% (185). Based on these findings, we hypothesized that other bile substrates, such as cholesterol and phospholipid, may have altered concentrations in bile, suggesting that ABC transporters responsible for their biliary secretion have altered expression. To identify possible differences between species, we used C57Bl/6 mice and Sprague Dawley rats at different stages of lactation to analyze transporter expression (Bsep, Abcb4 and Abcg5/g8) and concentrations of bile substrates, i.e., bile acids, phospholipids, and cholesterol, respectively.

The rodent is an excellent *in vivo* model to use in the study of metabolic changes during lactation. Rats have increased bile flow and increased expression of hepatic and intestinal bile acid transporters as discussed above. Both rats and mice have altricial offspring that have relatively short gestation periods. Because of large litter sizes and rapid growth of offspring after birth, there is a high energy demand on the dam to provide nutrition to the pups, whereas precocial offspring have longer gestation periods, smaller litter sizes, and the offspring is born more developed. Therefore rats and mice are ideal models to study metabolic adaptations during lactation. In addition, the lactating rodent is an excellent model to study changes in biliary secretion under normal physiological conditions. Other models used to study bile acids and bile flow utilize bile duct-ligation, altered diet, or knock out animals. Although these techniques are well established, they are more invasive and time consuming when compared to the lactating rodent model. Most lactation studies are performed in cows to study mechanisms of milk production in the mammary gland. We used the rat model because it is a well known species used in lactation studies and it is convenient and useful model
to study metabolic changes in other tissues besides the mammary gland. Also, we utilized the C57Bl/6 mouse model for possible future experiments in transgenic or knockout mice.

The most exciting finding from these studies was the absence of Abcg5 and Abcg8 expression in lactating rats that was seen as early as postpartum day three (Fig. 2.1), despite physiological biliary cholesterol levels, and evidence that G5G8 are required for cholesterol secretion (202). However, when bile secretion was maximized with infusion of increasing concentrations of TC, cholesterol secretion into bile could not be maintained at the highest concentrations of TC (Fig. 2.3) implying that G5G8 are needed for maximal cholesterol secretion. Also, cholesterol secretion was uncoupled from bile acid secretion, confirming decreased transport. These results show that cholesterol secretion in the absence of G5G8 is significantly blunted, but other transport mechanisms can maintain basal cholesterol secretion. As discussed in chapter 2, cholesterol secretion can occur in the presence of acceptor molecules (133, 135). Elevated levels of bile acids, resulting from increased synthesis and increased size and hydrophobicity of the bile acid pool, most likely promote cholesterol secretion by serving as acceptor molecules.

Bile acid synthesis and biliary secretion are the only methods of cholesterol elimination from the body (153). Cholesterol synthesis takes place in the liver and the rate-limiting enzyme in the synthesis of cholesterol is 3-hydroxy-3-methylglutaryl-coenzymeA (HMGCoA). HMGCoA expression is elevated during lactation (73, 189) resulting in increased cholesterol synthesis in the rat (61, 62). Normally, 50% of
cholesterol synthesized in the liver is catabolized by bile acid synthesis, where cholesterol is the precursor molecule (34). As we have shown previously, during lactation the bile acid pool size increases almost three fold, increasing the demand for bile acid synthesis (198). In addition, cholesterol is an essential component of milk to support membrane synthesis and neurodevelopment in the offspring (155). Roughly 40% of cholesterol is synthesized in the mammary gland, with about 11% coming from the rat’s diet (73), leaving 50% of the cholesterol in milk resulting from de novo synthesis in the liver. Taken together, increased demand for bile acid synthesis and cholesterol incorporation into milk provides a physiological explanation for the conservation of cholesterol and the down regulation of G5G8, potentially decreasing biliary secretion of cholesterol during lactation.

