EXPRESSION AND FUNCTION OF DRUG TRANSPORTERS IN AN *IN VITRO*

MODEL OF THE MAMMARY EPITHELIAL BARRIER (BME-UV)

by

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D.V.M., Jordan University of Science and Technology, 2000
M.S., Jordan University of Science and Technology, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition and health status of the udder. The changes in milk composition seem to match the changes in the expression of membrane proteins in secretory mammary epithelial cells that are needed for the movement of molecules from blood to milk and vice versa (Nouws and Ziv, 1982). Thus, an understanding of transporter expression, function and regulation in mammary epithelial cells can provide insight into mammary gland function and regulation.

The goal of this project was to elucidate (molecularly and functionally) the role of drug transporters in the barrier function of an epithelial monolayer cultured from an immortalized bovine mammary epithelial cell line (BME-UV). To characterize the regulation (expression and function) of these drug transporters in BME-UV cells after exposure to cytokine TNF-α for selected periods of time. Representative members of drug transporters of the *SLC* (OCT and OAT) and *ABC* (P-glycoprotein) superfamilies were chosen for this project.

In the first study, the involvement of a carrier-mediated transport system in the passage of organic cation (TEA) and anion (EsS) compounds was elucidated across the BME-UV monolayer. In the second study, molecular and functional expression of bOAT isoforms in BME-UV cells were studied. The final study characterized the effects of cytokine TNF-α on the expression and function of P-glycoprotein, an efflux pump, in BME-UV cells. Cytokine TNF-α exposure induced the expression of *ABCB1* mRNA and increased P-glycoprotein production in BME-UV cells, resulting in a greater efflux of digoxin, a known P-glycoprotein substrate, back into the apical fluid.

The expression, function, and regulation of these transporters in the mammary gland has important implications for understanding the barrier function of the mammary epithelium and, in more specific, for characterizing the role of these transporters in the accumulation and/or removal of specific substrates from milk and/or plasma. Moreover, this study provides an *in vitro* cell culture model of mammary epithelium to characterize mammary epithelial cell function during inflammation.

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Dedication

To my wonderful family, Saja, Omar and Nisreen, without whom none of this would have been possible. All my love.

CHAPTER 1 - INTRODUCTION

Milk

People have used milk and milk products from different species of domesticated animals as important foods for many years ago (Rusoff, 1970). Milk components provide major macronutrients (i.e., protein, fat, and carbohydrate) in a highly digestible form together with the micronutrients (i.e., minerals, trace elements, and vitamins) required for growth and development (Haug et al., 2007). Milk also contains an array of factors associated with the innate immune system that facilitate a neonate's transition from the relatively sterile environment of the uterus to a postnatal environment containing a multitude of microbes, including life-threatening pathogens (Ontsouka et al., 2003). Furthermore, milk contains hormones, growth factors, cytokines, nucleotides, peptides, polyamines, and enzymes (Haug et al., 2007).

Milk, also can be a source of xenobiotic exposure in the form of residues, such as drugs, natural and artificial hormones, and environmental contaminants that have public health and industrial implications. Potential adverse effects of xenobiotic residues to human consumers include acute toxicity, carcinogenicity, reproductive effects, and allergic reactions (Gehring et al., 2006). Moreover, transporter inhibition by xenobiotics may alter the flux of nutrients across the mammary secretory epithelia.

The Lactating Mammary Gland

The bovine lactating mammary gland is composed of a branching network of ducts lined by epithelial cells and ending in alveoli that are surrounded by a single layer of secretory epithelial cells connected by apical junctional complexes. The alveoli are surrounded by myoepithelial cells and a vascularized connective-tissue stroma containing lipid-depleted adipocytes and fibroblasts (McManaman and Neville, 2003). Milk synthesis occurs within clusters of differentiated mammary epithelial cells and is controlled by lactogenic hormones, stromal cells, and extracellular matrix interactions (German and Barash, 2002).

Mammary gland secretion routes

Various solute transport and secretion processes are involved in milk production. These processes also provide pathways for movement of xenobiotic molecules from plasma to milk and

vice versa. The exocytotic pathway is the primary mechanism for protein secretion by alveolar cells and for secretion of water, lactose, oligosaccharides, phosphate, calcium, and citrate. In the lipid secretion process, cytoplasmic lipid droplets are transported to the apical plasma membrane, where they are secreted by a unique budding process as membrane-enveloped structures called bound milk fat globules. Membrane protein transporters offer another pathway that is composed of several distinct, solute-specific mechanisms for transport of monovalent and polyvalent ions and small molecules such as glucose and amino acids across the apical and basal membranes of cells. The transcytotic pathway allows for vesicular transcytosis of proteins such as immunoglobulins and other macromolecules, which involves endocytic uptake of substances at the basal membrane, formation and maturation of endosomes, and sorting to lysosomes for degradation or to the apical recycling compartment for exocytosis at the apical membrane. And the paracellular transport pathway accounts for direct, bidirectional, extracellular movement of low-molecular-weight substances and macromolecular solutes between the alveolar lumen and the interstitial space during pregnancy, with mastitis states, or after involution (McManaman and Neville, 2003).

Mammary secretory epithelium as a barrier

Tight junctions between mammary epithelial cells limit the paracellular movement of molecules, but water-filled pores could offer a pathway for hydrophilic molecules to enter milk by bulk flow. Transcellular movement is another option that would require molecules to pass through the lipophilic apical and basolateral membranes of mammary epithelial cells. Such movement would be driven by the concentration gradient between plasma and milk and may be facilitated by ion- or substrate-selective transporters. Alternatively, xenobiotics could enter milk independent of the concentration gradient by one of the primary or secondary active transport mechanisms, some of which are important for the transfer of essential nutrients from plasma to milk (Berlin and Briggs, 2005; Wilson et al., 1980). Thus, the mammary secretory epithelium, which plays an important role in separating and maintaining differences between blood and milk, is likely a significant barrier to the movement of some xenobiotics into milk (McManaman and Neville, 2003).

CHAPTER 2 - LITERATURE REVIEW

Numerous compounds, such as pharmaceuticals, environmental substances, and metabolites of both exogenous and endogenous origins are charged at physiological pH (Miyazaki et al., 2004). The lipophilic plasma membrane surrounding animal cells impedes the passive diffusion of such charged molecules. Nevertheless, these substances can be concentrated in tissues to exert either therapeutic or toxic effects. The mechanism of cellular entry necessarily involves permeation through specialized membrane proteins called transporters.

In recent years, it has been recognized that transporters are important determinants of the disposition of endogenous, exogenous compounds and their metabolites in the body by altering their absorption, distribution, and elimination. Transporters also have been implicated in drug-drug interactions and toxicities (Audus et al., 1990). For example, P-glycoprotein is expressed in the intestinal tract at the brush border of epithelial cells, where it limits absorption of molecules after oral administration (Thiebaut et al., 1987). Moreover, different secretory epithelia, such as renal and hepatic epithelia, express a variety of membrane transporters, including members of organic anion transporters (OAT), organic cation transporters (OCT), multidrug resistance-associated protein (MRP), and P-glycoprotein (Borst et al., 2000; Kim, 2002; Klein et al., 1999; Koepsell, 1998; Sekine et al., 2000). These transporters play important roles in movement of molecules across the epithelial monolayer by facilitating uptake of molecules or efflux them back to the donor fluid.

Membrane Protein Transporters

Major membrane transporters have been classified into the solute carrier (*SLC*) and ATP-binding cassette (*ABC*) superfamilies (Goodman et al., 2006).

Solute carrier (SLC) superfamily

The SLC superfamily includes genes that encode facilitated transporters and ion-coupled secondary active transporters that reside in various cell membranes. Forty-three *SLC* families with approximately 300 transporters have been identified in the human genome. Many serve in drug absorption and disposition processes (Goodman et al., 2006). Within the *SLC* superfamily, the *SLC22* family, which has polyspecific members, has been identified as mediating the

transport of a variety of structurally diverse organic anion and cation compounds. The *SLC22* family consists of the following major transporter subfamilies: OAT (Sweet et al., 2001), OCT, the carnitine transporter (Wu et al., 1998), and the urate anion-exchanger (Enomoto et al., 2002).

Organic anion transporters (OAT)

Four significant mammal OAT isoforms have been identified; OAT-1 (Sekine et al., 1997), OAT-2 (Sekine et al., 1998a), OAT-3 (Kusuhara et al., 1999), and OAT-4 (Cha et al., 2000). These OAT subfamily members differ in substrate selectivity and localization. For instance, OAT-1, OAT-2, and OAT-3 were reported to be involved in uptake of various endogenous and exogenous substances via the basolateral membrane, whereas OAT-4 was reported to be involved in either uptake or excretion of different substances via the apical membrane (Giacomini et al., 2010; Miyazaki et al., 2004; Sekine et al., 2006; Takeda et al., 2001).

OAT-1 and OAT-3 have distinct substrate selectivity. For example, OAT-1 has high affinity for the classical renal OAT substrate *P*-aminohippuric acid (Pah), but OAT-3 has no affinity for Pah. In contrast, OAT-3 has high affinity for estrone sulfate (EsS), whereas OAT-1 has only a very slight affinity for EsS (Dantzler and Wright, 2003). Acetylsalicylate (Sal) is a selective substrate for OAT-2 (Miyazaki et al., 2004; Sekine et al., 2006), which mediated saturable uptake of Sal when expressed in *Xenopus laevis oocytes* (Sekine et al., 1998a). Also, EsS has been reported to interact, with high affinity, with OAT-4 (Sekine et al., 2006).

The OAT have pharmacological significance because of their selectivity to a wide variety of substrates, including clinically important compounds such as β-lactam and sulfonamide antibiotics, loop and thiazide diuretics, nonsteroidal anti-inflammatory drugs, antiviral agents, and environmental pollutants (e.g., anionic herbicide 2,4-dichlorophenoxyacetic acid) (Sekine et al., 2000; Sweet et al., 2001). All OAT isoforms possess a common structural feature12 putative transmembrane domains (TMD) with large hydrophobic loops between the first and second and between the sixth and seventh TMDs (Sekine et al., 2000).

Organic cation transporters (OCT)

The OCT subfamily contains three isoforms of facilitated transporters called OCT-1, OCT-2, and OCT-3 (Choi and Song, 2008). These OCT isoforms mediate the transport of small,

hydrophilic compounds with at least one positively charged amine moiety at physiological pH. Selective substrates for OCT include the model substrate tetraethylammonium (TEA), clinically important therapeutic drugs (e.g., metformin, procainamide, cisplatin, citalopram, cimetidine), and endogenous compounds such as dopamine and norepinephrine (Gorboulev et al., 1997; Koepsell et al., 2007; Zhang et al., 1997).

Tissue distribution of the OCT isoforms is distinctive. For example, OCT-1 is primarily expressed in the sinusoidal membrane of hepatocytes and is present in the epithelial membrane of the intestine at a low level, whereas OCT-2 is primarily expressed in the basolateral membrane of the kidney proximal tubules. Tissue distribution of OCT-3 is widespread and includes the brain, heart, skeletal muscle, blood vessels, placenta, and liver (Koepsell et al., 2007). Transcripts for OCT-1 and OCT-3, but not OCT-2, have been detected in the lactating rat mammary gland (Gerk et al., 2001b). The OCT isoforms have 12 TMD with a big extracellular loop with glycosylation sites between the first and second TMDs, and between the amino and carboxyl termini on the cytoplasmic side (Ciarimboli and Schlatter, 2005).

ATP-binding cassette (*ABC*) superfamily

Most *ABC* proteins are primary active transporters, which rely on ATP hydrolysis to actively pump their substrates across membranes. There are 49 known genes for ABC transporters that can be grouped into seven subclasses or families (*ABCA* to *ABCG*) (Goodman et al., 2006). One of the most recognized transporters in the *ABC* superfamily is P-glycoprotein (the MDR1 gene product, which is also classified as *ABCB1*), an ATP-driven efflux pump located on the apical side of cells (Chandra and Brouwer, 2004; Kim, 2002; Masereeuw et al., 2003; Mealey, 2004).

P-glycoprotein plays an important role in xenobiotic absorption, distribution, and elimination as a result of its tissue distribution and cellular localization. For example, P-glycoprotein is expressed at the brush borders of epithelial cells in the intestinal tract, where it limits absorption after oral administration and facilitates secretion into the intestinal lumen (Thiebaut et al., 1987). In the liver, P-glycoprotein is expressed at the canalicular (apical) surface of hepatocytes, where it facilitates excretion of drugs and toxins (or their metabolites) into the bile (Chandra and Brouwer, 2004). In the kidney, P-glycoprotein is expressed on the apical

surface of the epithelial cells of the proximal tubules, where it mediates elimination of endogenous metabolic waste products and xenobiotic metabolites (Masereeuw et al., 2003). At biological barriers such as the BBB, P-glycoprotein is expressed on the luminal plasma membrane of the capillary endothelium, where it prevents passage of drugs and toxins across the capillary endothelium into the brain (Fricker and Miller, 2004).

P-glycoprotein mediates active cellular efflux of a large number of drugs and toxic compounds and transports many structurally and pharmacologically unrelated hydrophobic compounds including anticancer agents, immunosuppressive agents, calcium channel blockers, β-blockers, cardiac glycosides, pesticides, anthelmintics, and antibiotics (Marzolini et al., 2004; Sakaeda et al., 2002; Schwab et al., 2003). P-glycoprotein is a large (170 Kd) homodimer with two TMD (each with six helices) and two nucleotide-binding domains (NBD) (Rosenberg et al., 2005). Higgins and Gottesman (1992) proposed a "hydrophobic vacuum cleaner" model for substrate interactions with P-glycoprotein. They suggested that substrates partition into the lipid bilayer before interacting with P-glycoprotein and being expelled into the extracellular aqueous phase. There is substantial experimental evidence to support this model, and it is widely accepted (Eckford and Sharom, 2009).

Transporter expression in mammary epithelial cells

During lactation, the mammary gland epithelium is differentiated into a highly active secretory tissue (Alcorn et al., 2002). mRNA transcripts for 19 different transporter genes have been reported in non-lactating and lactating human mammary epithelia. For example, OCT, OCTN, OATP, MRP, and P-glycoprotein are expressed by human mammary epithelial cells at levels comparable to those in liver and kidney (Alcorn et al., 2002; Ito et al., 2005). Using RT-PCR, different transporters were detected in rat whole mammary gland including Mdr1a, Mdr1b, Mrp1, Oct1, Pept1, and Petpt2 (Gilchrist and Alcorn, 2009) .

Although several membrane transporters have been extensively characterized in rat, mouse, and human (Alcorn et al., 2002; Buist and Klaassen, 2004; Matsushima et al., 2005; Sekine et al., 1997; Takeda et al., 2001), information on bovine membrane transporters is limited. To our knowledge, the only transporters that have been characterized in bovine mammary gland are breast cancer resistance protein (*BCRP*) (Jonker et al., 2005), glucose

transporters (Zhao and Keating, 2007), fatty acid transporter (Bionaz and Loor, 2008), lipid transporters (Mani et al., 2009), and monocarboxylate transporters (Kirat and Kato, 2009).

However, other membrane transporters have been reported in different bovine tissues: monocarboxylate transporters in adrenal gland (Kirat et al., 2009), cationic amino acid transporter and nucleoside transporters in small intestinal epithelia (Liao et al., 2008; Liao et al., 2009), P-glycoprotein in brain microvessel endothelial cells (Perloff et al., 2007), prostaglandin transporter in uterus and fetal membranes (Banu et al., 2005), carnitine transporters in brain capillary endothelial cells (Berezowski et al., 2004), and serotonin transporter in many tissues such as placenta, lung, kidney, and liver (Mortensen et al., 1999).

