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Gender-specific expression of ATP-binding cassette (*Abc*) transporters and cytoprotective genes in mouse choroid plexus

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ABSTRACT

The choroid plexus (CP) and blood-brain barrier (BBB) control the movement of several drugs and endogenous compounds between the brain and systemic circulation. The multidrug resistance associated protein (Mrp) efflux transporters form part of these barriers. Several Mrp transporters are positively regulated by the transcription factor nuclear factor erythroid-2-related factor (Nrf2) in liver. The Mrps, Nrf2 and Nrf2-dependent genes are cytoprotective and our aim was to examine basal gender differences in expression of Mrp transporters, Nrf2 and Nrf2-dependent genes (Nqo1 and Ho-1) in the brain-barriers. Previous studies have shown higher expression of Mrp1, Mrp2 and Mrp4 in female mouse liver and kidney. We hypothesized that similar renal/hepatic gender-specific patterns are present in the brain-barrier epithelia interfaces. qPCR and immunoblot analyses showed that Mrp4, Ho-1 and Nqo1 expression was higher in female CP. Mrp1, Mrp2 and Nrf2 expression in the CP had no gender pattern. Female Mrp1, Mrp2 and Mrp4 mouse brain expressions in remaining brain areas, excluding CP, were higher than male. Functional analysis of Mrp4 in CP revealed active accumulation of the Mrp4 model substrate fluo-cAMP. WT female CP had 10-fold higher accumulation in the vascular spaces than males and 60% higher than *Mrp4*$^{-/-}$ females. Probenecid blocked all transport. Methotrexate did as well except in *Mrp4*$^{-/-}$ females where it had no effect, suggesting compensatory induction of transport occurred in *Mrp4*$^{-/-}$. Collectively, our findings indicate significant gender differences in expression of Mrp transporters and cytoprotective genes in the CP and BBB.

1. Introduction

Gender can be an important factor in drug and xenobiotic toxicity through differences in absorption, metabolism, distribution, and excretion. Many of the genes associated with these processes have gender-specific patterns in liver and kidney. For example, multidrug resistance-associated protein 4 (Mrp4/*Abcc4*) is a membrane transport ATPase expressed at much higher levels in female than in male murine kidney and liver (Cheng et al., 2008; Lu and Klaassen, 2008). Its transported substrates include chemotherapeutic drugs, antiretroviral agents, and anti-cancer agents, as well as several endogenous ligands, such as uric acid, conjugated bile acids, glucuronidated estrogens, and leukotrienes. Mrp4, together with several other efflux transporters, is not only associated with cellular resistance to the toxicity of short-term exposure to, for example, methotrexate (MTX) (Chen et al., 2002; Hooijberg et al., 1999), but it also facilitates transepithelial transport and excretion by liver and kidney (Chen et al., 2002; Hooijberg et al., 1999; Reichel et al., 2007). Thus, the effectiveness of certain drugs as well as the excretion rate of xenobiotics and endogenous metabolites may be significantly impacted by gender. Sex differences in the gene expression of these efflux transporters may influence both tissue specific and whole body substrate clearance and, thus, aid in risk assessment and drug dosing decisions.

Efflux transporters are a critical component of the active barrier functions of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) as they protect the brain from endogenous and exogenous compounds, including a variety of therapeutics, by exporting them from the cerebrospinal fluid (CSF) or brain parenchyma interstitial space (IS) to the blood for ultimate excretion. The choroid plexus (CP) epithelium (BCSFB) and the brain parenchyma capillary endothelium (BBB) exert significant control over what goes into and comes out of the central nervous system. The CP is a highly vascularized

Abbreviations: CP, choroid plexus; BBB, blood-brain barrier; CSF, cerebrospinal fluid; Mrp, multidrug resistance-associated protein; *Abc*, ATP binding cassette; MTX, methotrexate; Mrp4, multidrug resistance-associated protein 4; BCSFB, blood-cerebrospinal fluid barrier; OA, organic anions; IS, interstitial space; Nrf2, nuclear factor erythroid-2-related factor; Nqo1, NAD(P)H quinone oxidoreductase-1; Ho-1, heme oxygenase-1; Gclc, glutamate cysteine ligase catalytic subunit; APAP, acetaminophen; ANOVA, analysis of variance

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epithelium that receives as much blood as the rest of the brain combined and secretes about 80% of the CSF. Because it assists in the clearance of various soluble compounds such as organic anions (OA) from CSF it is referred to as the "kidney" of the brain. The BBB has an important role as well in controlling movement of substrates between the blood and the brain IS. These barriers are highly active, dynamic and selective; however, the gender-specific characterization of the ATP binding cassette (*Abc*) efflux transporters in the brain is still limited.

The Abc-type transporters act as gatekeepers by allowing or limiting the entrance of an enormous variety of substrates across cellular membranes. In CP epithelium, Mrp1, Mrp4 (Leggas et al., 2004) and Mrp5 (*Abcc5*) (Roberts et al., 2008) are located in the basolateral membranes − an orientation that allows active transport from cytosol to an interstitium contiguous with the CP's fenestrated capillaries − whereas Mrp2 (*Abcc2)* is located in the apical membrane − transporting from cytosol to CSF. Mrps are also located in BBB endothelium of several species, including humans, rats and mice (Leggas et al., 2004; Rao et al., 1999; Roberts et al., 2008). P-glycoprotein (P-gp/*Abcb1*), breast cancer resistance protein (Bcrp/*Abcg2*), Mrp1, Mrp2, Mrp4 and Mrp5 are all localized to the BBB luminal membranes. In relatively lower amounts the *Abc* transporters are also expressed in astrocytes, microglia, neurons, and oligodendrocytes (Ballerini et al., 2002; Berezowski et al., 2004).