Further microarray analysis in our laboratory of control and lactating rats at PPd10-11 confirmed increased Cyp7a1 expression and decreased G5G8 mRNA expression in the liver and small intestine (11). Decreased G5G8 in the small intestine would facilitate increased cholesterol absorption from the diet, further contributing to the conservation of cholesterol. Also, increased bile acids in bile, which in turn would increase bile acids in the GI tract, have been correlated with increased cholesterol absorption (100). Microarray pathway analysis also identified the cholesterol biosynthetic pathway in the liver and intestine to be significantly up regulated (11). Sterol regulatory element binding protein (Srebp), a nuclear receptor in control of lipid and cholesterol biosynthetic genes, was also upregulated in the liver, as well as chaperone proteins that promote its transport to the Golgi and delivery to the nucleus
further elucidating possible mechanisms for increased cholesterol synthesis during lactation.

Other studies in our group have explored the mechanistic regulation of Cyp7a1, which may also regulate other changes in lactation, like decreased G5G8. The liver X receptor (LXR) is a positive regulator of Cyp7a1 and G5G8 and LXRα is increased during lactation in the rat (199). Studies in Cyp7a1 transgenic (Cyp7atg) mice overexpressing rat Cyp7a1, show that these animals have an increased hydrophobicity and size of the bile acid pool, as well as increased cholesterol synthesis and expression of G5G8 (120). These mice have many similarities of bile regulation when compared to the lactating rat, however, G5G8 expression is maintained, indicating there are other factors in lactation that contribute to decreased G5G8 expression. Also, there are also obvious species differences identified in this study, with mice have having a blunted effect with respect to G5G8 down regulation. Species differences may be a result of larger litter sizes in the rat placing a higher energy demand on the dam.

Lactation is a state of negative energy balance in the rodent due to the four- to five-fold increase in energy demand placed on the dam to nurture its offspring (185). As a result, lactation is characterized by hypothyroidism, hypoinsulinemia, and hypoleptinemia (185). Both leptin (154) and thyroid hormone (TH) (19) increase G5G8 expression in mice resulting in increased biliary cholesterol secretion. Insulin has been shown to directly negatively regulate G5G8 in rat hepatoma cells and to blunt the positive transcriptional regulation of forkhead box protein O1 (FOXO1) (17). Therefore, decreased levels of TH and leptin likely contribute to the lack of G5G8 expression in the
rat liver during lactation. Although decreased insulin levels would increase G5G8 expression, other adaptations to increase metabolic efficiency during lactation are sufficient to suppress expression.

In summary, this study identifies a novel mechanism of cholesterol conservation by decreased expression of G5G8 in the liver of lactating rats. Although cholesterol synthesis and absorption are increased, demand for bile acid synthesis and cholesterol incorporation into milk is met by decreased maximal cholesterol secretion. Further studies using the lactating rat model could identify the specific pathways of G5G8 regulation, increasing our understanding of G5G8 genetic defects like sitosterolemia.

**Protective role of Mrp1 in Doxorubicin-induced cardiac toxicity**

Doxorubicin is an anti-tumor antibiotic used in treating a wide variety of cancer and that produces a dose-dependent cardiac toxicity (53). A reactive quinone moiety in the DOX molecule undergoes redox cycling, producing ROS. The major lipid peroxidation product resulting from DOX-induced oxidative stress is HNE, which is detoxified by conjugation with GSH and subsequent efflux by Mrp1 (4). In chapter 3 we examined the role of the ABC transporter Mrp1 in protection against cardiac toxicity of DOX because DOX toxicity is thought to be mainly attributed to oxidative stress. Our group had previously shown that Mrp1 expression was increased at 6 and 24 h following DOX treatment in mice (109). We also showed that HNE adduction of proteins was increased in DOX treated mice and maximal at 72 h (109). Additionally, Mrp1 expression was increased in mitochondria and was shown to be functionally active (110). Based on these findings and previous evidence the Mrp1 can transport GS-HNE,
we hypothesized that Mrp1 could play a protective role in DOX toxicity and used the Mrp1^{-/-} mouse as a model to study these effects.

The C57Bl/6 mouse was a necessary model used in these studies. These mice show reproducible cardiac injury after DOX treatment (66) and are a common background for Mrp1^{-/-} and transgenic mice. The 20 mg/kg dose, which converts to a human dose of 60 mg/m^2, is clinically relevant to treatment of small cell lung cancer where patients receive between 45-72 mg/m^2 (144). Treatment time points of 24 h and 72 h were chosen, based on previous data showing substantial Mrp1 protein expression increase by 24 h and maximal HNE protein adduction at 72 h (109).