Regulation of mammary epithelial barrier functions

Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition and health status of the udder (Ontsouka et al., 2003). The changes in milk composition seem to match the changes in the expression of membrane proteins in secretory mammary epithelial cells that are needed for the movement of molecules from blood to milk and vice versa.

Pregnancy and lactation

The mammary gland undergoes dramatic functional and metabolic changes during the transition from late pregnancy to lactation. Rising estrogen levels in blood circulation during early stages of pregnancy in non-lactating cows stimulate mammary ductal morphogenesis, whereas the combined action of estrogen, progesterone, and prolactin induces the proliferative phase of alveolar morphogenesis (Neville et al., 2002). Lactogenesis, the initiation of milk synthesis and secretion, has two stages. Stage I begins in mid-pregnancy with the progressive expression of many, but not all, of the genes involved in synthesis of milk components. Stage II is initiated around parturition and characterized by closure of tight junctions between epithelial cells and secretion of colostrums, and then milk (Neville et al., 2002).

Besides the cellular proliferation and differentiation of mammary epithelium into a polarized epithelium (Neville et al., 2002), significant changes also occur in the transporter array that allow transport of nutrients and endogenous compounds across the mammary epithelium (Alcorn et al., 2002). This altered expression array includes changes in transporter expression

levels of members of the *ABC* and the *SLC* superfamilies (Alcorn et al., 2002). Using RT-PCR, significant changes in transporter expression were observed in rat whole mammary gland during late gestation and different periods of lactation; these changes were stage dependent and transporter dependent (Gilchrist and Alcorn, 2009). Furthermore, expression profiles of approximately 23,000 gene transcripts in bovine mammary tissue on day 5 before parturition and day 10 after parturition were analyzed using microarray analysis; 389 transcripts (1.6%) were significantly differentially expressed at the two stages (Finucane et al., 2008). Of the transcripts with significant changes, 105 were upregulated and 284 were downregulated. The main upregulated genes were involved in milk synthesis and transport activity (amino acid, glucose, and ion transporters), whereas the main downregulated genes were related to cell cycle and proliferation (cyclins, cell division cycle associated proteins, etc.).

Pathological conditions (Bovine mastitis)

Bovine mastitis, a major disease affecting dairy cattle worldwide and a major source of residues found in milk supplies (Booth and Harding, 1986), results from inflammation of the mammary gland. Inflammation severity can be classified into subclinical, clinical, and chronic forms, and the degree of severity depends on the nature of the causative pathogen as well as age, breed, immunological health, and lactation state of the animal (Viguier et al., 2009). The primary cause of mastitis is a wide spectrum of bacterial strains, however, viral, algal, and fungal mastitis also have been reported (Pyorala, 2003).

Mastitis can be associated with changes in mammary epithelial barrier functions that alter milk composition. The most severe alterations in milk composition due to subclinical mastitis, next to high somatic cell count (SCC), are elevated quantities of free fatty acids, a reduction in casein and concomitant increase in whey protein; a reduction in lactose concentration; changes in the concentration of minerals such as sodium, chloride, potassium, and calcium, and an increase in milk pH. The changes in milk constituents are caused by chemical mediators of inflammation (e.g., histamine, cytokines), bacterial toxins, and the activity of enzymes in milk (Brandt et al., 2010).

Proinflammatory cytokines

The mammary gland is protected by a variety of defense mechanisms, which can be separated into two distinct categories: innate immunity and specific immunity (acquired).

Interactions between these two branches of host defense are regulated by cells of the immune system and soluble factors, including cytokines (Sordillo et al., 1997). Cytokines are small proteins (less than 50 KDa) that act as intracellular communication signals in hematopoiesis, stress, inflammation, immunity, and tissue repair (Belardelli and Ferrantini, 2002). Proinflammatory cytokines regulate the activity of immune cells in the pathophysiology of bovine mastitis by increasing the bactericidal capacity of macrophages and neutrophils, promoting recruitment of neutrophils toward the site of infection, and inducing maturation of dendritic cells (Oviedo-Boyso et al., 2007).

Major groups of cytokines reported during intramammary infection of dairy cows include interleukin (IL), colony-stimulating factor (CSF), interferon (IFN), and tumor necrosis factor (TNF) (Alluwaimi, 2004; Bannerman, 2009). TNF-α, which has been detected and monitored in normal and infected bovine mammary glands, is produced by macrophages, neutrophils, and epithelial cells. Different levels of TNF-α have been detected in all lactation, involution, and periparturient periods except a short period before parturition, when it drops below a detectable level (Alluwaimi and Cullor, 2002). In natural coliform mastitis, experimental *Escherichia coli* infection, and a lipopolysaccharide-infused mammary gland model, TNF-α was significantly increased in milk and serum (Nakajima et al., 1997; Persson Waller et al., 2003).

Several *in vitro* and *in vivo* investigations have shown that inflammation and proinflammatory cytokines such as TNF-α alter the expression of membrane transporters (Evseenko et al., 2007; Hirsch-Ernst et al., 1998; Wedel-parlow et al., 2009). A significant increase in *ABCB1* mRNA and protein production of P-glycoprotein was observed in immortalized human brain capillary endothelial cells incubated with TNF-α (Poller et al., 2009). Effects of these cytokines appear at different levels of expression including transcriptional, translational, and post-translational (Englund et al., 2007; Sukhai et al., 2000; Theron et al., 2003).

Other factors

Additional factors such as drugs, toxic agents, and heat shock can modulate the expression of membrane transporters (Kim et al., 2003; Sukhai et al., 2000). It has been reported that the heat shock element may be involved in the alterations of *ABCB1* transcription rates through pathways that are dependent upon protein kinase A (PKA) and the raf oncogene (i.e. raf activation by heat shock, which stimulates the heat shock response, resulted in an induction of P-

glycoprotein activity, whereas inhibition of PKA activity, blocked the heat shock potentiation of P-glycoprotein activity) (Kim et al., 1996). On the other hand, chronic furosemide or hydrochlothiazide infusion caused increases in OAT-1 protein abundance in rat kidney (Kim et al., 2003). However, downregulation of OAT-1 and OAT-3 with impaired secretion of Pah was observed after ischemic acute renal failure in rats (Schneider et al., 2007).

Methods to assess transporter expression, function, and regulation

Providing assay systems that can be used for studying the role of transporters in movement of xenobiotics across biological barriers is becoming increasingly important. These systems can provide insight into function and regulation of these transporters and, possibly, act as screening tools for predicting drug-drug or drug-nutrient interactions. Currently available systems are described in the following paragraphs.

Membrane-based assay system

Inverted plasma membrane vesicles are equivalent to the hepatic microsomes used for *in vitro* drug metabolism studies and are used primarily to obtain information on affinity (K_m) and capacity (V_{max}) of various transporters to selected solutes. The principal advantage of the vesicle approach is evaluating membrane-specific drug transport without the effects of metabolism as a complicating factor (Hopfer, 1978; Lever, 1980). The major disadvantages of this assay system are vesicle heterogeneity, back diffusion of hydrophobic compounds, and the high degree of background binding to cell membranes in vesicle or cellular systems when uptake or efflux of hydrophobic compounds needs to be measured (Keppler et al., 1998).

Cell-based assay systems

These systems can be used in mechanistic studies to assess transport mechanisms, the rate-limiting step in transepithelial transport, and transporter-based drug-drug interactions.

Polarized cell lines without recombinant transporters

Wild-type cell lines provide the advantage of expressing multiple transporters simultaneously. Under appropriate culture conditions, some wild-type cell lines produce polarized monolayers, thus allowing for evaluation of molecular flux in both directions; apical-to-basolateral (Ap-to-BL), and BL-to-Ap. However, it is important that the chosen cell culture

model displays some of the morphological and functional properties that are representative of the corresponding *in vivo* cell layers.

Single- and double-transfected cell lines

A recombinant transporter that are stably, achieved by integration of the gene of interest into the target cell's chromosome, or transiently, not necessarily indicates integration of the gene into the host chromosome and is not passed onto the next generation, expressed in various cell lines can be used to characterize compound transport across the epithelial monolayer. Cultured cell lines used to study compound-transporter interactions include cell monolayers such as MDCK, LLC-PK1, and HEK293. These monolayers can be used to assess uptake and efflux by single recombinant transporters and obtain kinetic measurements (Giacomini et al., 2010).

Polarized cells that stably express multiple transporters (e.g., a recombinant uptake transporter in their basolateral membrane and an efflux transporter in their apical membrane) also have been developed (Matsushima et al., 2005; Sasaki et al., 2004). These cell lines may overcome the limitations of certain single-transfected cells that often lack the endogenous uptake or efflux transporters to provide a complete mechanism for transcellular transport of a molecular entity.

Primary cell-based assay

Primary cells are derived from intact tissues, which, at the time of isolation, express the full complement of transporters present in a given tissue. Thus, primary cells may be used to study drug disposition and clinically relevant drug interactions. However, because most primary cell cultures have a limited lifespan and can adapt to culture conditions over time, strict definition of culture methods is required to ensure proper polarization (Giacomini et al., 2010). Furthermore, primary cultures are often heterogeneous and detailed information on their morphology is lacking (Zhao et al., 2010).

Intact organ

Isolated whole-organ perfusions have an advantage in that they maintain the cellular architecture and *in vivo* environment of the organ. In the case of the mammary gland, which is grounded on interactions between milk-producing cells and their extracellular matrix environment (Howlett and Bissell, 1993; Parry et al., 1987), a whole-organ perfusion would

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seem like a reasonable system. But this technique also has disadvantages: it might require specialized surgical techniques, it has a low throughput capability, and the perfusion rate must match organ blood flow to maintain physiological conditions (Heyman et al., 2002).

In vivo models

Numerous transporter-gene knockout and naturally occurring transporter-deficient animal models have been characterized, and their commercial availability has been increasing steadily. Limitations of knockout and mutant models include species, strain, diet, and housing condition differences, as well as compensatory mechanisms, (Klaassen and Lu, 2008). All of these variables should be considered carefully in data interpretation and in attempts to extrapolate findings across species.

Need for an *in vitro* model

Use of cell culture *in vitro* model systems as an alternative to animal experimentation in basic science research has recently received more attention as a possible screening tool for xenobiotic transport and metabolism studies (Bock et al., 2004; Franke et al., 2000; Gruber and Hartung, 2004). Compared with animal systems, *in vitro* model systems are more cost-effective, have higher throughput, allow for more replicates (stronger statistical power), the mechanism of transport can be evaluated, early signs of cell toxicity can be recorded, and pathological conditions can be induced and molecular mechanisms evaluated (Lundquist et al., 2002). Most importantly, *in vitro* studies may provide scientists with new, basic information about transport mechanisms in biological barriers that will permit them to develop novel strategies for targeting drugs to specific tissue compartments or enhancing drug permeability through now-impermeable biological barriers (Audus et al., 1990).

Cell culture in vitro model

In vitro culture models of polarized epithelial cells grown on permeable supports are used by the pharmaceutical industry to study the movement of drugs across various epithelial barriers. These models can be used for mechanistic studies to assess transport mechanisms, the rate-limiting step in transepithelial transport, and transporter-based drug-drug interactions. These models are considered to be good predictors of the rate and extent of movement of compounds across specific epithelial barriers, as they incorporate not only simple passive diffusion but also

the various other transport mechanisms by which compounds may cross the epithelial barrier (Lehr, 2002).

Primary epithelial cell cultures and several epithelial cell lines, developed from mice, rats, cows and humans, have been used to study drug movement across epithelial monolayers, responses to inflammatory mediators, and other functions of epithelial barriers (Al-Bataineh et al., 2009; Bisbee, 1981; Bisbee et al., 1979; Gordon et al., 2000; Guidry et al., 1998; Huynh and Pollak, 1995; Huynh et al., 1991; Kimura et al., 2006; Taketani and Oka, 1986; Zavizion et al., 1996; Zucchi et al., 1999). One of the most extensively used cell lines is a human colorectal adenocarcinoma cell line (Caco-2). Caco-2 undergoes spontaneous enterocytic differentiation with time in culture and thus forms monolayers of differential epithelial cells joined by intercellular tight junctions (Sambuy et al., 2005). These cells exhibit remarkable morphological and biochemical similarity to the small intestinal columnar epithelium; they are extremely useful for mechanistic studies of drug absorption and are widely used in pharmaceutical companies as an absorption screening assay for pre-clinical drug selection (Maubon et al., 2007).

Another well-known epithelial cell line is MCF-7, a human mammary epithelial cell line that was isolated from a patient with breast cancer (Horwitz *et al.*, 1975b). Lines of normal human mammary epithelial cells include MCF-10 and T-HME (Kim *et al.*, 2002;Lee *et al.*, 2007). However, these cell culture models may not be appropriate for evaluation of the specific transport processes in the bovine because of transporter expression is species and organ dependent (McNamara et al., 1992; Sekine et al., 1998a; Sekine et al., 1998b). Therefore, a bovine-specific culture model of mammary epithelial cells is essential to evaluate the possible carrier-mediated transport of drugs between plasma and milk.

Besides primary culture, there exist some immortalized bovine mammary epithelial cell (BMEC) lines (Huynh and Pollak, 1995; Huynh et al., 1991; Zavizion et al., 1996). The MAC-T epithelial cell line was established from primary bovine MEC by immortalization with SV40 large-T antigen (Huynh et al., 1991). The MAC-T cells possess insulin-like growth factor-I (IGF-I) receptors and respond to IGF-I in terms of increased DNA synthesis (Woodward et al., 1996; Zhao et al., 1992). This cell line has been used mainly to study IGF-I metabolism (Robinson et al., 2000). However, the expression of milk protein by these cells is not stable. Furthermore, MAC-T cells do not respond to epidermal growth factor (EGF), a known modulator of mammary function (Zavizion et al., 1996). Another bovine cell line has also been generated; the HH2A

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which is a spontaneously immortalized bovine mammary epithelial cell line. The HH2A cells express the gene encoding for mammary derived growth inhibitor (MDGI) and contain keratin intermediate filaments and desmosomes that make it useful in investigations of the roles of cell-cell interactions in regulation of epithelial cell proliferation (Huynh and Pollak, 1995). However, the synthesis of casein, the predominant milk protein, has not been reported for this HH2A cells (Zavizion et al., 1996). Another BME cell line is L-1 which is also a spontaneously immortalized bovine mammary epithelial cell line that may only synthesize a spot of β-lactoglobulin when cultured on Matrigel, but the synthesis of casein, the predominant milk protein, has not been reported for this cell line as well (German and Barash, 2002).

The BME-UV cell line was established from primary bovine mammary epithelial cells by stable transfection with a plasmid encoding the thermolabile large T-antigen. The BME-UV cells expressed functional markers such as microvilli and desmosomes and biochemical markers of mammary epithelial cells such as a repertoire of cytokeratins. Moreover, the BME-UV cells are capable of synthesizing α-lactalbumin and α_{s1}-casein. Also, the BME-UV cells showed enhanced proliferation in the presence of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I). The BME-UV cell line is the only known bovine mammary epithelial cell line responsive to EGF (Zavizion et al., 1996). Furthermore, this cell line establish a cell polarity with well-formed tight junctions between adjacent cells, exhibit a transepithelial electrical resistance, and show responsiveness to steroid hormones (Quesnell et al., 2007; Schmidt et al., 2001). It has been also shown that BME-UV cells express metabolizing enzymes that have catalytic activity (Caruso et al., 2009), suggesting BME-UV cell line as a potential *in vitro* model for studying biotransformation in bovine mammary gland. The relatively low monolayer integrity, long cell culture period (14 days), and the expensive components of the media are the main disadvantages of this cells line.

