Sphingolipid Signaling Reduces Basal P-Glycoprotein Activity in Renal Proximal Tubule

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ABSTRACT

P-glycoprotein is an ATP-driven xenobiotic export pump that is highly expressed in barrier and excretory tissues, where it greatly influences drug pharmacokinetics. Recent studies in the blood-brain and spinal cord barriers identified a sphingolipid-based signaling pathway that regulates basal activity of P-glycoprotein. Here we use an established comparative renal model that permits direct measurement of P-glycoprotein activity to determine whether such signaling occurs in another tissue, killifish renal proximal tubule. Isolated killifish tubules exposed to 0.01–1.0 μ M sphingosine-1-phosphate (S1P) exhibited a profound decrease in P-glycoprotein transport activity, measured as specific accumulation of a fluorescent cyclosporine

A derivative in the tubule lumen. Loss of activity had a rapid onset and was fully reversible when the S1P was removed. Transport mediated by multidrug resistance-associated protein 2 (Mrp2) or a teleost fish organic anion transporter (Oat) was not affected. S1P effects were blocked by a specific S1P receptor 1 (S1PR1) antagonist and mimicked by a S1PR agonist. Sphingosine also reduced P-glycoprotein transport activity and those effects were blocked by an inhibitor of sphingosine kinase and by the S1PR1 antagonist. These results for a comparative renal model suggest that sphingolipid signaling to P-glycoprotein is not just restricted to the blood-brain and blood-spinal cord barriers, but occurs in other excretory and barrier tissues.

Introduction

P-glycoprotein is an ATP-powered drug efflux pump that is highly expressed in certain multidrug-resistant tumors and in the cells of many normal barrier and excretory tissues. For many drugs, it is a major determinant of drug pharmacokinetics, driving excretion from liver, gut, and kidney and exclusion from tumors and the CNS. We recently found that basal P-glycoprotein transport activity (but not expression) at the blood-brain and blood-spinal cord barriers could be rapidly and reversibly reduced through sphingolipid signaling (Cannon et al., 2012; Cartwright et al., 2013). Transport activity of other ABC transporters is not affected. In intact rats, reducing P-glycoprotein activity at the blood-brain barrier increases brain accumulation of three drugs that are P-glycoprotein substrates. In isolated brain and spinal cord capillaries in vitro and in vivo, these effects are mediated through a sphingosine-1-phosphate (S1P) receptor (S1PR1) (Cannon et al., 2012; Cartwright et al., 2013). Thus, sphingosine, S1P, and S1PR agonists decrease P-glycoprotein transport activity, and such effects are blocked by S1PR1

antagonists. Consistent with sphingosine conversion to S1P, the effects of sphingosine are blocked when sphingosine kinase (SK) is inhibited.

Given these recent findings for blood-brain barrier, we wondered whether sphingolipid signaling could also reduce P-glycoprotein activity in other tissues where the transporter is highly expressed. To answer this question, we turned to a comparative system that allows direct measurement of ABC transporter activity in intact, renal proximal tubules from a teleost fish. In mammalian renal proximal tubule, S1P acts through multiple S1PRs to contribute to a number of renal diseases, including diabetic nephropathy, renal fibrosis, and ischemia perfusion injury (Jo et al., 2008, 2009; Koch et al., 2013). S1PRs are widely expressed across species and tissues (Oskouian and Saba, 2004; Donati and Bruni, 2006). In the genome of zebrafish *Danio rerio*, a fresh water teleost fish, multiple S1PR and enzymes involved in sphingolipid metabolism are highly conserved (Mendelson et al., 2013). In that model organism, S1P acts through S1PR to regulate several processes, including angiogenesis and endothelial barrier integrity (Tobia et al., 2012; Mendelson et al., 2013).