In the current study, we found that mitochondrial ultrastructural damage in both WT and Mrp1^{-/-} mice treated with DOX was similar, with the only significant difference being increased cytosolic damage in Mrp1^{-/-} mice at 72 h. This led to the discovery of advanced edema and apoptotic nuclei in Mrp1^{-/-} mice at 72 h. We confirmed previous studies (147) that showed Mrp1^{-/-} mice to have elevated cellular levels of GSH, as well as increased mRNA expression of Pgp in mice after 72 h DOX treatment (24). Pgp protein expression was unchanged in the WT mice but significantly increased in Mrp1^{-/-} mice 72 h after DOX treatment. Sod1, a cytosolic antioxidant, expression was increased in Mrp1^{-/-} mice 24 h after saline treatment as well, suggesting that Mrp1^{-/-} mice have increased basal levels of Sod1. These data imply that the combination of higher GSH levels, increased Sod1 and increased Pgp protein expression in Mrp1^{-/-} mice blunts the overall cellular damage after DOX treatment. However, the lack of Mrp1 increases intracellular and cell junction edema, as well as apoptotic nuclei, indicating advanced
toxicity at 72 h. Further studies are needed to elucidate the mechanism of edema and apoptosis in the absence of Mrp1.

The up regulation of Pgp in Mrp1−/− mice may play a protective role by decreasing or preventing DOX accumulation within the cell, as DOX is a substrate for Pgp. However, when we examined DOX concentrations at 24 and 72 h, there were no differences between WT and Mrp1−/− mice. This could be due to clearance of DOX by 72 h. Earlier time points are needed to study changes in DOX accumulation in the heart. Also, the concentration of DOX measured by fluorescence includes fluorescent DOX metabolites, which may not be substrates for Pgp. In addition, DOX causes ATP depletion (141), which may lower the functional capacity of ATP-dependent transporters like Pgp. It is also possible that Pgp adduction with HNE may be taking place, as we previously showed this occurred in the case of Mrp1 (109).

As discussed in Chapter 3, increased edema in DOX treated Mrp1−/− mice could result from increased inflammation. Mrp1 expression has been shown to be up regulated when inflammation is caused by LPS in microglia (74), where other studies show Pgp expression is decreased (8, 89). Studies have also shown Mrp1−/− mice have decreased inflammatory responses, most likely due to decreased LTC4 transport (194). Dystrophin, a structural protein in muscle, was found to have decreased membrane localization upon DOX treatment in rats (29), indicative of sarcomeric actin/myosin protein disruption. In the same study, sarcolemmal permeability was also increased. Taken together, altered responses to inflammation and decreased membrane integrity may play a role in enhancing edema in Mrp1−/− mice.
Glutathione plays an important role in cellular apoptosis. GSH release from cells during apoptosis causes intracellular depletion of GSH (71, 72, 87, 181). Apoptosis induced by damaging agents depends on an imbalance of the redox state within the cell and GSH efflux can be sufficient to cause cytochrome c release from the mitochondria and subsequent apoptosis (43). Mrp1 expression is essential for GSH export (87, 204). Over expression of Mrp1 decreases intracellular GSH and increases GSH export (204), and results in increased cytotoxicity (178, 194). Despite increased efflux of GSH, HEK293 cells over expressing Mrp1 that are treated with an apoptosis-inducing agent appear to be protected by increased synthesis of GSH (130). As we have shown, GSH levels in Mrp1/− mice are increased above basal levels in normal mice and remain higher through 72 h of DOX treatment. Although increased intracellular levels of GSH would appear to be protective, we did observe increased apoptotic nuclei in Mrp1/− mice that were not present in WT mice upon DOX treatment. There is some evidence that GSH conjugate efflux is essential for increased synthesis of GSH. In MCF-7 cells, efflux of GS-conjugates lowered intracellular levels, resulting in oxidative stress and increased Nrf2 signaling to restore GSH levels in the cell (167). However, this effect was lost when Mrp1 inhibitors were used. Therefore, increasing GSH levels in the cell to protect against oxidative stress may depend on the presence of a GSH transporter. In this study, although GSH levels were elevated in Mrp1/− mice, upon DOX treatment, the GSH concentrations inside the cell remained fairly constant, as did the concentration in WT mice. Although WT mice express Mrp1, it is plausible that by 24 h or 72 h there is decreased function because of its adduction by HNE.
**Future Directions:** ABC transporters play important roles in cellular function and toxicity