Conclusion

The roles of members of the solute carrier (*SLC*) and ATP-binding cassette (*ABC*) superfamilies in molecule transport have been elucidated in many polarized epithelial barriers (e.g., kidney, liver, blood-brain barrier, and intestine) in many species. But despite the large amount of effort devoted to evaluating these transporters, limited work has been conducted to evaluate the roles of these transporters in the mammary gland. Research described in this

dissertation was focused on the carrier-mediated transport processes of xenobiotic molecules and provides perspective on how the proinflammatory cytokine TNF-α regulates expression and function of these transporters.

CHAPTER 3 - CULTURED MAMMARY EPITHELIAL MONOLAYERS (BME-UV) EXPRESS FUNCTIONAL ORGANIC ANION AND CATION TRANSPORTERS

Abstract

There is ongoing concern about the potential adverse effects of xenobiotic residues in cows' milk to the human consumer. Although drugs that are intentionally administered to lactating dairy cattle are rigorously regulated to prevent harmful residues, there are numerous other potential sources of exposure that are not as easily controlled. For example, cattle may be exposed to mycotoxins, pesticides and/or persistent organic pollutants through feed, water and inhalation of polluted air. Accurate estimates of the rate and extent of excretion of these compounds into milk is important to assess the risk of exposure through cows' milk. In the present study, the expression of carrier mediated transport processes in cultured monolayers of an immortalized bovine mammary epithelial cell line (BME-UV) was determined using a flow-through diffusion cell system, selective substrates and inhibitors of organic cation transporters (OCT) and organic anion transporters (OAT). The basal-to-apical (BL-to-Ap) flux of tetraethylammonium and estrone sulfate significantly exceeded their flux in the opposite direction. The addition of selective inhibitors to the donor compartment significantly decreased the BL-to-Ap flux of either selective substrate. These results suggest that both OCT and OAT are functionally expressed by BME-UV cells.

Introduction

Milk and milk products from different species of domesticated animals have been used as important foods by man for thousands of years (Rusoff, 1970). The United States produced more than 185 billion pounds of milk in 2007. Approximately 60% or 111 billion pounds of this total was consumed as fluid milk (USDA, 2007). This high level of consumption, often by vulnerable sub-populations such as infants and children, makes it very important that milk is safe and free of potentially harmful residues. Potential adverse effects of xenobiotic residues to the human consumer include acute toxicity, carcinogenicity, teratogenicity, allergic reactions and the selection of resistant bacteria (Gehring et al., 2006; Przyrembel et al., 2000). Residues can also interfere with the manufacture of dairy products, such as cheese, which depend on bacterial fermentation for their production (Molina et al., 2003).

An integral part of ensuring that consumed milk is the conduct of mandatory residue depletion studies prior to the approval of drugs for use in lactating dairy cattle (FDA). These studies ensure that there are no harmful residues as a result of intentional administration of veterinary drugs, according to label instructions. But there are numerous other potential sources of harmful residues in milk, including exposure to environmental contaminants through feed, water and inhaled air, as well as extra-label use of veterinary drugs. Drug-drug and drug-feed interactions may also lead to changes in the rate and extent of xenobiotic excretion into milk. Environmental contaminants that may result in harmful milk residues include mycotoxins, heavy metals, pesticides and persistent organic pollutants such as polychlorinated biphenyls and dioxins (Markaki and Melissari, 1997; Patra et al., 2008; Przyrembel et al., 2000; Westin, 1993)

Milk concentrations of xenobiotic residues, and therefore the risk of exposure to the human consumer, depend on the rate and extent to which these compounds are excreted into the milk. This, in turn, is dependent on the physicochemical properties of the xenobiotic and how these interact with the biological environment (Gehring and Smith, 2006). Several authors have developed mathematical models to predict milk concentrations based compounds' physicochemical properties, including lipophilicity, pK_a and molecular weight (Atkinson and Begg, 1990; Begg et al., 1992). The extent of ionization and protein binding are also considered in these models. Although these models are successful in predicting exposure to some compounds, *in vivo* studies in a variety of species have identified drugs that are present in milk at higher than predicted concentrations. Among these are acyclovir (Lau et al., 1987), cimetidine (McNamara et al., 1996) and nitrofurantoin (Kari et al., 1997). Contribution of carrier mediated

transport systems to the movement of xenobiotics from plasma to milk is the most likely explanation for these higher concentrations (Kimura et al., 2006). Messenger-RNA coding for different members of organic anion and organic cation transporter families have been isolated from human mammary epithelial cells purified from pooled fresh breast milk samples (Alcorn et al., 2002) or from cell cultures (Kimura et al., 2006).

In vitro culture models of polarized epithelial cells grown on permeable supports are used by the pharmaceutical industry to study the movement of drugs across various epithelial barriers. Models have been developed for the gut, respiratory and ocular epithelia, amongst others. These models are considered to be good predictors of the rate and extent of movement of compounds across specific epithelial barriers, since they incorporate not only simple passive diffusion, but also the various other transport mechanisms by which compounds may cross the epithelial barrier (Lehr, 2002). It is, however, important that the chosen cell culture model displays some of the morphological and functional properties that are representative the corresponding *in vivo* cell layers were established (Wilson, 1990). Since the most likely rate-limiting barrier to xenobiotic movement from plasma to milk is the mammary secretory epithelium (Nguyen and Neville, 1998; Shennan and Peaker, 2000), Kimura *et al.(2006)* investigated the feasibility of developing an *in vitro* model of this barrier using a human mammary epithelial cell line (HMEC). Kimura *et al*. (2006) found OCTs to be expressed in this cell line. Among these is the human mammary epithelial cell (HMEC) culture model that was developed by Kimura *et al.,* (2006). To the authors' knowledge, a model using bovine mammary epithelial cells has not yet been developed, despite the importance of bovine milk as a source of human nutrition, especially for children and infants. The study described in this manuscript was designed to investigate functional expression of OATs and OCTs in the mammary epithelial cell line (BME-UV) which may play a role in the movement of xenobiotic agents from plasma to milk as well as from milk to plasma. Some of these agents are benzylpenicillin, tetracycline, salicylate, acyclovir, cidofovir, and p-aminohippurate which were classified as substrates for OAT, while verapamil, pyrilamine, quinidine, ganciclovir and cimetidine were classified as substrates for OCT (Sai and Tsuji, 2004).

Materials and Methods

Chemicals

Tetraethylammonium bromide, estrone sulfate, probenecid, 1,1-Diethyl-2,2-cyanine iodide, disodium ethylenediaminetetraacetic acid and Perdrogen® (30% H_2O_2 w/w) were purchased from Sigma-Aldrich (St Louis, MO, USA). [^{3}H]-mannitol (20 Ci/mmol), [^{14}C]-mannitol (55 mCi/mmol), [^{14}C]-tetraethylammonium bromide (55 mCi/mmol) and [^{3}H]-estrone sulfate (50 Ci/mmol) were purchased from American Radiolabelled Chemicals, Inc. (St Louis, MO, USA). Biosol® and Bioscint® were purchased from National Diagnostics, Inc. (Atlanta, GA, USA).

Cell culture

The BME-UV cells were cultured under conditions similar to those described previously (Quesnell et al., 2007a; Quesnell et al., 2007b; Schmidt et al., 2001). Briefly, stock cultures were grown to 65–75% confluency in 25 cm^2 plastic culture flasks (Corning, Inc., NY, USA). Cells were dissociated for passage using a solution containing 0.25% trypsin and 2.65 mm disodium ethylenediaminetetraacetic acid (EDTA, Gibco) in phosphate-buffered saline. Then, dissociated and dispersed cells were seeded on permeable polyester inserts, Transwells® (Corning, Inc.). These inserts measure 24 mm in diameter and have a pore size of 0.4 μm. Typical bovine medium (TBM), which contains little lactose and has concentrations of electrolytes that closely mirror serum, bathed the basolateral aspect of the cells throughout all experiments. The apical aspect was exposed to apical bovine medium (ApM) of low electrolyte-high lactose composition that resembles the ionic composition of milk. Composition of TBM and ApM were reported previously (Quesnell et al., 2007a; Quesnell et al., 2007b; Schmidt et al., 2001). ApM (1.5 mL) and TBM (2.5 mL) were added to the top and bottom compartments that were formed by the monolayers of cells respectively. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Media on both the apical and basolateral aspects of the cells were refreshed daily. Cells were maintained in culture on permeable supports for 2 weeks to form a confluent, polarised and electrically tight monolayer.

Flow-through diffusion cell system

The confluent monolayer of BME-UV cells on the permeable Transwell® support was mounted as a barrier membrane in a flow-through diffusion cell system (Permegear®, Bethlehem,

PA, USA). The system had 14 diffusion chambers, automated receptor fluid collection and a high precision multichannel dispenser (Ismatec SA, Switzerland). The flow rate of receptor fluid through the receiver compartment of each diffusion chamber was controlled at 4 mL/h. Test compounds were added to the donor side of the chamber, and sequential samples were collected from the receiver side at predetermined times over a period of 4 h. Donor compartments of diffusion chambers were capped directly after adding test solutions. A constant temperature was maintained by circulating 37 °C water through the diffusion chambers holder.

Transport studies

The flow-through system was used to determine the rate of permeation of selective substrates for OCTs and OATs across the BME-UV monolayer. To characterise the role of transporters in the substrates' permeation across the monolayer, rates were compared from the apical to the basolateral side and vice versa, in the presence and absence of selective transporter inhibitors and over a wide range of concentrations. In the case of carrier mediated transport, the permeation rate constant is expected to be dependent on the direction of the substrate movement across the mammary epithelial cells [apical-to-basolateral (Ap-to-BL) or basal-to-apical (BL-to-Ap)] and concentration (saturable at higher level) and to decrease significantly in the presence of selective inhibitors.

Test compounds were prepared in both TBM and ApM together with mannitol as a cell monolayer integrity marker (Kimura et al., 2006). The transport experiment was initiated by adding 1.5 mL of test solutions containing 100 μm of either [14C]-tetraethylammonium bromide, a known selective substrate for OCT (Goralski et al., 2002; Goralski and Sitar, 1999; Kimura et al., 2006; Schomig et al., 1993) or [3H]-estrone sulfate, a known selective substrate for OAT (Dantzler and Wright, 2003; Kusuhara et al., 1999; Sai and Tsuji, 2004; Takeda et al., 2001) to the upper compartment (donor) together with 2 μm of [3H]- or [14C]-mannitol (Mnt) depending on the radiolabelled test compound that was used in the experiment. Mannitol was used as a marker for paracellular movement to monitor the integrity of the mammary epithelial monolayer as it is hydrophilic and relatively small molecule (mol. wt 182) (Kimura et al., 2006).

To test, if the permeability of the selective substrates was dependent on direction, the cell monolayer was mounted with either the apical or the basolateral side facing the donor compartment. Each of the 14 diffusion chambers was randomly assigned to a direction.

If directionality was observed in the permeation rate constant, the involvement of transporters was confirmed by examining the effect of an inhibitor of OCT (cyanine) (Goralski et al., 2002; Schomig et al., 1993) and an inhibitor of OAT (probenecid) (Lash et al., 2007; Takeda et al., 2001) on the permeation rate constants of tetraethylammonium (TEA) and estrone sulfate (EsS) in both directions. Finally, if the effect of inhibitors was found to be significant, then permeation rate constants for both TEA and EsS were determined for a range of concentrations.

Three replicates were performed for each treatment in the above experiments. Two control diffusion chambers with only the permeable support as a separator between the donor and receiver compartments were also included in each experiment. Sequential samples of the receptor fluid were collected at 15-min intervals for the first hour, 30-min intervals for the next 2 h and finally at the fourth-hour of the experiment. At the end of the fourth hour, the residual media in the diffusion chambers were aspirated.

Sample analysis

Substrate concentrations were measured in both the donor and acceptor media for each sampling time. One millilitre of sample was transferred into a glass scintillation vial and 0.3 mL of 30% H_2O_2 added for decolorization purposes. After incubating the sample at 55 °C for 1 h, the sample was cooled to room temperature and 15 mL of liquid scintillation cocktail (Bioscint®) were added. To determine the intracellular content of radioactive isotopes, BME-UV cells were lysed by incubating them with 1 ml of Biosol® at 55 °C for 2 h. Fifteen millilitre of liquid scintillation cocktail (Bioscint®) were then added to the lysed cells after the samples cooled to room temperature. Samples of either [^{14}C]- or [^{3}H]-radiolabelled test compounds and mannitol were assayed by dual labelled β-scintillation counting with quench correction (Beckman LS6500; Beckman Coulter, Inc., CA, USA). Automatic calculation of disintegrations per minute was used for each sample.

Data analysis

The permeation rate constant (P_{app}, cm/s) of each test compound and mannitol was calculated using the following equation (Yamashita et al., 2000):

$$Papp = \frac{dX}{dt} x \frac{1}{A \cdot C_0}$$

where (dX)/(dt) (μmol/s) is the slope of the graph obtained by plotting the cumulative amount of the test compound in the receiver compartment vs. time. The slope was measured between time points at which the system was judged to be at pseudo-equilibrium as evidenced by the slope forming a straight line on a linear scale. It can be accepted that sink conditions were maintained throughout the experiment as <10% of the test compound deposited in the donor compartment appeared in receiver compartment and the test compound was continuously removed from the receiver compartment by receptor fluid flow. A (cm^2) is the effective diffusion area of the cell culture insert and C_0 (μm) is the compound's initial concentration in the donor compartment.

Statistical analysis

All statistical analyses were performed using the commercially available software sigmastat[®] (Systat Software Inc., San Jose, CA, USA). Group comparisons were made by repeated measures analysis of variance (ANOVA), followed by Tukey's test when anova showed significant differences among treatments to determine the specific pairs of treatments between which statistically significant differences occurred. Comparison between control and other treatments was made by Student's t-test. The level of significance for all tests was set at $P < 0.05$.

Results

Barrier function of the BME-UV monolayer

The permeation rate constant for both TEA (161×10^{-6} cm/s) and EsS (122×10^{-6} cm/s) in the control diffusion chambers that had only a permeable support was at least 30 and 80 times greater than BL-to-Ap and Ap-to-BL permeation rate constants respectively of either test compounds in the diffusion chambers with the monolayer of BME-UV cells as a separator between donor and receiver compartments. In addition, the permeation rate constant of mannitol across monolayer of BME-UV cells was $0.91 \pm 0.4 \times 10^{-6}$ cm/s. These results indicate that the monolayer of BME-UV cells forms an effective barrier to the movement of these compounds.

Transport study

The cumulative concentrations of TEA and EsS in the receiver compartment as a function of time in the absence or presence of inhibitors are shown in Figs 3-1 & 3-2 respectively. The permeation rate constants of TEA and EsS across BME-UV monolayer under different conditions are summarized in Table 1. Transport of TEA in the BL-to-Ap direction was significantly greater by a factor of 2.6 than that in the Ap-to-BL direction (Table 1). Similar directionality was also observed in the EsS transport experiment (the BL-to-Ap permeation rate constant was 3.3 times greater than in the other direction). The permeation rate constants for TEA were three and two times greater than for EsS in the Ap-to-BL and BL-to-Ap directions respectively.

The permeation rate constant of TEA was decreased significantly by increasing the initial concentration of TEA in the donor compartment from 100 to 500 μm and then to 1000 μm (Fig. 3-3). While for EsS, it was decreased significantly only when the initial concentration of EsS was increased in the donor compartment from 100 to 500 μm. When the concentration was increased from 500 to 1000 μm, there was no significant difference in the permeation rate constant (Fig.3-4).