Renal tissue from certain marine teleost fish offers several advantages for the study of mechanisms of xenobiotic secretion (Pritchard and Miller, 1980). The nephron of these animals is composed primarily of proximal tubules, which are

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ABBREVIATIONS: Akt, protein kinase B; BODIPY, boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene); DMSO, dimethylsulfoxide; ETBR, endothelin receptor B; FL, fluorescein; FL-MTX, fluorescein methotrexate; FTY-720, fingolimod (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol); FTY-720P, fingolimod phosphate; MTS, marine teleost saline; Mrp, multidrug resistance-associated protein; NBD-CSA, [N-(4-nitrobenzofurazan-7-yl)-D-Lys8]-cyclosporin A; Oat, organic anion transporter; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PSC833, valsopodar; S1P, sphingosine-1-phosphate; S1PR1, sphingosine-1-phosphate receptor 1; SK, sphingosine kinase.

easily isolated and which retain viability for long periods when maintained in a simple physiologic saline. During tubule isolation, broken ends reseal and form a closed, fluid-filled luminal compartment that is separated from the medium by the epithelium. With fluorescent substrates, confocal microscopy, and image analysis, xenobiotic accumulation in cells and secretion into the lumen can be visualized and measured (Schramm et al., 1995; Masereeuw et al., 2000). We previously identified fluorescent substrates and nonfluorescent inhibitors that can be used as tools to measure changes in transport activity of specific ABC transporters in killifish tubules, e.g., P-glycoprotein, multidrug resistance protein (Mrp)2 (Schramm et al., 1995; Masereeuw et al., 2000).

By use of killifish renal tubules, we show here that P-glycoprotein transport activity was rapidly and reversibly reduced through sphingolipid activation of a teleost S1PR. These results in a comparative model suggest that sphingolipid signaling to P-glycoprotein is not just restricted to the blood-brain and blood-spinal cord barriers but also occurs in other excretory and barrier tissues.

Materials and Methods

Chemicals. NBD-CSA ([*N*- ϵ (4-nitrobenzofurazan-7-yl)-D-Lys⁸]-cyclosporin A) was custom-synthesized by R. Wenger (Basel, Switzerland). Fluorescein methotrexate (FL-MTX), BODIPY-verapamil, and fluorescein (FL) were purchased from Life Technologies/Molecular Probes (Grand Island, NY). PSC833 (valspodar) was a gift from Novartis (Basel, Switzerland). S1P was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were obtained from Sigma-Aldrich and Cayman Chemical (Ann Arbor, MI).

Animals. All animal studies were performed in accordance with institutional regulations for animal protection and approved by the Animal Care and Use Committee of the Mount Desert Island Biologic Laboratory (MDIBL). Killifish (*Fundulus heteroclitus*) were wild-caught on Mount Desert Island, Maine. The fish were maintained in tanks with free flowing natural seawater at the MDIBL. Both males and females were used as the source of renal tissue.

Transport Experiments. All procedures for tubule isolation, incubation, and imaging have been described previously (Masereeuw et al., 2000; Prevoo et al., 2011). In brief, killifish were decapitated and renal tubular masses were collected and transferred into a dish containing marine teleost saline (MTS): 140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, and 20 mM Tris at pH 7.8. Under a dissecting microscope the masses were teased with fine forceps to remove adherent hematopoietic tissue and free the tubules. Renal tubules were then transferred to imaging chambers containing MTS containing fluorescent substrates and potential effectors. Chemicals were added to MTS from stock solutions in MTS or dimethylsulfoxide (DMSO). The final DMSO concentration in the chamber never exceeded 0.3%; preliminary experiments showed that DMSO concentrations $\leq$ 0.5% had no effect on transport activity. All experiments were conducted at room temperature (20–22°C).

Confocal Microscopy. For imaging, chambers were placed on an inverted confocal microscope (Zeiss LSM 510; Jena, Germany) and viewed with a 20 $\times$ dry objective (NA = 0.8). Under transmitted light, intact, undamaged tubules were selected. Confocal fluorescent images and phase contrast images were acquired using the 488-nm line of an argon ion laser, a 510-nm dichroic filter, and a 515-nm long-pass emission filter (Fig. 1). Four scans were averaged to provide a final image (512 $\times$ 512 $\times$ 8 bits). Stored images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Data Analysis. Data are presented as mean fluorescence intensity, variability as S.E.M. For statistical analysis, one-way

Fig. 1. Representative confocal and phase-contrast images of killifish renal tubules incubated to steady state (60 minutes) in MTS with 2 μ M NBD-CSA (A and B; control) or 2 μ M NBD-CSA plus 5 μ M PSC833 (C and D) or 1 μ M S1P (E and F). Note the high fluorescence in the lumens of the control tubules and the greatly reduced fluorescence in the lumens of the tubules exposed to PSC833 or S1P.

analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test were applied using GraphPad Prism (version 6 for Mac; GraphPad Software, San Diego, CA). Means were considered significantly different when $P < 0.05$. Each experiment used pooled tissue from three to four killifish. Experiments were repeated one to two times.