The human genome project has been used to identify single nucleotide polymorphisms and their effects on gene expression and function. There are currently 13 genetic diseases associated with ABC transporters (20). These SNPs result in changes in protein expression, function of the transporter, and drug disposition and response. This information helps to identify individuals that may be at risk for adverse drug reactions or hereditary diseases (102). It is important to investigate the physiological role that ABC transporters play, including normal cell function and toxicity, to fully appreciate their contribution to cell function and cell survival. It is apparent from previous studies and those presented here, that regulation of ABC transporters and outcomes of their altered expression are complicated. For example, in the lactating rat, expression of G5G8 is drastically reduced with no negative effects, whereas in humans and other animal models, absence of G5G8 results in sitosterolemia (15). These are obviously two completely different physiological states that can be used to learn key regulation pathways of G5G8. Although women usually only have one child at a time, compared to rodents who have litters, the lactating rat is still an essential tool in learning more about risk factors occurring in pregnancy and lactation. Lactating women have been shown to have hyperlipidaemia beginning during the second trimester and remaining elevated through four weeks postpartum (146). However, lactating women had a more rapid decrease in triglycerides and cholesterol after that time than women who bottle-fed (146). With increasing number of pregnancies, women are more likely
to develop gallstones as well (179). Women who do not breastfeed, or do so only for a short period of time, have greater weight retention and fat mass accumulation. This results in higher blood pressure, serum glucose, and insulin resistance, all of which can be associated with cardio-metabolic disorders later in life (196). Therefore, metabolic changes that we see in the rat, like decreased leptin, insulin, and thyroid hormone, as well increased cholesterol synthesis and altered transport, may help us understand metabolic diseases seen in women who do not breastfeed and thus provide strategies to reverse the effects.

Next we used a disease state model to look at the role of ABC transporters using a chemotherapeutic drug, doxorubicin. The ABC transporters Pgp, Abcg2, and Mrp1 have been studied extensively based on their ability to transport DOX and subsequent drug resistance effects. Although these transporters are best illustrated in causing MDR in tumors, there are substantial studies on cardiac toxicity in normal tissue after DOX treatment. Here we used Mrp1\(^{-/-}\) mice to see if Mrp1 plays a protective role in DOX-induced cardiac toxicity. As discussed above, GSH appears to be playing a pivotal role in the toxicity we see after DOX treatment, since Mrp1\(^{-/-}\) mice have elevated intracellular levels. Several SNPs have been identified, one in particular is the G671V variant, located in the Walker A motif in the first NBD. Patients with the G671V SNP have increased cardiac toxicity and work in our laboratory has shown 85% decrease in GS-HNE transport as well as increased intracellular DOX accumulation in HEK293 cells (111). However, we have had confounding data when trying to measure GS-HNE levels in heart tissue of WT and Mrp1\(^{-/-}\) mice at 72 h, possibly due to an acute inflammatory response from the i.p.
injection, whether treated with saline or DOX. Also, the Mrp1−/− mice appear to have blunted response to oxidative stress-induced levels of GS-HNE in both DOX and saline treatment groups, possibly from the inability to efflux GSH conjugates. Despite these effects at 24 h, by 72 h the Mrp1−/− mice have more cytotoxic characteristics that the WT mice. These data are essential for understanding the importance of GSH efflux as well as GS-conjugation in instances of oxidative stress.