Inhibition study

The permeation rate constant of TEA was decreased significantly (approximately 49%) only in the BL-to-Ap direction by adding cyanine (OCT-inhibitor) to the donor compartment (Table 3-1). The permeation rate constant of TEA was not changed significantly by adding probenecid (OAT-inhibitor) to the donor compartment in either direction (data not shown). Similar results, but not identical were found for EsS in which the permeation rate constant was decreased significantly (approximately 36%) only in the BL-to-Ap direction by adding probenecid (OAT-inhibitor) to the donor compartment (Table 3-1). The permeation rate constant of EsS was not changed significantly by adding cyanine (OCT-inhibitor) to the donor compartment in either direction (data not shown).

Discussion

The immortalised bovine mammary epithelial cell line (BME-UV) that was used for the present study was selected for its ability to establish a cell polarity and form tight junctions

between adjacent cells when grown as a monolayer on a filter support that acts as a barrier between dissimilar fluid compartments (Schmidt et al., 2001). Accepted criteria for monitoring the quality of monolayers in transport studies include transepithelial electrical resistance (R_{te}), and permeability to hydrophilic markers like mannitol. The diffusion of mannitol across the epithelial monolayers is limited to the paracellular route (Shaw, 1996). In the present study, the P_{app} of mannitol ($0.91 \pm 0.4 \times 10^{-6}$ cm/s) across monolayer of BME-UV cells was comparable with those obtained in mouse mammary epithelial monolayer (1.94×10^{-6} cm/s) (Toddywalla et al., 1997) and in human mammary epithelial monolayer (2.6×10^{-6} cm/s) (Kimura et al., 2006), indicating that the monolayer of BME-UV cells is tight enough to evaluate transcellular drug transport.

The Bronaugh-type flow-through diffusion cell system that was used in this study has numerous advantages. The system allows for automatic sampling of receiver fluid to accurately monitor the absorption/time profile. Multiple replicates of treatment conditions could be obtained cost-effectively. In addition, the permeability to a test compound in two directions, from the Ap-to-BL and BL-to-Ap directions and both passive and active transport processes could be studied. Most importantly, receiver fluid flow maintained effective infinite sink conditions in the receiver compartment throughout the experiment, which reduces the effect of test compound molecules diffusing back from the basolateral to the apical side or vice versa, thereby isolating directional processes of drug movement across the epithelial barrier (Bronaugh and Stewart, 1985).

The directionality of both TEA and EsS transport suggests the functional expression of OCT and OAT, respectively, in the BME-UV cells. A similar polarised transport process for TEA has been identified previously in human mammary epithelial monolayers and attributed to the human OCT (Kimura et al., 2006). Several *in vivo* studies have also suggested that organic anions (N^4-acetylated *P*-aminohippuric acid, N^4-acetylated sulfanilamide and nitrofurantoin) are actively transported across the bovine, caprine and rat mammary epithelium into milk, respectively, as their observed milk/plasma ratio was 3–30 times higher than the predicted value which calculated based on the pKa of drug and the pH of both milk and plasma (Kari et al., 1997; Rasmussen, 1969a, b). Other studies confirmed the presence of a cimetidine transport system in the rat mammary epithelium that is saturable and inhibited by ranitidine (McNamara et al., 1996).

Decreasing values of the permeation rate constant with increasing concentrations being added to the donor compartment serves as further evidence for the involvement of carrier mediated transport in the movement of TEA and EsS across the BME-UV monolayer. This is because saturation of the carriers at higher concentrations leads to the rate of movement no longer being proportional to the concentration gradient. The permeation rate therefore decreases as it is normalised to the concentration gradient. The permeation rate constant of TEA decreased significantly when initial concentrations were increased from 100 to 500 μm and then 1000 μm, suggesting that the carriers were saturated at these high concentrations. Increasing the initial concentration of EsS from 100 to 500 μm also decreased the permeation rate. However, an increase from 500 to 1000 μm in the donor compartment had no effect on the permeation rate constant of EsS, This may explained by a significant contribution of passive diffusion to the movement of EsS across the monolayer of BME-UV cells. The same conclusion was also found by (Koljonen et al., 2008) and (Neuhoff et al., 2005) who studied passive and active transport mechanisms of acidic drugs across Caco-2 cells.

The significant decrease in the permeation rate constant of both TEA and EsS in the BL-to-Ap direction after adding OCT and OAT inhibitors, respectively, also supports the hypothesis that the directional movement of organic cations and anions across the BME-UV monolayer is mediated by transporters. As the test compounds crossed the monolayer in the presence of inhibitors, albeit at a significantly lower rate, passive diffusion may play a role in the movement of these compounds across the mammary epithelium. Further studies are needed to determine the concentrations of inhibitors that are required to saturate the transporters.

This is the first report of the presence of carrier-mediated directional transport in functionally differentiated bovine mammary epithelial monolayers. The BME-UV monolayer appeared to be more permeable to TEA compared with EsS, as evidenced by a lower value for P_{app} for the latter. This could be explained by differences in the number of organic cation and anion transporters per BME-UV cell. It may also be explained by the differences in the physicochemical properties of these compounds and their affinity to the transporters that are present in the BME-UV cells.

Transporters play a key role in governing bodily drug absorption, distribution and elimination. They also play a role in drug–drug interactions and interindividual differences in pharmacokinetic profiles (Ito et al., 2005). Knowledge of transporter expression in the mammary

epithelium and their apical or basolateral directionality is therefore a necessary step to study the mechanisms that result in xenobiotic residues in milk. This is an important concern to the scientists in both pharmaceutical industry and public health field.

A better understanding of the mechanisms that contribute to the movement of xenobiotics across the mammary epithelium would enhance our ability to predict milk xenobiotic concentrations at specific times following drug administration, or after accidental exposure to environmental contaminants, such as mycotoxins, pesticides and toxic metals. The effect of drug–drug interactions and changes in milk composition could also be explored. Milk loss could thereby be minimised by determining the exact amount of milk that should be discarded based on early information about the transport characteristics of potential xenobiotics into milk. Specific transporters responsible for the excretion of these xenobiotics into milk could also be blocked.

The expression of members of the OCT and OAT families on the membrane of the BME-UV cells may relate to a physiological role of these transporters in milk production. Conversely, they may impart a protective role to the lactating mammary gland extruding potentially toxic xenobiotics into milk. But this increases the risk of milk residues that are potentially harmful to suckling off-spring or the human consumer. The potential involvement of drug efflux transporters, such as P-glycoprotein and multidrug resistance associated protein, on the monolayer of BME-UV cells remains unknown and further studies should be performed. Further studies are also required to quantify the activity of these transporters and determine the substrate concentrations that lead to saturation.

Acknowledgment

Figures and tables

Table 3-1.The permeation rate constants (P_{app}) for model substrates across monolayer of BME-UV cells or support alone from apical-to-basolateral (Ap-to-BL) or BL-to-Ap directions in the absence or presence of inhibitors (Api-to-BL) and (BLi-to-Ap).

	$P_{app} \pm$ SD (x 10^{-6} cm/s)				
	Ap-to-BL[¥]	Api-to-BL[¥]	BL-to-Ap[¥]	BLi-to-Ap[¥]	Support
TEA	$2.0 \pm .02$	$1.9 \pm .06$	$5.1 \pm .4*$	$2.6 \pm .2**$	$161 \pm 18***$
EsS	$0.66 \pm .06$	$0.62 \pm .07$	$2.2 \pm .1^{✳}$	$1.4 \pm .08^{✳✳}$	$122 \pm 34^{✳✳✳}$

Different numbers of stars & daggers indicate the permeation rate constants are significantly different (P < 0.05).

[‡]indicates Ap-to-BL: apical to basolateral and BL-to-Ap: basal to apical directions in the absence or presence of inhibitors (Api-to-BL) and (BLi-to-Ap).

Figure 3-1. *In vitro* cumulative concentration–time profiles of tetraethylammonium (TEA). Permeation of TEA across monolayer of BME-UV cells from apical-to-basolateral (Ap-TEA) and BL-to-Ap (BL-TEA) directions in the presence or absence of inhibitors (Ap-(+Cyn)) and (BL-(+Cyn)). Data represent mean ± SD of three replicates.
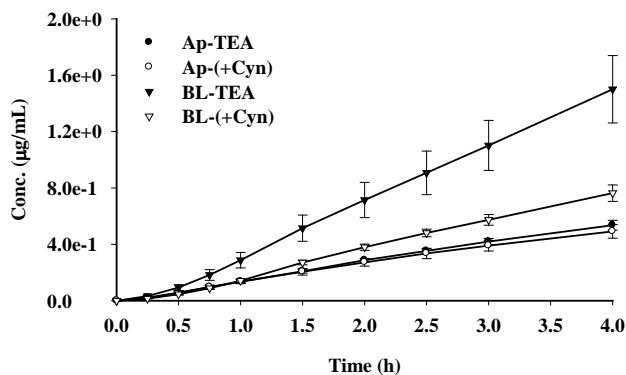
Figure 3-2. *In vitro* cumulative concentration–time profiles of estrone sulphate (EsS). Permeation of EsS across monolayer of BME-UV cells from apical-to-basolateral (Ap-EsS) and BL-to-Ap (BL-EsS) directions in the presence or absence of inhibitors (Ap-(+Prob)) and (BL-(+Prob)). Data represent mean ± SD of three replicates.
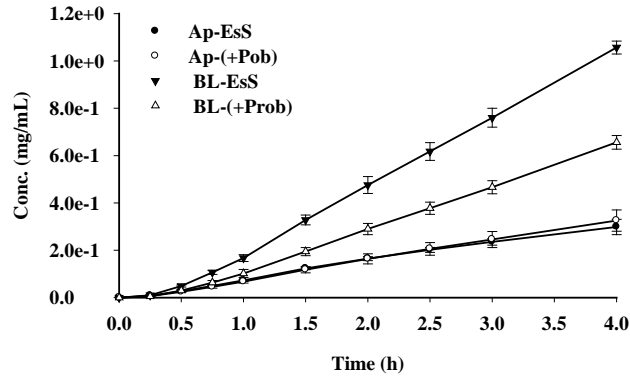
Figure 3-3. Permeation rate constant of tetraethylammonium across monolayer of BME-UV cells from BL-to-Ap (BL) direction. Data represent mean ± SD of three replicates. Different number of stars indicates the permeation rate constants are significantly different ($P < 0.05$).
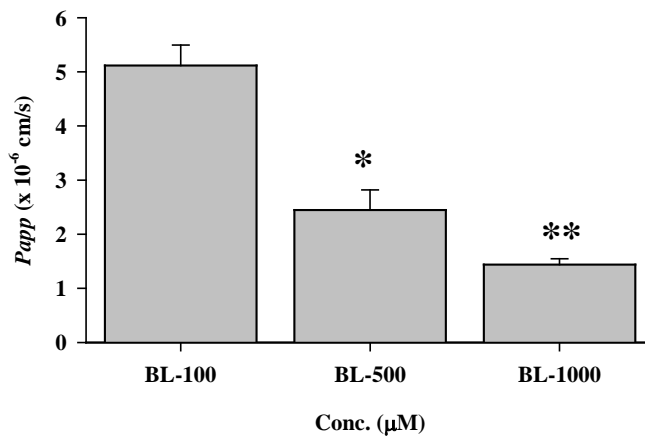
Figure 3-4. Permeation rate constant of estrone sulphate across monolayer of BME-UV cells from BL-to-Ap direction. Data represent mean ± SD of three replicates. Different number of stars indicates the permeation rate constants are significantly different ($P < 0.05$).
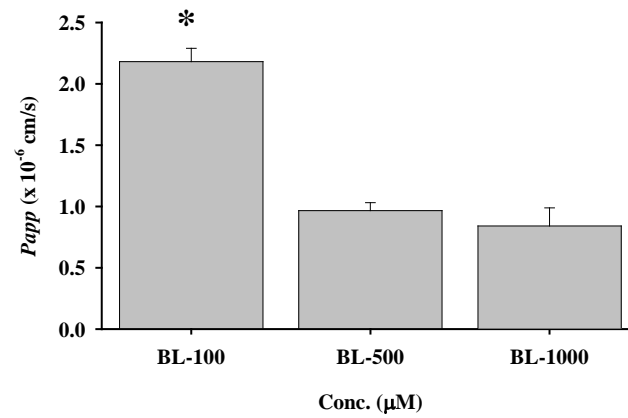
CHAPTER 4 - MOLECULAR AND FUNCTIONAL IDENTIFICATION OF ORGANIC ANION TRANSPORTER ISOFORMS IN CULTURED BOVINE MAMMARY EPITHELIAL CELLS (BME-UV)

Abstract

Mammary epithelial cells express a diversity of membrane transporters including members of organic cation and organic anion (OAT) transporter subfamilies. Four mammal OAT isoforms have been identified: OAT-1, OAT-2, OAT-3, and OAT-4. The pharmacological significance of OAT isoforms has been emphasized because of their role in the movement of a wide variety of substrates across epithelial barriers. The present study identified (molecularly and functionally) bOAT isoforms in BME-UV cells. mRNA expression levels of all tested transporters in BME-UV cells were less than expression levels of the corresponding transporters in bovine kidney. Directionality in the flux of *P*-aminohippuric acid and acetylsalicylate, compounds known to interact with OAT-1 and OAT-2, respectively, across BME-UV monolayers was not observed at the concentrations used in this study. Directionality was, however, observed in the flux of estrone sulfate. Adding probenecid, penicillin G or non-radiolabeled estrone sulfate to the apical donor compartment significantly increased the apical to basolateral flux of estrone sulfate across the BME-UV monolayer. These results suggest that BME-UV cells express an organic anion transport system, making it a potentially useful model to study the role of this transport system in the mammary epithelial barrier.

Introduction

Numerous compounds, such as drugs, environmental pollutants, and metabolites of both exogenous and endogenous origins, exist as organic anions at physiological pH (Miyazaki et al., 2004). These compounds are transported by the organic anion transporter (OAT) system expressed in different epithelial cells, including mammary epithelial cells (Al-Bataineh et al., 2009; Alcorn et al., 2002; Gilchrist and Alcorn, 2009; Sekine et al., 2006). The OAT subfamily,

encoded by the *SLC22A*, is a group within the organic ion transporter family that includes organic cation transporter (OCT), OCT novel type/carnitine, and OAT subfamilies (Koepsell et al., 1999; Sekine et al., 1998a; Sekine et al., 1998b; Tamai et al., 1998; Tamai et al., 1997).

Four mammal OAT isoforms have been identified: OAT-1 (Sekine et al., 1997), OAT-2 (Sekine et al., 1998a), OAT-3 (Kusuhara et al., 1999), and OAT-4 (Cha et al., 2000). These OAT subfamily members differ in substrate selectivity and localization. For instance, OAT-1, OAT-2, and OAT-3 are involved in the uptake of various endogenous and exogenous substances via the basolateral membrane, whereas OAT-4 is involved in either the uptake or the secretion of different substances via the apical membrane (Giacomini et al., 2010; Miyazaki et al., 2004; Sekine et al., 2006; Takeda et al., 2001).