In addition to Mrps, several drug metabolizing enzymes are expressed in the CP and BBB including glutathione S-transferases and several isoforms of the cytochrome P450 family (Decleves et al., 2011; Miksys et al., 2000; Tyndale et al., 1999). There are transcription factors known to positively regulate the expression and activity of several cytoprotective genes during periods of oxidative stress. For example, the transcription factor Nrf2 is involved in many cellular functions including drug metabolism, oxidative stress mitigation and regulation of efflux transporter pathways. These genes include NAD(P)H quinone oxidoreductase 1 (Nqo1), heme-oxygenase 1 (Ho-1) and glutamate cysteine ligase catalytic subunit (Gclc), as well as certain efflux transporters such as Mrp4 (Aleksunes et al., 2008, 2010). Biotransformation of foreign compounds via these phase I and II enzymes can result in metabolites which can be transported by Mrps. Consequently, metabolic enzymes and transporters can act together to eliminate harmful compounds from the brain to the blood for eventual body excretion. The extent to which metabolism contributes to the BCSFB and to the BBB remains to be established. Furthermore, the gender-specific patterns of detoxification enzyme profiles and the influence of Nrf2 on transporter expression have not been well characterized in CP.

There are numerous reports of sexual dimorphism of Abc-type transporters in kidney and liver of adult mice, rats and humans (Cheng et al., 2008; Lu and Klaassen, 2008), but data on sex differences in CP are extremely limited. Our primary goal was to test the hypothesis that *Abc* transporters and cytoprotective genes in adult mouse CP share kidney- and liver-like gender expression patterns. Here, gender specific expression patterns of several key cytoprotective genes and proteins in the CP and brain are presented together with functional data for Mrp4.

2. Methods

2.1. Animal tissue collection

All animal studies were performed in accordance with institutional regulations for animal protection and were approved by the Institutional Animal Care and Use Committee of the University of Connecticut (Protocol A12-050). Age-matched male and female C57BL/6J mice (10–12 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *Mrp4*$^{-/-}$ mice with a C57BL/6J background were kindly provided by Dr. John Schuetz from St. Jude's Children's Hospital, Memphis, TN. A colony of these mice is maintained at the University of Connecticut. All mice were housed in temperature, light, and humidity controlled conditions approved by the Association for Assessment and Accreditation of Laboratory Animal Care. All mice were provided standard laboratory chow (Harlan) and water *ad libitum*. Following euthanasia via decapitation, brains were removed and immersed in ice-cold PBS. The two lateral CPs of each animal were removed from the ventricles under a dissecting microscope. Tissues were then transferred into 0.5 ml tubes containing either RNA later (Qiagen, Valencia, CA, USA), neutral phosphate buffered 10% formalin fixative or Laemmli sample buffer for future analyses.

Table 1
Description of primary and secondary antibodies for immunoblots.

Protein	Primary Antibody Conc. Tm/T	Primary Antibody Source	Molecular weight (kDa)	2 Antibody Conc. Tm/T	Primary and secondary blocking buffer solution
Mrp1	1 ab24102 1:500 4° C/ON	Abcam	190	Anti-rat[S] 1: 2000 RT/1hr	5% NFDM
Mrp2	ab3373 1:500 4° C/ON	Abcam	190	Anti-mouse[S] 1:2000 RT/1hr	5% NFDM
Mrp4	ab15602 1:500 4° C/ON	Abcam	160	Anti-rat[S] 1:2000 RT/1hr	5% NFDM
Mrp5	2 ab24107 1:500 4° C/ON	Abcam	160	Anti-rat[S] 1:2000 RT/1hr	5% NFDM
Nrf2	8882S 1:500 4° C/ON	Cell Signaling	~100	Anti-rat[S] 1:2000 RT/1hr	5% BSA
Gclc	– 1:10000 4° C/ON	Washington	~75	Anti-rabbit[S] 1:20000 RT/1hr	5% NFDM
Ho-1	SPA-895 1:5000 4° C/ON	Stressgen Bioreagents	~33	Anti-rabbit 1:2000 RT/1hr	5% NFDM
Nqo1	ab2346 1:1000 4° C/ON	Abcam	~32	Anti-goat[S] 1:10000 RT/1hr	5% NFDM
β-actin	ab8227 1:3500 4° C/1hr	Abcam	42	Anti-rabbit[S] 1:2000 RT/1hr	5% NFDM

NFDM, non-fat dry milk.
BSA, bovine serum albumin.
[S] Antibody purchase from Sigma.
Con, Antibody concentration.
Tm, incubation temperature.
T, incubation time.
ON, overnight.
RT, room temperature.

2.2. Solutions and chemicals

8- (2- [Fluoresceinyl]aminoethylthio)adenosine- 3′, 5′- cyclic monophosphate (Fluo-cAMP) was purchased from Biolog Life Science Institute (Bremen, Germany). This fluorescent cAMP analogue is highly resistant to metabolic degradation by cAMP-specific phosphodiesterase. Probenecid and methotrexate (MTX) were purchased from Sigma (St. Louis, MO, USA). Information on primary and secondary antibodies for immunoblots is listed in Table 1. All other chemicals were obtained from Sigma.

2.3. SDS-PAGE and immunoblotting

The two lateral CPs from each animal were suspended in 20 µl of Laemmli sample buffer (2.3% SDS, 5% ß −mercaptoethanol, 0.5% bromophenol blue, 62.5 mM Tris HCL, pH 6.8) and stored at −20° C. Equal amounts of sample were loaded on 10% SDS- Polyacrylamide gels