In conclusion, ABC transporters play essential roles in normal physiological function of cells, as well as disease states. Here we studied different instances of ABC transporter regulation and involvement in efflux of substrates such as cholesterol and GSH. Although these two systems are unique, they both provide insight into the complicated regulation of transporters and their substrates. Further studies to elucidate the mechanism of decreased G5G8 during lactation and DOX-induced oxidative stress in Mrp1−/− mice will provide essential information in determining treatment of metabolic disorders and DOX-induced cardiac toxicity, respectively.
Appendix A: Membrane preparation from liver tissue

**Solutions:**

**Buffer: A**

<table>
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<tr>
<td>20 mM Tris-HCl (pH 7.5)</td>
<td>10 ml 1 M Tris</td>
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<tr>
<td>2 mM MgCl₂</td>
<td>1 ml 1 M MgCl₂</td>
</tr>
<tr>
<td>0.25 M sucrose</td>
<td>42.8 g</td>
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</tbody>
</table>

Add Tris and MgCl₂ to 400 ml water, pH to 7.5. Add sucrose and qs to 500 ml. Store at 4°C for up to 1 month.

**Buffer B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>25 ml 1 M Tris</td>
</tr>
<tr>
<td>80 mM NaCl</td>
<td>2.34 g</td>
</tr>
<tr>
<td>2 mM CaCl₂</td>
<td>50 mL 20 mM CaCl₂</td>
</tr>
<tr>
<td>1% Triton X-100</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Add Tris, NaCl, CaCl₂, and Triton X-100 to 400 water and pH to 8, qa to 500 mL. Store at 4°C for up to 1 month.

**Protocol:**

1. Harvest liver tissue (100-200 mg). Use fresh or freeze at -80°C until use.
2. Homogenize on ice in Buffer A (Polytron homogenizer). Sample volume can be increased with Buffer A to appropriate tube size.
3. Spin for 10 min., 2,000 x g @ 4°C (SS-34 Sorvall rotor)
4. Collect supernatant. Do not take any of the pellet or fat that may be on top of supernatant.
5. Place in centrifuge tube for Ti 72 rotor.
6. Centrifuge at 100,000 x g 45 min, 4°C.
7. Decant supernatant. Leave tube upside down to drain for 1 min.
8. Resuspend pellet in Buffer B. Use pipette or insulin syringe (Baxter 59505-1) to completely resuspend pellet. Transfer to 1.5 ml eppendorf tube. Store at −20°C.
9. Use BCA protein assay. Add sample loading buffer and immediately prior to SDS-PAGE, add β-ME to a final concentration of 1.2% and heat at 95°C for 5 min.
Appendix B.

Isolated Liver Perfusion Protocol

Krebs-Henseleit Perfusate Buffer

Stock Solutions

I. 138.5g NaCl in 1 L dH₂O
II. 41.8 g NaHCO₃ in 1 L dH₂O
III. 3.23g KH₂PO₄
    5.84g MgSO₄
    7.07g KCl in 1 L dH₂O
IV. 3.73g CaCl₂ in 1 L dH₂O

Final Solution: 50 mL of I, II, III, VI
5 mM glucose (0.901g)
qs to 1 L with dH₂O
pH to 7.4 with HCl

Final Concentrations: 118 mM NaCl
25 mM NaHCO₃
1.2 mM KH₂PO₄
1.19 mM MgSO₄
4.74 mM KCl
1.27 mM CaCl₂

Protocol for liver perfusion and bile collection in mice and rats:

- Prepare Krebs-Henseleit perfusion buffer and maintain in 37°C and oxygenate perfusate with 95% O₂-5% CO₂ for duration of perfusion.

- Anesthetize mouse or rat with Urethane (1g/kg ip).

**Maintain body temperature throughout experiment by placing the mouse on a metal tray on top of a heating pad and use wet/warm gauze on top of the wound and periodically moisten with warm saline. Alternatively aluminum foil can be used.**

Gallbladder Cannulation in mice:

1. Make medial incision along midline of the abdomen (caudal to the cranial direction) stopping at the sternum. Then cut skin laterally and set it out of the way.