OAT-1 and OAT-3 have distinct substrate selectivity. For example, OAT-1 has a high affinity (Km = 14 μM) for the classical renal OAT substrate *P*-aminohippuric acid (Pah), but OAT-3 has no affinity for Pah. In contrast, OAT-3 has a high affinity (Km = 2.3 μM) for estrone sulfate (EsS), whereas OAT-1 has only a very slight affinity for EsS (Dantzler and Wright, 2003; Sekine et al., 2000). Acetylsalicylate (Sal) is a selective substrate for OAT-2 (Miyazaki et al., 2004; Sekine et al., 2006), which mediates saturable uptake of Sal when expressed in *Xenopus laevis oocytes* (Sekine et al., 1998a). Also, EsS has been reported to interact, with high affinity, with OAT-4 (Sekine et al., 2006). These differences and similarities in substrate affinities make it possible to map transporters functionally that are involved in processes across the epithelial monolayer.

The pharmacological significance of OAT isoforms has been emphasized because of their role in the movement of a wide variety of substrates across the epithelial barrier including clinically important compounds such as loop and thiazide diuretics, nonsteroidal anti-inflammatory drugs, antiviral agents, environmental pollutants (e.g., the anionic herbicide 2,4-dichlorophenoxyacetic acid), as well as sulfonamide and β-lactam antibiotics, (Sekine et al., 2000; Sweet et al., 2001).

Different cell culture *in vitro* models have been established to study drug flux across mammary epithelial cells (Kimura et al., 2006; Toddywalla et al., 1997). An immortalized bovine mammary epithelial cell line (BME-UV) was used in the present study. This cell line was selected as a model for the blood-milk barrier due to its ability to express transport proteins (Al-Bataineh et al., 2009), establish a cell polarity with well-formed tight junctions between adjacent

cells, and exhibit a transepithelial electrical resistance (Quesnell et al., 2007a; Schmidt et al., 2001). The aim of this study was to identify molecularly and functionally bOAT isoforms in BME-UV cells.

Materials and Methods

Expression of genes encoding for the different bOAT isoforms in cultured BME-UV cells were determined using real time-PCR technique (RT-PCR). Transport studies using compounds known to interact with the OAT isoforms were conducted to determine whether the transporters were functional and significantly influenced the flux of substances across the epithelial barrier.

Cell Culture

The BME-UV cells were cultured under conditions similar to those described previously in detail (Quesnell et al., 2007a; Schmidt et al., 2001). Briefly, the BME-UV cells were grown to 65-75% confluency in 25 cm^2 plastic culture flasks (Corning, Inc., Corning, NY). Then, dissociated and dispersed cells were seeded on permeable polyester inserts, (Transwell, Corning, Inc.,) and fed with asymmetrical media. Typical bovine medium (TBM), which contains little lactose and has concentrations of electrolytes that closely mirror serum, bathed the basolateral aspect of the cells throughout all experiments. The apical aspect was exposed to apical bovine medium (ApM) of low electrolyte-high lactose composition that resembles the ionic composition of milk. Composition of TBM and ApM were reported previously (Quesnell et al., 2007a; Schmidt et al., 2001). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Media on both the apical and basolateral aspects of the cells were refreshed daily. Cells were maintained in culture on permeable supports for 2 weeks to form a confluent, polarized and electrically tight monolayer prior to all experiments.

Identification of bOAT transporters in BME-UV cells

RT-PCR

Expression levels of bOAT isoforms (bOAT-1, bOAT-2, bOAT-3, and bOAT-4) in BME-UV cells were determined and compared with expression levels of the corresponding transporters in bovine kidney, one of the major routes of organic anion elimination.

RNA Extraction

RNA was isolated from confluent monolayers of BME-UV cells and bovine kidney lysates using RNeasy Mini kit (QIAGEN, Foster City, California) according to the manufacturer's directions. RNA concentrations were determined by the measurement of optical density at 260 nm (NanoDrop 8000, Thermo Scientific, Rockford, IL). Isolated total RNA integrity was verified by an OD_{260}/OD_{280} absorption ratio greater than 1.8 and less than 2.0. Non-degraded and sufficiently concentrated RNA (~0.5 to 1.0 µg/µl) was used in RT-PCR assays.

Primer Design for RT-PCR

Bovine sequences for each gene were obtained from the National Center for Biotechnology Information Genebank (http://www.ncbi.nih.gov). Primer pairs for target genes (table 4-1) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/), a web-based primer design program. Both primer pairs were custom synthesized by Integrated DNA Technologies (Integrated DNA Technologies (http://www.idtdna.com/Home/Home.aspx).

RT-PCR

The expression of target bOAT isoforms in mammary epithelial cells was determined by RT-PCR (iCycler, Bio-Rad Laboratories, Hercules, CA) with the use of a commercial kit (Quantitect SYBR Green RT-PCR kit and reagents, Qiagen). Reactions were performed in a final volume of 25 µL containing 100 ng of template RNA, 0.5 µM forward and reverse primers, 0.25 µL Quantitect RT Mix, 12.5 µL Quantitect SYBR Green (1x) and 8.25 µL RNase-free Water. Triplicate determinations were performed for each combination of RNA isolation and target sequence in a 96-well optical plate. This experiment was repeated five times (i.e. RNA isolated in 5 different occasions from different passage numbers) with RT-PCR conducted on separate days. RT-PCR assay was conducted in the manner where samples of bovine kidney, no-template reaction and no reverse transcriptase were included in the assay.

RT-PCR Protocol

The RT-PCR protocol consisted of reverse transcription (50°C for 30 minutes), PCR initial activation (95°C for 15 minutes), followed by 40 cycles of denaturing at 94°C for 15s, annealing at 58°C for 30s, and extension at 72°C for 30s. A melt curve analysis from 65°C-95°C at 0.5°C/s was conducted immediately following PCR. Relative gene expression in different treatments to the average untreated was calculated using $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

Transport studies

To study the function of bOAT members in the BME-UV monolayer, we compared the flux of different anionic compounds known to interact with bOAT isoforms from apical-to-basolateral (Ap-to-BL) side and vice versa in the presence and absence of other compounds known to interact with different drug transporters including bOAT. Test compounds were prepared in TBM or ApM together with ^{3}H-mannitol (^{3}H-Mnt). Because Mnt is a hydrophilic and relatively small molecule (mol. wt. 182), it was used as a marker for paracellular movement to monitor the integrity of the mammary epithelial monolayer (Kimura et al., 2006).

Flow-Through Diffusion Cell System

Confluent monolayers of BME-UV cells on permeable supports were mounted as a barrier in a flow-through diffusion cell system (Permegear, Bethlehem, PA). The system has automated receptor fluid collection and a high precision multichannel dispenser (Ismatec SA, Switzerland). The flow rate of receptor fluid through the receiver compartment of each diffusion chamber was controlled at 4 ml/h. Temperature was maintained at 37°C by circulating water through the diffusion chambers' holders. Donor compartments of diffusion chambers were capped directly after adding test solutions. Sequential samples were collected from the receiver side at regular intervals (15 min) over a period of 120 min. Samples of either [^{14}C]- or [^{3}H]-radiolabeled test compounds were assayed by dual labeled β-scintillation counting with quench correction (Beckman LS6500, Beckman Coulter, Inc., CA). Automatic calculation of disintegrations per minute (DPM) was used for each sample.

bOAT-1

The flux of 2.0 µM *P*-aminohippuric acid ([^{14}C]-Pah), a compound known to interact with OAT-1 (Sekine et al., 2006), together with 0.025 µM [^{3}H]-Mnt was studied across the BME-UV monolayer from Ap-to-BL side and vice versa in the presence and absence of probenecid (1 mM), a compound known to interact with OATs (Lash et al., 2007), Verapamil (50 µM), a compound known to interact with P-glycoprotein (Kim, 2002), and EsS (100 µM), a compound known to interact with OAT-3 (Dantzler and Wright, 2003). These compounds were added separately into the donor compartment together with Pah at the start of each experiment.

bOAT-2

The flux of acetylsalicylate (Sal), a compound known to interact with OAT-2 (Miyazaki et al., 2004; Sekine et al., 2006) was studied across the BME-UV monolayer from Ap-to-BL side and vice versa in the presence and absence of probenecid (1 mM). The transport experiment was initiated by adding 1 ml of test solution containing 1.0 µM [^{14}C]-Sal together with 0.025 µM [^{3}H]-Mnt into the donor compartment.

bOAT-4

The flux of EsS, a compound that has been reported to interact with OAT-4 with high affinity (Sai and Tsuji, 2004; Sekine et al., 2006; Takeda et al., 2002), was studied across the BME-UV monolayer in both directions (Ap-to-BL and BL-to-Ap). The transport experiment was initiated by adding 1 mL of test solution containing 0.02 µM [^{3}H]-EsS together with 2 µM [^{14}C]-Mnt to the donor compartment. Moreover, the Ap-to-BL flux of [^{3}H]-EsS across the BME-UV monolayer was determined in the presence and absence of 1 mM probenecid, 100 µM PenG, and 20 µM of non-radiolabeled EsS. These compounds were added separately into the donor compartment together with EsS.

Data Analysis

The flux (µmol/cm^2 per hour) of both tested compound and mannitol were calculated based on the amounts of compound recovered in the receiver compartment per unit time and then were normalized by the effective diffusion area (cm^2) of the cell culture insert. Then, the flux of tested compound was divided by mannitol flux at each measured time point to account for changes in overall epithelial integrity.

Statistical Analysis

All statistical analyses were performed using the commercially available software SIGMASTAT (Systat Software Inc., San Jose, CA). Group comparisons were made by repeated measures ANOVA. When the ANOVA showed significant differences among treatments, the Holm-Sidak posttest was performed to determine the specific pairs of treatments between which statistically significant differences occurred. The level of significance for all tests was set at $P < 0.05$.

Results

mRNA expression of bOAT isoforms in BME-UV cells

Expression levels of all tested transporters in BME-UV cells were less than expression levels of the corresponding transporters in bovine kidney by at least 2 orders of magnitude (Table 4-2). The relative expression of bOAT-4 in BME-UV cells to that in bovine kidney was the highest among tested bOAT isoforms (bOAT-4: $3.1 \pm 0.7 \times 10^{-2}$ fold; n = 5).

Transport studies of organic anion compounds across BME-UV monolayers

The Mnt-normalized flux of Pah across the BME-UV monolayer in the Ap-to-BL direction was not significantly different from the Pah flux in the opposite direction (BL-to-Ap) at the concentration used in the current study (Fig. 4-1). Adding probenecid or verapamil together with Pah to the apical donor compartment had no distinguishable effect on the Ap-to-BL flux of Pah. The same results were observed in the Pah flux in the opposite direction (BL-to-Ap) after adding probenecid or EsS together with Pah to the basolateral donor compartment.

Directionality in the Mnt-normalized flux of Sal across the BME-UV monolayer was not observed at the concentration used in the current study (Fig. 4-2). Furthermore, adding probenecid together with Sal to the donor compartment had no distinguishable effect on the Sal flux in either direction.

The Mnt-normalized flux of EsS across the BME-UV monolayer from BL-to-Ap direction was more than 2 times the flux in the opposite direction (Fig. 4-3). The Ap-to-BL flux of EsS across the BME-UV monolayer was significantly increased by adding probenecid together with EsS to the apical donor compartment (Fig. 4-4). Adding PenG also increased the Ap-to-BL flux of EsS but to a lesser degree than when probenecid was added. The maximum

increase in the Ap-to-BL flux of EsS was observed after adding non-radiolabeled EsS together with [^{3}H]-EsS to the donor compartment.

The amount of EsS that crossed BME-UV monolayers in the presence of probenecid, PenG or non-radiolabeled EsS was significantly higher than the amount of EsS that crossed the BME-UV monolayer when [^{3}H]-EsS was added alone to the apical donor compartment. The highest amount value ($5.4 \pm 0.1 \times 10^{-3}$ nmol) was when non-radiolabeled EsS was added together with [^{3}H]-EsS to the donor compartment, which was double the amount value of EsS that crossed BME-UV monolayer when [^{3}H]-EsS was added alone. Furthermore, the detected intracellular content of EsS, when probenecid, PenG or non-radiolabeled EsS were added together with [^{3}H]-EsS to the apical donor compartment, was higher than when EsS was added alone. The recovery of EsS from the entire system at the conclusion of experiment was 93 ± 2 %.

Discussion

The expression levels of all tested transporters in BME-UV cells were less than the expression levels of the corresponding transporters in bovine kidney, one of the major routes of organic anion elimination (You, 2002). The fluxes of either Pah or Sal across the BME-UV monolayer are similar in both directions (Ap-to-BL or BL-to-Ap) at the concentrations used in this study. Directionality was, however, observed in the flux of EsS across the BME-UV monolayer, this could be attributed to the involvement of the organic anion transport system in the molecules' passage across BME-UV cells.

EsS is a substrate for both OAT-3 and OAT-4. Both of these transporters were expressed in BME-UV cells, with bOAT-4 having the highest relative expression compared to the kidney, where it plays an important role in the excretion of anionic substances. In a previous study (Al-Bataineh et al., 2009), we elucidated the involvement of a carrier-mediated transport system in the passage of EsS across the BME-UV monolayer in the BL-to-Ap direction that was inhibited by probenecid and became saturated at higher concentration.

The excretion of EsS across the apical membrane may involve more than one transport system as demonstrated by the differences in the increase in the EsS flux when probenecid and non-radiolabeled EsS were added. Probenecid is a compound known to interact with OAT, while non-radiolabeled EsS may interact with bOAT as well as other transporters including BCRP and this may explain the higher EsS flux after adding non-radiolabeled EsS than after adding

probenecid to the donor compartment. Probenecid, PenG and non-radiolabeled-EsS increased the Ap-to-BL flux of EsS by either competing with EsS or inhibiting directly the transporters that involved in EsS flux. The concentration of 0.02 µM EsS was selected because it is well below the Km value of EsS when it is up taken by OAT-4 that expressed in *Xenopus laevis oocytes* (Km = 1 µM) (Cha et al., 2001).

Pah and Sal were added to the donor compartment at concentrations of 2 µM and 1 µM, respectively, which are well below the Km values of both compounds (Km = 14.3 µM, and 89 µM, respectively) as determined by uptake into OAT expressing *Xenopus laevis oocytes* (Sekine et al., 1998a; Sekine et al., 1997). The absence of directionality in the flux of either test compounds might be explained by low expression of the transporters for these compounds in BME-UV cells. This conclusion is further supported by the unchanged flux of Pah and Sal when other anionic compounds (e.g. probenecid) known to interact with OAT were added to the donor compartment. The observed flux of both Pah and Sal across the BME-UV monolayer is therefore most likely attributed to saturated transporters-mediated processes with the possible addition of some passive diffusion. Studies with a range of concentrations would be needed to confirm this hypothesis.

Results from the transport studies reported in this manuscript are consistent with low observed mRNA expression of bOAT in BME-UV cells as well as with observations reported from other species (Alcorn et al., 2002; Gerk et al., 2001b). OAT-1 and bOAT-2 were not detected in human mammary epithelial cells purified from pooled fresh breast milk samples (Alcorn et al., 2002), or lactating rat mammary tissue (Gerk et al., 2001b). These authors did not detect OAT-3 and OAT-4 either, although we did detect relatively higher bOAT-4 expression. The most likely explanation for this disagreement is experimental method. We used cell culture (higher amount of mRNA), while tissue and collected cells in milk samples may have yielded less mRNA and was therefore less sensitive. Another reason is species differences in OAT expression (Buist and Klaassen, 2004).

The flux of different anionic compounds known to interact with OAT in our *in vitro* cell culture model (BME-UV) is consistent with other observations reported in lactating cows and other species such as goat, mouse and rat. Earlier *in vivo* studies in lactating cows and rats showed that experimental and theoretical (calculated on the basis of pH partition concept) milk-to-plasma ultra-filtrate ratios were in close agreement for Pah and Sal and not altered by drug

concentrations or probenecid (Gerk et al., 2001a; Miller et al., 1967). Thus, a passive diffusion process may govern the movement of both Pah and Sal from plasma to milk.