Results

Previous studies from this laboratory showed that luminal accumulation of a fluorescent cyclosporin A derivative (NBD-CSA) provides a way to measure changes in P-glycoprotein transport activity in living, intact killifish renal tubules (Schramm et al., 1995; Masereeuw et al., 2000). Such transport is concentrative, energy-dependent and inhibited by P-glycoprotein substrates, but not by inhibitors of Oats and Mrps. Figure 1A shows a representative confocal micrograph of killifish renal tubules incubated to steady state (60 minutes) in MTS medium containing 2 μ M NBD-CSA. In these control tubules, cellular fluorescence is low and luminal fluorescence, however, is substantially higher than both cellular and bath fluorescence. This pattern indicates concentrative accumulation of the fluorescent compound in the urinary space. Addition to the bath of PSC833, an inhibitor of P-glycoprotein-mediated transport, substantially reduced luminal fluorescence (Fig. 1C). Cellular fluorescence increased in PSC833-treated tubules, a finding consistent with inhibited P-glycoprotein-mediated efflux at the luminal membrane.

Addition of 1 μM S1P to the bath also reduced luminal NBD-CSA fluorescence and increased cellular fluorescence (Fig. 1E). Quantitation of luminal fluorescence showed that 0.1–1.0 μM S1P had nearly the same effect as 5 μM PSC833 (50% and 70% reduction with S1P and PSC833, respectively; Fig. 2A). The effects of S1P were concentration-dependent. Even a S1P concentration as low as 10 nM significantly reduced luminal NBD-CSA fluorescence (Fig. 2B). When we used BODIPY-verapamil, another P-glycoprotein substrate, to assess changes in transport activity, we again found that 1.0 μM S1P significantly reduced luminal fluorescence (Fig. 2C). Finally, inhibition of protein synthesis with cycloheximide did not diminish the effects of 1.0 μM S1P on luminal NBD-CSA fluorescence, indicating that the effects on transport were independent of translation (Fig. 2D).

To establish the time course of S1P action, we first incubated tubules to steady state (60 minutes) in MTS with 2 μM NBD-CSA and then added 1.0 μM S1P to the bath. As shown in Fig. 3A, luminal fluorescence rapidly declined after S1P addition. Luminal fluorescence was significantly lower within 5 minutes and even lower within 30 minutes. After 30 minutes exposure to S1P, replacing the bath with S1P-free MTS returned luminal fluorescence to control levels within 30 minutes (Fig. 3B). Thus, S1P rapidly and reversibly reduced P-glycoprotein transport activity in killifish renal proximal tubules.

Not all xenobiotic transporters are affected by S1P. In experiments with FL-MTX or FL as substrates, S1P did not decrease luminal fluorescence (Fig. 4). We previously showed that FL-MTX is a substrate for a killifish Mrp2 ortholog (Masereeuw et al., 1996) and that FL is a substrate for a killifish organic anion transporter (Oat) ortholog (Miller and Pritchard, 1997). Both of these transporters function to drive concentrative,

energy-dependent processes, Mrp2 through ATP splitting and Oat indirectly through sodium gradient-dependent processes. This lack of effect of S1P on FL-MTX and FL transport indicates that S1P effects on P-glycoprotein-mediated transport were specific and that S1P did not reduce energy supply to the transporter. Of course, we do not know whether S1P would alter the activity of untested transporters.

In rat and mouse brain capillaries, S1P acts through S1PR1 to reduce P-glycoprotein transport activity (Cannon et al., 2012; Cartwright et al., 2013). This appeared to be the case in killifish tubules, since W146, a specific S1PR1 antagonist, blocked the effects of S1P on NBD-CSA transport (Fig. 5A). In cells, S1P is generated from sphingosine through the action of SK. Addition of 1.0 μM sphingosine to the bath significantly reduced luminal accumulation of NBD-CSA (Fig. 5B). Consistent with added sphingosine being converted to S1P and the S1P acting through a S1PR, the effects of sphingosine were blocked by an inhibitor of SK and by W146 (Fig. 5B); neither of these drugs affected transport in tubules not exposed to sphingolipids (Fig. 5C). Finally, FTY720 is a prodrug that is phosphorylated intracellularly to a nonselective S1PR agonist (FTY720P). Adding FTY720 to the bath significantly reduced P-glycoprotein transport activity; as with sphingosine, the effects of FTY were blocked by the SK inhibitor and by W146 (Fig. 5D).