2. Locate the duodenum and common bile duct by gently moving intestine from animal cavity.
3. Ligate the common bile duct (redirects bile to the gallbladder)

4. Use forceps to pull up gently on the sternum

5. Locate the gallbladder. There is a piece of connective tissue that connects the gallbladder to the sternum. Place a suture around the connective tissue, tie a loose knot, and pull the loose suture down to about the midline of the gallbladder. Use at least 2 sutures around the gallbladder.

6. Hold the top of the gallbladder with tweezers and make a small incision in the upperpart of the gallbladder with vannas scissors

7. Keep holding the gallbladder with tweezers and insert PE-10 tubing through the incision until the tubing is close, but not at, the bottom of the gallbladder.

8. Very carefully tie the loose suture(s) around the horizontal midline of the gallbladder. Bile flow should be immediately apparent in the tubing. If bile stops flowing, pick up the outflow end of the cannula and gently reposition until the bile starts flowing again

(30g mouse (~1g liver), expect 10 uL of bile every 10 min (1uL/min/g liver))

**Bile duct cannulation in rats:**

1. Make medial incision along midline of the abdomen (caudal to the cranial direction) stopping at the sternum. Then cut skin laterally and set it out of the way.

2. Locate the duodenum and common bile duct by gently moving intestine from animal cavity.

3. Tie 2 loose sutures around bile duct.

4. Gently pull up on bile duct and use 26G needle to make a small puncture in bile duct.

5. Keep holding the bile duct with tweezers and insert PE-10 tubing through the puncture, slipping the tubing into the bile duct towards the liver.

6. Once tubing is inserted, tighten sutures around bile duct. Bile flow should be immediately apparent in the tubing. If bile stops flowing, adjust tubing to reestablish bile flow.
**Liver Perfusion:**

1. Locate portal vein next to common bile duct
2. Suture intestinal branch of portal vein so as to not lose perfusate to intestine
3. If collecting perfusate from superior vena cava, tie a lose suture around the inferior vena cava
4. Tie 2 loose sutures around portal vein
5. Insert 25G (blue) needle for mice, 18G (pink) needle for rats, with perfusate already flowing into portal vein (Perfuse with oxygenated Krebs-Henseleit Buffer at a rate of 5mL/min/g liver). Once you see the liver start to turn yellow, immediately cut the inferior vena cava below where you have sutured it.
6. The animal will bleed out. Be careful not to let animal convulse and cause the needle in the portal vein to slip out.
7. Once the animal has bled out, tie the 2 loose sutures around the portal vein to secure the needle.
8. Collect bile in 10 minute intervals using pre-weighed eppendorf tubes. Assume density of water (1g/cm$^3$) to calculate bile volume.

**Perfusate collection through the superior vena cava:**

** If you are not collecting perfusate you can stop here and just let perfusate flow out of the inferior vena cava onto the metal tray.

1. Cut ribcage up the left and right side to open up the chest cavity (be careful not to disturb gallbladder cannula in mice). Cut all connective tissue. Use forceps to hold chest cavity open.
2. Locate the superior vena cava and tie a loose suture around it
3. Use vannas scissors to cut small hole in the top of heart where superior vena cava enters and insert PE60 tubing. Carefully secure by tightening suture.
4. Tighten suture around inferior vena cava. You should see perfusate flow being redirected to the PE60 tubing immediately. Readjust tubing as needed to obtain perfusate out flow.
5. Tie suture around inferior vena cava branch to kidney to prevent loss of perfusate.
Appendix C:

Measurement of Doxorubicin Concentration in Tissue.

Adapted from Laginha et al. 2005 (115)

Extract DOX with acidified isopropanol for each sample:

- 200 µL heart homogenate (diluted 1/10 (wt/v))
- 100 µL 10% v/v Triton X-100
- 200 µL water
- 1.5 mL acidified isopropanol (0.75N HCl)

Total volume: 2 mL

Procedure:

1. Add all mixture components to 2 mL tube.
2. Vortex
3. Extract overnight at -20°C
4. Next day: warm to room temperature
5. Vortex 5 min (or vortex and then rotate tubes 10-15 minutes)
6. Centrifuge 15,000xg for 20 minutes
7. Quantify fluorometrically (λ<sub>ex</sub>=470, λ<sub>em</sub>=590)