The milk concentration of endogenous EsS and EsS infused close-arterially into the mammary gland in bovine and a non-pregnant goat, respectively, was significantly greater than that in plasma, indicating that EsS was actively transferred from blood to milk against a concentration gradient (i.e. EsS cross mammary epithelial barrier by a carrier mediated process) (Heap et al., 1983, 1984).

In summary, the results from the current study indicate that EsS flux across BME-UV cells is transporter-mediated. The transporter that most likely plays a role in this flux is bOAT-4, due to its relatively higher expression compared to other members of this transporter family. bOAT-3 and other transporters such as BCRB may also play a role in EsS flux across the BME-UV cells. Though, the relative importance of the different bOAT isoforms in the milk excretion of compounds *in vivo* remains unknown and will require further studies. The expression of organic anion transport system in mammary epithelial cells has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of bOAT system in the accumulation and/or removal of anionic molecules from milk and/or plasma.

Acknowledgements

Figure and Tables

Table 4-1. Primer sequences and amplicon size (base pairs) for each transporter gene and GAPDH gene.

Transporter gene	Forward primer	Reversed primer	Amplicon size
bOAT-1	GGTGTACCCCACAATGATCC	CCACGTCTTGCACTGTGTCT	218
bOAT-2	CCCTGAACAAAGTAGCTGCC	CACCGACAGATAGACCAGCA	239
bOAT-3	ATTTGATGACCCTGAGGACG	GTGTCTCAGGCAGGAAGAGG	270
bOAT-4	CACCTCCACCATTGTGACTG	TGTAACCCTAGCCCCAGATG	194
bGAPDH	AAGATTGTCAGCAATGCC	ACAGACACGTTGGGAG	297

Table 4-2**.** Relative expression of mRNA coding for bOATs in BME-UV cells compared to the expression of the corresponding transporters in bovine kidney. Data represent mean $\pm$ SE of five replicates.

	Transporter			
	OAT-1	OAT-2	OAT-3	OAT-4
mRNA expression[1]	9.2E-04	33E-04	7E-04	310E-04
SEM[2]	4.5E-04	14E-04	2E-04	70E-04

[1] Expression of mRNA transporter in BME-UV cells relative to mRNA expression in bovine kidney.

[2]SEM: standard error of the mean.

Figure 4-1. Flux of Pah across the BME-UV monolayer in both directions; apical to basolateral (Ap) and basolateral to apical (BL) side.

No directionality is observed in the mannitol-normalized flux of *P*-aminohippuric acid (Pah) across BME-UV monolayers. Pah (2.0 µM) flux across the BME-UV monolayer from apical to basolateral side (Ap) and from basolateral to apical side (BL) in the presence and absence of probenecid (+Prob), verapamil (+Verp), and estrone sulfate (+EsS). Data represent mean ± SE of three replicates.
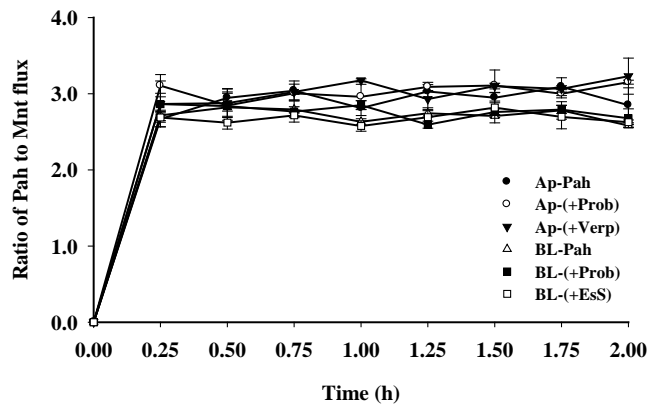
Figure 4-2. Flux of Sal across the BME-UV monolayer in both directions; apical to basolateral (Ap) and basolateral to apical (BL) side.

No directionality observed in the mannitol-normalized flux of Acetylsalicylate (Sal) across the BME-UV monolayer. Sal (1.0 µM) flux across the BME-UV monolayer from the apical to the basolateral side (Ap) and from the basolateral to the apical side (BL) in the presence and absence of probenecid (+Prob). Data represent mean ± SE of three replicates.
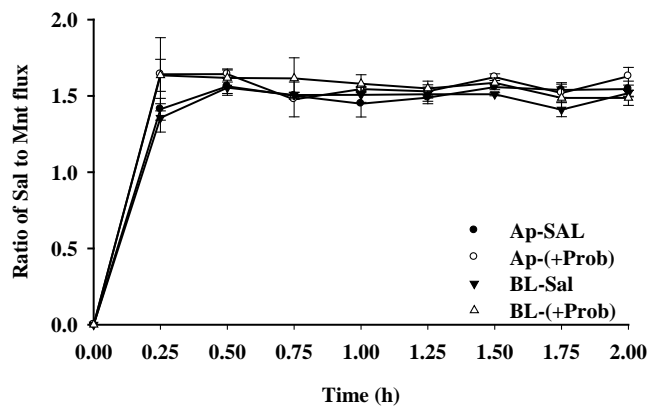
Figure 4-3. BL-to-Ap flux of EsS across the BME-UV monolayer.

BL-to-Ap flux of EsS across the BME-UV monolayer was significantly higher than EsS flux in the opposite direction (Ap-to-BL) ($P < 0.05$). Mannitol-normalized flux of 0.02 µM EsS across the BME-UV monolayer from apical to basolateral side (Ap), and from the basolateral to the apical side (BL). Data represent mean ± SE of three replicates.
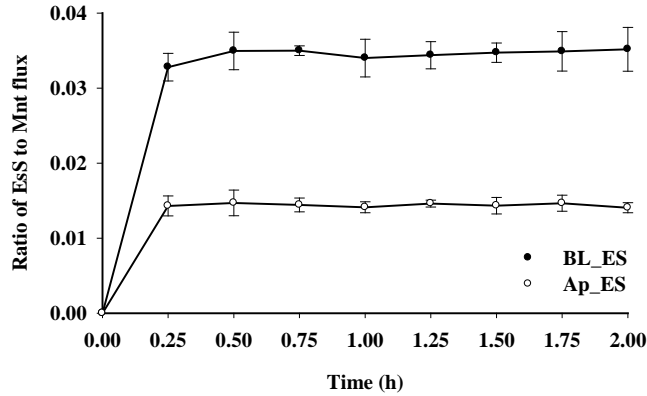
Figure 4-4. Flux of EsS across the BME-UV monolayer in both directions; apical to basolateral (Ap) and basolateral to apical (BL) side.

Probenecid, penicillin G, and non-radiolabeled estrone sulfate (EsS) significantly ($P < 0.05$) increased [³H]-EsS flux across the BME-UV monolayer. Mannitol-normalized flux of 0.02 µM [³H]-EsS across the BME-UV monolayer from apical to basolateral side (Ap) in the presence and absence of probenecid (+Prob), penicillin G (+PenG), and non-radiolabeled EsS (+EsS). Data represent mean ± SE of three replicates.
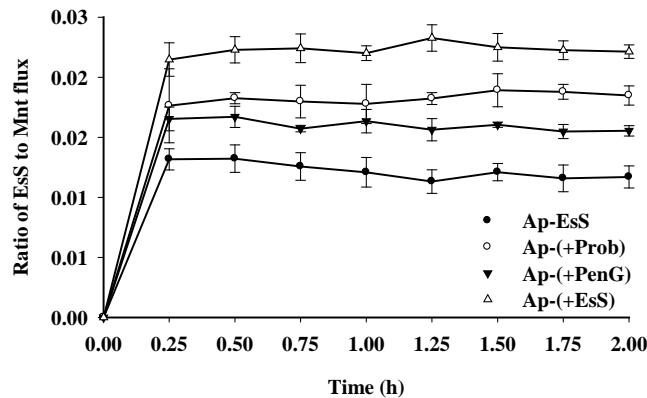
CHAPTER 5 - TUMOR NECROSIS FACTOR ALPHA INCREASES P-GLYCOPROTEIN EXPRESSION IN A BME-UV IN VITRO MODEL OF MAMMARY EPITHELIAL CELLS

Abstract

P-glycoprotein is an efflux pump belonging to the ATP-binding cassette super-family that influences the bioavailability and disposition of many drugs. Mammary epithelial cells express various drug transporters including P-glycoprotein, albeit at low level during lactation. During inflammatory reactions, which can be associated with changes in epithelial barrier functions, pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) are elevated in milk and serum. In this study, the role of TNF-α in the regulation of P-glycoprotein was determined in cultured BME-UV cells, an immortalized bovine mammary epithelial cell line. The protein production of P-glycoprotein and mRNA expression of *bABCB1*, the gene encoding bovine P-glycoprotein, were increased after 24 hours of TNF-α exposure. The highest observed effects for TNF-α on the regulation of P-glycoprotein was after 72 hours of exposure. Protein and mRNA expression remained elevated after 120 hours of TNF-α exposure, but it was lower than the level that observed in the cells exposed to TNF-α for 72 hours. The apical to basolateral flux of digoxin was decreased in the TNF-α-exposed epithelium. This effect was reversed when verapamil or ketoconazole, compounds known to interact with P-glycoprotein, were added together with digoxin into the donor compartment. Probenecid, a compound known to interact with organic anion transporters, but not P-glycoprotein, did not increase the flux of digoxin. This model has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of P-glycoprotein in the accumulation and/or removal of specific substrates from milk and/or plasma.

Introduction

The body of complex animals is separated into different compartments by biological barriers that control the contents of these compartments by including or excluding substances.

The functional concept of biological barriers has shifted from that of a static barrier to a complex of transport systems that include various efflux pumps. Efflux transporters are a family of ATP-dependent transport proteins that are known to be present within several major physiological barriers including the blood-brain barrier (BBB), kidney, intestine, and placenta (Alcorn et al., 2002; Madlova et al., 2009; Schrickx and Fink-Gremmels, 2007). The mammary epithelium, the most likely rate-limiting barrier between plasma and milk, expresses various drug transporters including P-glycoprotein (Al-Bataineh et al., 2009; Alcorn et al., 2002; Gilchrist and Alcorn, 2009).

P-glycoprotein (the *ABCB1* gene product, which was formally known as MDR1) is an ATP-hydrolyzing efflux pump belonging to the ATP binding cassette super-family (ABC family) (Kim, 2002; Mealey, 2004). P-glycoprotein plays an important role in xenobiotic absorption, distribution, and elimination as a result of its tissue distribution and cellular localization. For example, P-glycoprotein is expressed at the brush borders of epithelial cells in the intestinal tract, limiting absorption after oral administration and facilitating secretion into the intestinal lumen (Thiebaut et al., 1987). In the liver, P-glycoprotein is expressed at the canalicular (apical) surface of hepatocytes, facilitating the excretion of drug molecules, toxins and their metabolites into the bile (Chandra and Brouwer, 2004). In the kidney, P-glycoprotein is expressed on the apical surface of epithelial cells lining the proximal tubules where it mediates the elimination of endogenous metabolic waste products and xenobiotic metabolites (Masereeuw et al., 2003). At biological barriers such as the BBB, P-glycoprotein is expressed on the luminal plasma membrane of the capillary endothelium, where it prevents the passage of drugs and toxins across the capillary endothelium into the brain (Fricker and Miller, 2004).

P-glycoprotein expression can be modulated by different factors, including drugs, toxic agents, heat shock, inflammation, pregnancy and lactation (Alcorn et al., 2002; Sukhai and Piquette-Miller, 2000; Sukhai et al., 2000). Several *in vitro* and *in vivo* investigations have shown that inflammation and pro-inflammatory cytokines such as TNF-α alter the expression of P-glycoprotein. The effects of these cytokines appear at different levels of regulation including transcriptional, translational, and /or post-translational level (Englund et al., 2007; Sukhai et al., 2000; Theron et al., 2003).

During inflammatory reactions, which can be associated with changes in mammary epithelial barrier functions, pro-inflammatory cytokines such as TNF-α are elevated in serum and

milk. TNF-α is significantly increased in milk and serum in coliform mastitis and *Escherichia coli*-derived lipopolysaccharide-infused mammary gland (Hoeben et al., 2000; Nakajima et al., 1997; Persson Waller et al., 2003). Furthermore, exposure of bovine peripheral blood mononuclear cells to *Staphylococcal aureus* enterotoxins for 3 hours resulted in the rapid production of TNF-α that reached maximal levels by 48 hours then slowly declined by 96 hours after stimulation (Yokomizo et al., 1995). The influence of the pro-inflammatory cytokine TNF-α on the regulation of efflux pump in the mammary epithelial cells has not yet been reported.

Whole-animal models used to study the effects of pro-inflammatory cytokines on the expression and activity of P-glycoprotein provide information on the overall effects of inflammation on the regulation of P-glycoprotein. However, the effects of specific cytokines on the regulation of P-glycoprotein are complicated by the release of a complex series of endogenous cytokines. Thus, tractable *in vitro* models provide answers to elucidate the specific roles of inflammatory modulators. An immortalized bovine mammary epithelial cell line (BME-UV) was used in the present study. This cell line was selected as a model for the blood-milk barrier due to its ability to express transport proteins (Al-Bataineh et al., 2009), establish a cell polarity with well-formed tight junctions between adjacent cells, exhibit a transepithelial electrical resistance, and its responsiveness to steroid hormones (Quesnell et al., 2007a; Schmidt et al., 2001). The aim of the present study was to determine the effects of cytokine TNF-α on the expression and function of P-glycoprotein in BME-UV cells.

Materials and Methods

Modulatory effects of TNF-α on gene expression in cultured BME-UV cells were determined by measuring mRNA and protein levels following exposure to TNF-α for different periods of time. Effects on the permeation of epithelial barrier were examined by performing diffusion studies using compounds known to interact with P-glycoprotein.

Cell Culture

The BME-UV cells were cultured under conditions similar to those described previously in details (Quesnell et al., 2007a; Schmidt et al., 2001). Briefly, the BME-UV cells were grown to 65-75% confluency in 25 cm^2 plastic culture flasks (Corning, Corning, NY). Then, dissociated and dispersed cells were seeded on permeable polyester inserts, (Transwell, Corning) and fed with asymmetrical media. Typical bovine medium (TBM), which contains little lactose and has

concentrations of electrolytes that closely mirror serum, bathed the basolateral aspect of the cells throughout all experiments. The apical aspect was exposed to apical bovine medium (ApM) of low electrolyte-high lactose composition that resembles the ionic composition of milk. Composition of TBM and ApM were reported previously (Quesnell et al., 2007a; Schmidt et al., 2001). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Media on both the apical and basolateral aspects of the cells were refreshed daily. Cells were maintained in culture on permeable supports for 2 weeks to form a confluent, polarized and electrically tight monolayer prior to all experiments.

Incubation Conditions with Pro-Inflammatory Cytokine (TNF-α)

Cells were grown to confluence and exposed to pathophysiologically relevant concentrations (200 ng/ml) of recombinant bovine TNF-α (Thermo Scientific, Rockford, IL) for 24, 72, and 120 hours prior harvesting the cells for RNA isolation and protein production investigations, or using them in transport studies. TNF-α concentration within this range have been reported in dairy cows with mastitis (Bannerman, 2009; Hoeben et al., 2000). Paired cells were maintained without exposure to TNF-α (unexposed).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA Extraction

RNA was isolated from confluent monolayers of BME-UV cells that were exposed to TNF-α for 24, 72 and 120 hour prior to harvesting the cells using RNeasy Mini kit (QIAGEN, Foster City, CA) according to the manufacturer's directions. RNA concentrations were determined by the measurement of optical density at 260 nm (NanoDrop 8000, Thermo Scientific). Isolated total RNA integrity was verified by an OD_{260}/OD_{280} absorption ratio greater than 1.8 and less than 2.0. Non-degraded and sufficiently concentrated RNA (~0.5 to 1.0 μg/μl) was used in RT-PCR assays.

Primer Design for RT-PCR

Bovine sequences of mRNA for each gene were obtained from the National Center for Biotechnology Information Genebank. Primer pairs (GGAGCCCATTCTGTTTGACT and TCCTTTGTCCCCTACTCTGG) to detect mRNA coding for bovine P-glycoprotein (*bABCB1*) and (AAGATTGTCAGCAATGCC and ACAGACACGTTGGGAG) to detect mRNA coding for

bovine GAPDH (*bGAPDH*) were chosen to give amplification product between 150 and 300 base pairs using Primer3 software, a web-based primer design program. Both primer pairs were custom synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA).

RT-PCR

The expression of *bABCB1* in mammary epithelial cells was determined by RT-PCR (iCycler, Bio-Rad Laboratories, Hercules, CA) with the use of a commercial kit (Quantitect SYBR Green RT-PCR kit and reagents, Qiagen). Reactions were performed in a final volume of 25 μL containing 100 ng of template RNA, 0.5 μM forward and reverse primers, 0.25 μL Quantitect RT Mix, 12.5 μL Quantitect SYBR Green (1x) and 8.25 μL RNase-free Water. Triplicate determinations were performed for each sample (three samples total) of each target gene (*bABCB1* and *bGAPDH*) in a 96-well optical plate. This experiment was repeated four times on separate days. RT-PCR assays were conducted in the manner where samples of bovine kidney, no-template reaction and no reverse transcriptase were included in each assay.

RT-PCR Protocol

The RT-PCR protocol consisted of reverse transcription (50°C for 30 minutes), PCR initial activation (95°C for 15 minutes), followed by 40 cycles of denaturing at 94°C for 15s, annealing at 58°C for 30s, and extension at 72°C for 30s. A melt curve analysis from 65°C-95°C at 0.5°C/s was conducted immediately following PCR. Relative gene expression in different treatments to the average untreated was calculated using $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

Western Blot

The immunoreactive bands for P-glycoprotein were measured in plasma membrane fractions isolated from both unexposed and TNF-α exposed BME-UV cells on western blots by modifying a previously described method (Poller et al., 2010). Briefly, protein content was determined using a bicinchoninic assay (Thermo Scientific) and 30 μg of protein were resolved on 4-20% SDS-prepoured polyacrylamide gels (Thermo Scientific) and transferred to PVDF transfer membrane (Thermo Scientific). Membranes were blocked for 1 hour at room temperature with 5% powdered non-fat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA) in

phosphate buffered saline containing 0.1% Tween 20 (PBS-T). Washed membranes were incubated overnight at 4ºC with the monoclonal antibody, C219 (Covance, Denver, PA). Then, washed membranes were incubated for 1 hour at room temperature with horse-radish peroxidase-conjugated goat anti-mouse IgG (1:1500) in PBS-T containing 5% non-fat dry milk powder. P-glycoprotein immunorecativity was visualized using Femto-chemiluminescence substrate (Thermo Scientific). Gels were scanned (Kodak Image Station 4000R, Rochester, NY) and the intensity of the immunoreactive bands was quantified (Molecular Imaging software, Rochester, NY).

Transport Studies

Flow-Through Diffusion Cell System

Confluent monolayers of BME-UV cells on permeable supports were mounted as a barrier in a flow-through diffusion cell system (Permegear, Bethlehem, PA). The system has automated receptor fluid collection and a high precision multichannel dispenser (Ismatec SA, Switzerland). The flow rate of receptor fluid through the receiver compartment of each diffusion chamber was controlled at 4 ml/h. Temperature was maintained at 37°C by circulating water through the diffusion chambers holder. Donor compartments of diffusion chambers were capped directly after adding test solutions. Sequential samples were collected from the receiver side at regular time intervals (15 min) over a period of 120 min. Samples of either [^{14}C]- or [^{3}H]-radiolabeled test compounds were assayed by dual labeled β-scintillation counting with quench correction (Beckman LS6500, Beckman Coulter, Inc., CA). Automatic calculation of disintegrations per minute (DPM) was used for each sample.

Digoxin Transport Studies

[^{3}H]-digoxin ([^{3}H]-Digx), (American Radiolabeled Chemicals, MO), a compound known to interact with P-glycoprotein, was prepared in ApM together with [^{14}C]-mannitol ([^{14}C]-Mnt), (American Radiolabeled Chemicals). [^{14}C]-Mnt was used as a marker for paracellular movement (Kimura et al., 2006).

Study 1

The apical to basolateral (Ap-to-BL) flux of digoxin across BME-UV monolayers that were exposed to pro-inflammatory cytokine TNF-α for 24, 72, and 120 hours was determined

and compared to unexposed cells. Transport experiments were initiated by adding 1 mL of test solution containing 0.05 µM of [^{3}H]-Digx to the upper compartment (donor) together with 2µM [^{14}C]-Mnt where the apical side of the epithelial monolayer facing the donor compartment with or without verapamil (Verp, 50 µM), (Sigma-Aldrich, St. Louis, MO), a compound known to interact with P-glycoprotein (Kim, 2002).

Study 2

The Ap-to-BL flux of digoxin across BME-UV monolayers that were exposed to pro-inflammatory cytokine TNF-α for 72 hours was examined and compared to unexposed cells. This study was performed in the presence or absence of ketoconazole (Keto, 200 µM), (Sigma-Aldrich), which is also known to interact with P-glycoprotein (Elsby et al., 2008; Keogh and Kunta, 2006) or probenecid (Prob, 200 µM), (Sigma-Aldrich), a compound known to interact with organic anion transporters (OAT) (Lash et al., 2007; Takeda et al., 2001).

Data Analysis

The digoxin flux (µmol/cm^2 per hour) was calculated based on the amounts of digoxin recovered in the receiver compartment per unit time and then divided by mannitol flux at each measured time point to account for changes in overall epithelial integrity. Then, it was normalized by the effective diffusion area (cm^2) of the cell culture insert.

Statistical Analysis

All statistical analyses were performed using the commercially available software SIGMASTAT (Systat Software Inc., San Jose, CA). Group comparisons were made by repeated measures ANOVA. When the ANOVA showed significant differences among treatments, the Holm-Sidak posttest (versus control) was performed to determine the specific treatment that is statistically significant different from the control (unexposed). The level of significance for all tests was set at $P < 0.05$.

Results

Influence of TNF-α on Monolayer Integrity

The flux of mannitol across the BME-UV monolayer increased significantly after 24 and 72 hours of TNF-α exposure (Table 5-1). However, the flux of mannitol decreased to values

indistinguishable from untreated after exposure to TNF-α for a longer time (120 hours), which may indicate the epithelial monolayers were able to restore their integrity. The lowest flux value of mannitol was in the unexposed monolayers ($6.5 \pm 0.7 \times 10^{-6}$ µmol/cm^2 per hour), while the highest value was in the TNF-α exposed monolayers for 24 hours ($15.5 \pm 0.8 \times 10^{-6}$ µmol/cm^2 per hour).

Influence of TNF-α on ABCB1 mRNA Expression

The results from RT-PCR analysis demonstrate a significant increase in the *bABCB1* mRNA expression after 24 hours of TNF-α exposure (Fig. 5-1). The mRNA levels were further upregulated after 72 hours of TNF-α exposure where it reached the maximum increase in mRNA expression (32 ± 2 %) compared to unexposed cells during the experiment. After 120 hours treatment with TNF-α, the increase in mRNA expression was 25 ± 6 %.

Influence of TNF-α on the Expression of P-glycoprotein

BME-UV cells exhibited different immunodetectable levels of P-glycoprotein after exposure to TNF-α for different periods of time as shown in Figs 5-2. A significant increase in P-glycoprotein immunoreactive level was observed after 24 hours of TNF-α exposure. Longer exposure to TNF-α led to a further increase in immunoreactive protein levels that reached the maximum observed effect after 72 hours of TNF-α exposure. After 120 hours exposure of TNF-α, the immunoreactive protein levels was increased significantly compared to the level of unexposed cells but it was lower than the level that was observed in the cells exposed to TNF-α for 72 hours.

Influence of TNF-α on the Ap-to-BL flux of digoxin

The apical to basolateral flux/time curves of digoxin across BME-UV cells treated with TNF-α for different durations are shown in the Fig. 5-3. The Ap-to-BL flux of digoxin across the epithelial monolayer was significantly decreased after 24 hours of TNF-α exposure. Similar consequences were observed after longer exposure to TNF-α (72 hours) where the flux of digoxin was decreased 43 ± 3 % compared to unexposed monolayers. The Ap-to-BL flux of digoxin after 120 hours of TNF-α exposure was decreased by 18 ± 8 %, which was not significantly different from digoxin flux across the unexposed cells (Fig. 5-3).

The normalized amount of digoxin that crossed unexposed BME-UV monolayers was nearly double the amount that crossed TNF-α exposed BME-UV monolayers. The highest intracellular content of digoxin was detected in the unexposed BME-UV cells (6.3 ± 2.6 x10^{-7} μmol), while a lower intracellular content was observed in the TNF-α exposed cells for 24 hours (4.6 ± 1.1 x10^{-7} μmol) and the lowest level was observed after treating the BME-UV cells with TNF-α for 72 hrs (3.0 ± 0.2 x10^{-7} μmol). The recovery of digoxin from the entire system at the conclusion of experiment was 96 ± 2 %.

Addition of verapamil to the donor compartment increased digoxin flux across TNF-α exposed BME-UV monolayers to the extent that there were no observable differences between exposed and unexposed cells (Fig. 5-4). Ketoconazole also increased digoxin flux across BME-UV monolayers exposed to TNF-α for 72 hours. Probenecid (a compound known to interact with OAT) had no observable effect on digoxin flux across BME-UV monolayers, regardless of TNF-α exposure (Figures 5-5).

Rapid steady state conditions were attained quickly in less than 15 minutes. The whole system exhibits steady state activities over the experimental period of 2 hours. The results provide consistent evidence that the observations are related to the effect of TNF-α on P-glycoprotein expression. The correlation between digoxin flux and mRNA expression or P-glycoprotein immunoreactivity were both consistent as supported by regression ($r^2 = 0.71$ and 0.65, respectively).

Discussion

Cytokine TNF-α exposure induced the expression of *ABCB1* mRNA and increased P-glycoprotein production in BME-UV cells. A significant decrease in the Ap-to-BL flux of digoxin across the TNF-α-exposed BME-UV monolayer was also observed. This latter observation can be attributed to the upregulation of P-glycoprotein in mammary epithelial cells, resulting in a greater efflux of digoxin back into the donor fluid. *bABCB1* mRNA and P-glycoprotein levels increased depending on the duration of exposure to TNF-α, with maximum levels at 72 hours. The observed changes in the expression level of both mRNA and P-glycoprotein suggests that the effect of cytokine TNF-α on P-glycoprotein expression is at the transcription and translation level.

Mannitol flux increased across the BME-UV monolayer after exposure to TNF-α, suggesting that barrier function was compromised leading to an increase in paracellular solute flux. Similar results were observed by Quesnell et al (2007a), who studied the effects of pro-inflammatory cytokines on the integrity of mammary epithelial monolayer (BME-UV) via analysis of transepithelial electrical resistance (R_{te}). They observed a 30% decrease in R_{te} value after exposure to TNF-α for 8 hours. By adding mannitol together with digoxin, we could account for this decrease in monolayer integrity by normalizing digoxin to mannitol flux. The resulting observations were therefore indicative of TNF-α associated changes in transcellular digoxin flux.

Enhanced P-glycoprotein expression can explain the significant decrease in transcellular digoxin flux across the TNF-α-exposed BME-UV monolayers. The rate of digoxin efflux into the apical solution is proportioned to P-glycoprotein expression and activity or both, and the appearance of digoxin in the receiver compartment is inversely proportioned to P-glycoprotein activity. Verapamil and ketoconazole decrease this effect of TNF-α by either competing with digoxin or inhibiting the P-glycoprotein pump directly (Johnson et al., 2003; Kim, 2002). Probenecid reportedly has no effect on P-glycoprotein, but is known to affect the activity of other drug transporters including OAT. Thus, the absence of a probenecid effect further supports the notion that P-glycoprotein is responsible for digoxin flux across BME-UV cells. The differences in the intracellular contents of digoxin at the conclusion of experiment among different treatments were consistent with the other results in the current study. Enhanced P-glycoprotein expression in TNF-α-exposed mammary epithelial cells would pump more digoxin molecules out of the cells. Similarly, higher expression of P-glycoprotein would result in lower percentages of the administered digoxin dose crossing the BME-UV monolayer by the transcellular route, which is consistent with the observations in this study.

Two molecular mechanisms account for the changes in P-glycoprotein transport activity and expression induced by TNF-α; changes in the dynamics of transporter trafficking, release from the internalized inactive state (vesicle), or increased synthesis of transporter protein (Bauer et al., 2007). Furthermore, the expressed protein can be in active or inactive states. Thus, understanding the molecular mechanisms of TNF-α induced effects may help us in explaining the inconsistency between the expression and function of P-glycoprotein in BME-UV cells.

A significant increase (different levels) in *ABCB1* mRNA or protein production of P-glycoprotein or both were observed in different cell culture models when cells were exposed to TNF-α for different periods of time. For example, immortalized human brain capillary endothelial cells (Poller et al., 2010), primary porcine brain capillary endothelial cells (Wedel-parlow et al., 2009), and primary rat hepatocytes (Hirsch-Ernst et al., 1998). These results agree with our observations that the effect of TNF-α on the regulation of P-glycoprotein is dependent on the duration of TNF-α exposure.

There are also studies that show opposing results. A decrease in *ABCB1* mRNA and protein levels under the influence of TNF-α was reported in isolated primary placental trophoblasts (Evseenko et al., 2007) and human colon carcinoma cell lines, HCT15 and HCT116 (Stein et al., 1996). These observations may be explained by the inter-species differences, the cell type in which transporter regulation was studied, as well as dose and exposure time of TNF-α (McRae et al., 2003). Another important factor is the differences in the distribution of cytokines receptors among different types of cells (Yasumoto et al., 1992).

Conclusion

The results from the current study indicate that TNF-α induced *bABCB1* mRNA and protein production of P-glycoprotein and decreased significantly the flux of digoxin across BME-UV cells. The regulation of P-glycoprotein in the mammary epithelium has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of P-glycoprotein in the accumulation and/or removal of specific substrates from milk and/or plasma. In the non-lactating mammary gland, P-glycoprotein levels may affect the mammary epithelial cells' exposure to xenotoxins as they accumulate on the apical side of the epithelial barrier. Moreover, this study provides an *in vitro* cell culture model of mammary epithelium to characterize mammary epithelial cell function during inflammation.

Acknowledgements

Figures and Tables

Table 5-1. Mannitol flux across BME-UV monolayer from Ap-to-BL side

	Treatments[1]			
	Unexposed	TNF-24H	TNF-72H	TNF-120H
Flux, μmol/cm^2/hr	6.5×10^{-6a}	15.5×10^{-6b}	14.1×10^{-6b}	9.6×10^{-6a}
SE[2]	0.7×10^{-6}	0.8×10^{-6}	1.1×10^{-6}	1.5×10^{-6}

[a–b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: BME-UV cells were exposed to cytokine TNF-α for 24- (TNF-24h); 72- (TNF-72h) and 120-hr (TNF-120H) prior to performing the transport study.