Standard Curve:

1. Add known amounts of DOX to acidified isopropanol extracts of homogenized tissue of untreated mice or saline treated mouse homogenate
2. Stock solution of DOX (0.1µg/µL)
3. Samples for standard curve: added 1, 3, 5, 7, and 10 µL of stock to produce standard curve concentrations between 0.01 and 1 µg DOX.
Appendix D:

Reverse phase HPLC analysis of GSH and GSSG

Adapted from Senft et al. 2000 (159)

Material and Reagents:

1. 2x RQB: 12 N HCl 3.33 mL, diethyl triamine penta acetic acid (DTPA) (FW 393.35) 3.9335 g, ascorbic acid (FW 176.1) 3.522 g (Sodium Ascorbate FW 198.1, 3.962 g) – Add DTPA in water and HCl and adjust pH to 6.8 with 0.5 M Tris to dissolve, then add ascorbic acid (pH will decrease to 4.08) qs with ddH2O to 1 L, Filter 0.22, Store at 4°C and protect from light.
2. 1X RQB, dilute from 2x RQB in HPLC water
3. 1 M Triethanolamine (TEA) buffer, pH 8.2. TEA (Sigma T-1502, FW 185.7): 18.57 g per 100 mL. Adjust pH with 5N KOH
4. 10 mM NEM in RQB: NEM (FW 125.1) 12.51 mg/10 mL RQB. Store at 4°C. N-ethylmaleimide (NEM) is added to GSH containing sample to trap thiols and block their oxidation.
5. 100 mM dithiothreitol (DTT) in RQB: prepare 1 M DT (174.1 mg/mL), aliquot and store at -20°C. Dilute 1 to 10 in RQB to get 100 mM DT for working solution.
6. 10 mM GSSG standard in TCA-RQB: GSSG (FW 656.6) 32.83 mg in 5 mL, aliquot and store at -20°C.
7. 10 mM GSH – prepare 5 mL of 10 mM GSH in TCA-RQB with 1.25 g zinc dust, mix, centrifuge, and filter though 0.22 µm cellulose acetate syringe filter. GSH (FW 307.3) 15.36 mg in 5 mL, aliquot and store at -20°C.
8. 50 mM monobromobimane (MBB, FW 271.11 – Sigma B4380): 25 mg in 1.844 mL acetonitrile, aliquot and store at -20°C.
9. 6 N HCl: dilute 1:1 from concentrated HCl
10. 0.5 N NaOH
11. C-18 reverse-phase column and C-18 precolumn filter
12. Mobile phase A - 10% Methanol, 0.25% HOAc
13. Mobile phase B - 50% Methanol, 0.25% HOAc

Sample preparation

1. Aliquot sample for protein concentration Use 40 µg protein for GSH and 250 µg protein for GSSG samples.
2. Bring sample volume up to 54 µL with RQB buffer(one heart per sample).
GSH/GSSG standards

1. Dilute GSH, GSSG standard in RQB to 2.5 and 1.25 mM, respectively
2. For GSH (protocol A)
   a. Add GSH standard (0-1-2-3-4 µL) + RQB qs 54 µL (54-53-52-51-50 µL), 6 µL 0.5 N NaOH, 10 µL HPLC water, 25 µL TEA, 5 µL MBB.
   b. Mix incubate at 45°C in dark for 15 min
   c. Add 10 µL 6N HCl to stop reaction.
   d. Centrifuge 5,000 x g at 4°C for 5 min.
3. For GSSG (protocol B)
   a. Add GSSG standard (0-1-2-3-4 µL) + RQB qs 54 µL (54-53-52-51-50 µL), 6 µL 0.5 N NaOH, 5 µL NEM, incubate 5' RT
   b. Add 25 µL TEA, 5 µL DTT, mix, incubate at 45°C for 30 min
   c. Add 5 µL MBB, mix, incubate at 45°C in dark for 15 min
   d. Add 10 µL 6N HCl to stop reaction
   e. Centrifuge at 5,000 x g at 4°C for 5 min.