[2]SE: standard error of the mean.

Figure 5-1. *bABCB1* mRNA expression in confluent TNF-α exposed BME-UV monolayers. TNF-α exposure increases *bABCB1* mRNA expression in confluent BME-UV monolayers. Monolayers were exposed to 200 ng/ml of TNF-α for 24, 72, and 120 hours before cells were harvested for RNA isolation. Star indicates the outcomes are statistically significant different from the vehicle. Data represents mean ± SE for n = 4, ($P < 0.05$).
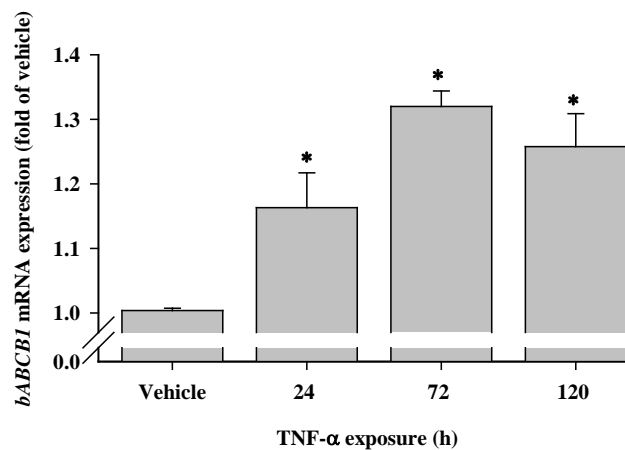
Figure 5-2. Western blot analysis for P-glycoprotein band intensity in confluent TNF-α exposed BME-UV monolayers.

Western blot analysis of total protein lysates identified significant changes in immunoreactive bands intensity of P-glycoprotein due to the exposure to TNF-α. Confluent BME-UV monolayers were exposed to TNF-α for 24, 72, and 120 hours before cells were harvested. Star indicates the outcomes are statistically significant different from the vehicle. Data represents mean ± SE for n = 5, ($P < 0.05$).
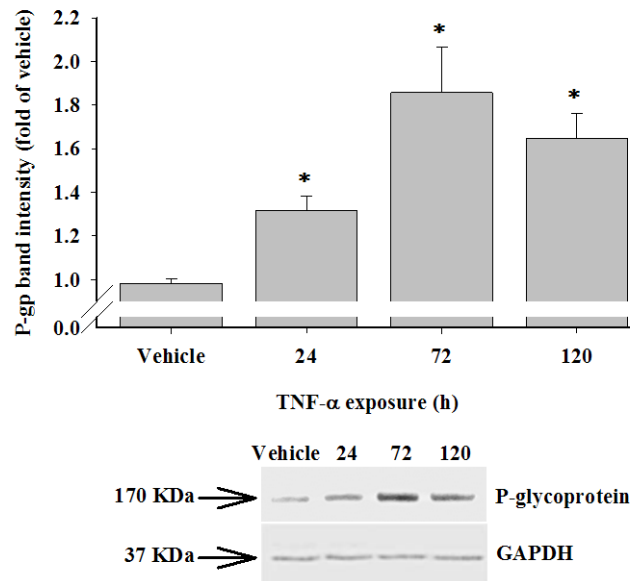
Figure 5-3. Ap-to-BL flux of digoxin across TNF-α exposed BME-UV monolayers.
TNF-α exposure, for 24 and 72 h, significantly ($P < 0.05$) decreases the mannitol-
normalized flux of digoxin across BME-UV monolayers. Digoxin flux across the BME-
UV monolayer from Ap-to-BL side after treating BME-UV cells with TNF-α for 24
(TNF-24H); 72 (TNF-72H) and 120 hours (TNF-120H) prior to performing the
transport study. Data represents mean ± SE for n = 3, ($P < 0.05$).
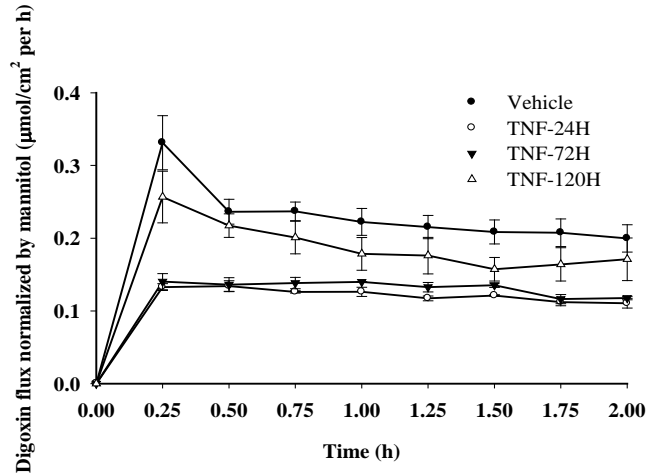
Figure 5-4. Ap-to-BL flux of digoxin across TNF-α exposed BME-UV monolayers in the
presence of verapamil.

Following exposure to TNF-α for the indicated durations, there were no significant
differences in digoxin flux across the BME-UV monolayers in the presence of verapamil,
a compound known to interact with P-glycoprotein. BME-UV cells were exposed to TNF-
α for 24 (TNF-24H); 72 (TNF-72H) and 120 hours (TNF-120H) prior to performing the
transport study. Data represents mean ± SE for n = 3, ($P < 0.05$).
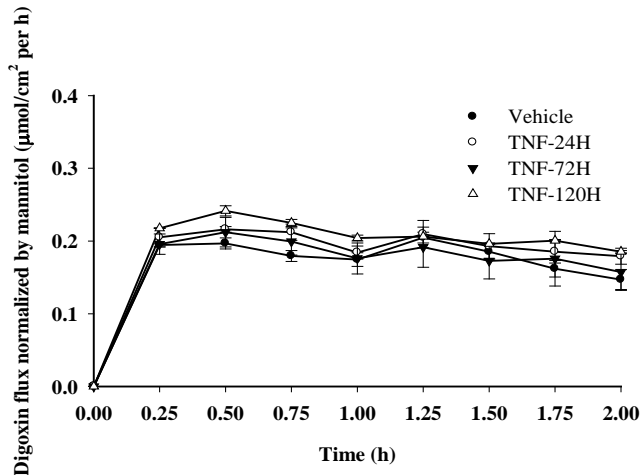
Figure 5-5. Ap-to-BL flux of digoxin across TNF-α-exposed and unexposed BME-UV monolayers in the presence and absence of ketoconazole and probenecid. Ketoconazole but not probenecid increased significantly ($P < 0.05$) the mannitol-normalized flux of digoxin across the TNF-α-exposed and unexposed BME-UV monolayers. Mannitol-normalized flux of digoxin (TNF_Digx or Vehicle_Digx) across the BME-UV monolayer from Ap-to-BL side. BME-UV cells were exposed to TNF-α for 72 hours prior to performing the transport study in the presence or absence of ketoconazole (TNF_Keto or Vehicle_Keto) or probenecid (TNF_Prob or Vehicle_Prob). Data represent mean ± SE for n = 3, ($P < 0.05$).
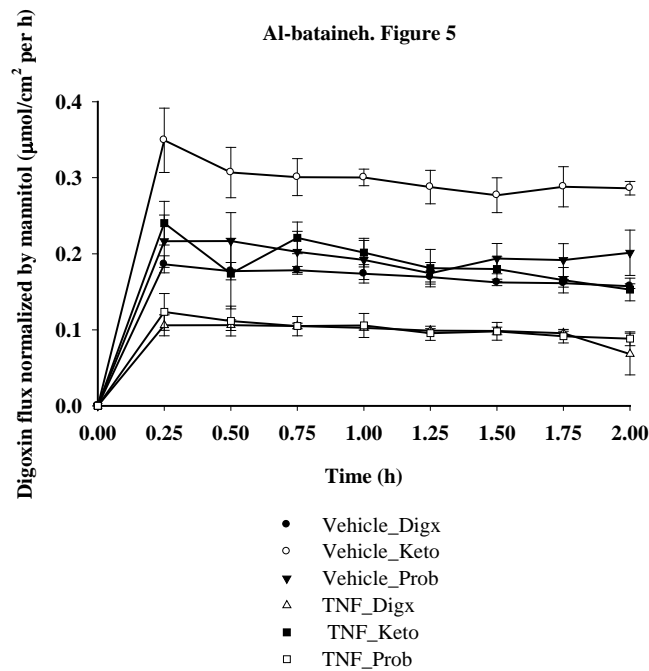
CHAPTER 6 - RESEARCH CONCLUSION AND FUTURE DIRECTIONS

The study described in chapter 3 was designed to study the potential contributions of bOAT and bOCT in the movements of compounds across BME-UV cells. By using a flow-through diffusion cell system, functional expression of organic anion and cation transport systems was demonstrated in BME-UV cells. A similar polarized transport process for TEA has been identified previously in human mammary epithelial monolayers and attributed to the human OCT (Kimura et al., 2006). Furthermore, mRNA transcripts for 19 different transporter genes including OCT have been reported in rat whole mammary gland, and in non-lactating and lactating human mammary epithelia (Alcorn et al., 2002; Gilchrist and Alcorn, 2009). The expression of members of the OCT and OAT family members on the membrane of the BME-UV cells may relate to a physiological role of these transporters in milk production. Conversely, they may impart a protective role to the lactating mammary gland exuding potentially toxic xenobiotics into milk. But this increases the risk of milk residues that are potentially harmful to suckling off-spring or the human consumer.

The Bronaugh-type flow-through diffusion cell system that was used in this study has numerous advantages over the static systems. The system allows for automatic sampling of receiver fluid to monitor the absorption/time profile accurately. Most importantly, receiver fluid flow maintained effective infinite sink conditions in the receiver compartment throughout the experiment, which reduces the effect of test compound molecules diffusing back from the basolateral to the apical side or vice versa (Bronaugh and Stewart, 1985). This is the first study using a cell culture *in vitro* model of bovine mammary epithelial cells to study the mechanisms of xenobiotic movement across epithelial barrier.

To characterize the organic anion transport system expressed in BME-UV cells (chapter 3), molecular and functional approaches were used to identify the isoforms of organic anion transporter (bOAT-1, bOAT-2, bOAT-3 and bOAT-4) (chapter 4). Expression levels of all tested transporters in BME-UV cells were less than expression levels of the corresponding transporters in bovine kidney, one of the major routes of organic anion elimination, by at least 2 orders of magnitude. Directionality in the flux of *P*-aminohippuric acid (Pah) and acetylsalicylate (Sal), compounds known to interact with OAT-1 and OAT-2 respectively, across the BME-UV

monolayer was not observed at the concentrations used in this study. This may be explained by low expression of the transporters for these compounds in BME-UV cells. Directionality was, however, observed in the flux of EsS across the BME-UV monolayer; this could be attributed to the involvement of the organic anion transport system in the molecules' passage across BME-UV cells.

The flux of different anionic compounds known to interact with OAT in the *in vitro* cell culture model (BME-UV) is consistent with other observations regarding lactating cows and other species such as the rat. Experimental and theoretical (calculated on the basis of pH partition concept) milk-to-plasma ultra-filtrate ratios were in close agreement for Pah and Sal and not altered by drug concentrations or probenecid (Gerk et al., 2001a; Miller et al., 1967), which agree with our results in which directionality in the flux of both tests compounds was not observed. The milk concentration of endogenous EsS in bovine was significantly greater than that in plasma, indicating that EsS was actively transferred from blood to milk against a concentration gradient (Heap et al., 1983, 1984), which supports our hypothesis that EsS cross mammary epithelial barrier by a carrier mediated process.

P-glycoprotein (the *ABCB1* gene product, which was formally known as *MDR1*) plays an important role in xenobiotic absorption, distribution, and elimination as a result of its tissue distribution and cellular localization. In chapter 5, modulatory effects of TNF-α on P-glycoprotein expression in cultured BME-UV cells were determined by measuring mRNA and protein levels following exposure to TNF-α for different periods of time. Effects on the permeation of the epithelial barrier were examined by performing transport studies using compounds known to interact with P-glycoprotein. Cytokine TNF-α exposure induced the expression of *ABCB1* mRNA and increased P-glycoprotein production in BME-UV cells. A significant decrease in the Ap-to-BL flux of digoxin (P-glycoprotein substrate) across the TNF-α-exposed BME-UV monolayer was also observed.

A significant increase (different levels) in *ABCB1* mRNA and/or protein production of P-glycoprotein was observed in different cell culture models when cells were exposed to TNF-α for different periods of time (Hirsch-Ernst et al., 1998; Poller et al., 2010; Wedel-parlow et al., 2009). These results agree with our observations that the effect of TNF-α on the regulation of P-glycoprotein is dependent on the duration of TNF-α exposure.

Implications

Taking the results together, the dynamic nature of the BME-UV monolayer has been confirmed in the current research by providing data regarding the expression, function, and regulation of members of OCT, OAT, and P-glycoprotein in BME-UV cells, a model for the blood-milk barrier.

The functional expression of members of bOCT and bOAT subfamilies in BME-UV cells may indicate the pivotal role of these transport systems in the function of mammary epithelial cells, translocation of molecules, endogenous and exogenous compounds and their metabolites, between plasma and milk.

The expression and regulation of P-glycoprotein in the mammary epithelium, expressed at low levels during lactation and at high levels during the dry period (Alcorn et al., 2002; Gilchrist and Alcorn, 2009), has important implications for understanding the barrier function of the mammary epithelium and its role in protecting the mother (mainly during lactation) from potentially harmful xenobiotics. The active efflux of P-glycoprotein substrates into milk may expose nursing infants and milk consumers to potentially harmful compounds. This exposure may be increased if the mammary gland was to become inflamed. In the non-lactating mammary gland, P-glycoprotein levels may affect the mammary epithelial cells' exposure to xenotoxins as they accumulate on the apical side of the epithelial barrier. This exposure may lead to dangerous consequences because mammary tissue possess enzymes capable of activating drugs and xenobiotic agents that may contribute to breast cancer and other diseases through formation of genotoxic agents in breast tissue (McManaman and Neville, 2003).

Exposure to pro-inflammatory cytokine TNF-α led to changes in the function of the mammary epithelial barrier as demonstrated by changes in the expression of P-glycoprotein (up-regulation) and the flux of mannitol, a paracellular marker, and other test compounds.

The relative importance of different membrane transporters in the milk excretion of compounds *in vivo* remains unknown and will require further studies. This study provides a cell culture *in vitro* model of bovine mammary epithelium that may be considered as a first step on the pathway of improving our understanding of the mechanisms by which xenobiotics move across the mammary secretary epithelium.

Future directions

Further investigations in the role of mammary gland transporters in the transepithelial movement of nutrients into the milk are greatly needed. Knowing the substrate (xenobiotic or nutrient) profile of mammary gland transporters will provide insight into the possibilities of xenobiotic-nutrient transporter interactions. For example, one of the physiological functions of *ABCG2* has been observed to be the secretion of vitamin B2 (riboflavin) into breast milk (Vlaming et al., 2009). Moreover, repeated exposure to dietary ingredients such as capsaicin, daidzein, piperine and sesamin can cause drug-nutrient interactions by affecting the function and mRNA expression of intestinal transporters such as P-glycoprotein (Okura et al., 2010). Thus, screening for these interactions will add another assessment tool to help safeguard infant and milk consumer health by preserving the high quality composition of milk.

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