HPLC Assay Procedure

1. Supernatant was assayed for thiolbimane fluorescence (GS-MB) by HPLC with fluorescent detector Ex394/Em477
2. Inject standard 10 µL, tissue samples 20 µL.
3. Flow rate 0.8 mL/min (2000 psi). Run on gradient protocol

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
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<td>29</td>
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<tr>
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<td>40</td>
<td>100</td>
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</table>

4. Fluorescence intensities versus time of elution are collected, and peak areas integrated and converted to nmol GSH equivalents from the integrated areas under the GSH/GSSG standard curve.
### Appendix E:

#### List of Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein (ABCG2/Abcg2)</td>
</tr>
<tr>
<td>BSEP/Bsep</td>
<td>Bile salt export pump</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
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<td>CHO</td>
<td>Chinese hamster ovary cells</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DOX</td>
<td>Doxorubicin</td>
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<tr>
<td>E₂₁₇G</td>
<td>Estradiol-17β-glucuronide</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>FXR</td>
<td>Farnesoid X Receptor</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>GS-HNE</td>
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<td>HCl</td>
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<td>HNE</td>
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<td>ICL</td>
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<td>JNK</td>
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<td>MSD</td>
<td>Membrane spanning domain</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Nuclear transcription factor Y</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor-like 2</td>
</tr>
<tr>
<td>NTCP</td>
<td>Na/Taurocholate cotransporting polypeptide</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylincholine</td>
</tr>
<tr>
<td>PFIC</td>
<td>Progressive familial intrahepatic cholestasis</td>
</tr>
<tr>
<td>PGP</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylnsulfonyl fluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Postpartum</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane-X-receptor</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper-Zinc superoxide dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>SP-1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>TC</td>
<td>Taurocholate</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Vita

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Education
August 2006-Present: PhD candidate, Graduate Center for Toxicology, University of Kentucky.

August 2001-December 2005- B.S (Forensic Science) Eastern Kentucky University

Research Experience:
Graduate Research Assistant: University of Kentucky:
Project 1: Alterations in biliary secretion of bile components and their transporters during lactation
Project 2: Protective role of Mrp1 in Doxorubicin-induced cardiac toxicity
Internship: Toxicology laboratory, Kentucky State Police, Frankfort Kentucky. Mass Spectometry analysis of amphetamines.

Professional Skills:
HPLC techniques and troubleshooting
Rodent research: small animal surgery, echocardiograms
Animal husbandry
Molecular biology techniques

Other Experience:
Lecture
Introductory lecture to TOX 509, University of Kentucky 2009-2010
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Treasurer: 2010-2012
President: 2009-2010
Vice President: 2008-2009
Secretary: 2007-2008
1st Year Representative: 2006-2007

Research Publications:

**Oral Presentations**

Donna Coy *Alterations in biliary secretion in lactating rodents* Reproductive Sciences and Women’s Health Forum, University of Kentucky, April 2008

**Poster Presentations**

Donna Coy, Paiboon Junsuwadee, Teresa Noel, Luksana Chaswig, Terry Oberley, Daret St. Clair, Mary Vore *Mrp1 protects against Doxorubicin-induced Cardiotoxicity.* Experimental Biology Meeting 2012, San Diego, California

Donna Coy, Paiboon Junsuwadee, Teresa Noel, Rukhsana Sultana, David A Butterfield, Terry Oberley, Daret St. Clair, Mary Vore *Mrp1 protects against Doxorubicin-induced Cardiotoxicity.* Markey Cancer Center Research Day 2011, University of Kentucky


Antony Athippozhy, Tianyong Zhao, Donna Coy, Clavia Ruth Wooton-Kee, Paiboon Junsuwadee, Arnold J Stromberg, Mary Vore *Detection of differential splicing of Abcg8 in liver of lactating rats compared to virgin controls using exon arrays.* Experimental Biology Meeting 2010, San Diego, California

Jones, G., Jones, D., Coy, D. and Ratnamalala, N. *Farnesoid hormone binding, reception and action in development of Drosophila melanogaster.* Poster Session with . Symposium to Honor Dr. Lynn Riddiford, National Meeting, Entomological Society of America, Dec. 12, 2006