Discussion

In the present study, we took advantage of a comparative renal model to determine whether sphingolipid signaling regulates basal activity of the ABC transporter, P-glycoprotein. Marine teleost renal tubules hold an important place in the history of renal physiology, providing some of the first

Fig. 2. Effects of S1P on luminal NBD-CSA (A, B, and D) and Bodipy-verapamil (Bodipy-verap; C) accumulation in killifish renal tubules. Tubules were incubated to steady state (60 minutes) in MTS containing 2 μM NBD-CSA (control) or 2 μM NBD-CSA plus the indicated additions. Shown are mean $\pm$ S.E. for 12–20 tubules collected from three fish. Statistical comparisons: ***Significantly lower than controls, $P < 0.001$.

Fig. 3. Time course of S1P action in killifish renal tubules. Tubules were incubated to steady state (60 minutes) in MTS containing 2 μ M NBD-CSA. At that time, 1 μ M S1P (A and B) or 5 μ M PSC833 (B) was added to the MTS. In (B), S1P-containing MTS was replaced with S1P-free medium after 30 minutes to monitor the effect of washout. In both experiments, NBD-CSA was present in the medium at all times. Each point shows the mean $\pm$ S.E. for 8–10 tubules collected from three fish. Statistical comparisons: ***Significantly lower than controls, $P < 0.001$.

evidence for the general process of tubular secretion (Kinter, 1975). They continue to provide important models for the study of excretory xenobiotic transporter function and regulation in intact proximal tubules (Miller, 1987, 2002). One reason for this is that in marine teleost proximal tubules the tubular lumen remains patent and all the steps in the excretory transport of fluorescent substrates can be visualized for several hours in the living tubules. Indeed, studies with teleost renal tubules have identified fluorescent substrates for specific ABC transporters, e.g., P-glycoprotein and Mrp4 (Abcc4), that have translated to mammalian systems (Schramm et al., 1995; Miller et al., 2000; Reichel et al., 2007, 2010). Moreover, with regard to P-glycoprotein, there are clear molecular-level similarities between the killifish and human transporters. The full sequence of killifish P-glycoprotein has not been published. However, an alignment of the partial sequence of killifish P-glycoprotein with that of the human transporter shows 64% identity and 80% homology at the amino acid level (R.E. Cannon, unpublished data).

We show here that all of the basic characteristics of S1P signaling in mammalian brain and spinal cord capillaries are recapitulated in killifish renal proximal tubules. These include: 1) a rapid onset of S1P effects that were fully reversible when the S1P was removed; 2) no effect on transport mediated by other transporters; 3) blockade by a S1PR1 antagonist; 4) loss of transport activity by the S1P precursor, sphingosine, and by FTY720, a prodrug that is metabolized (phosphorylated) to a S1P mimic; effects of both were blocked by a SK inhibitor and by a S1PR1 antagonist.

It is likely that sphingolipid signaling to P-glycoprotein is connected upstream and downstream to other signaling

events within proximal tubule epithelial cells. In brain and spinal cord capillaries from rat and mouse, an extended signaling pathway leads to release of S1P and activation of S1PR1 (Cannon et al., 2012; Cartwright et al., 2013). Signaling upstream of sphingolipids involves tumor necrosis factor receptor 1 (TNFR1), endothelin receptor B (ET_BR), inducible nitric-oxide synthase (iNOS), and protein kinase C (PKC) β 1 (Miller and Cannon, 2013). Previous studies in killifish renal proximal tubules showed that endothelin-1 (ET-1) acting through ET_BR reduces transport mediated by P-glycoprotein and Mrp2 (Masereeuw et al., 2000). For Mrp2, we found signaling through NOS, PKC and guanylyl cyclase to be downstream of ET_BR (Masereeuw et al., 2000; Notenboom et al., 2002, 2004). At present, it is not clear whether NOS, PKC, and guanylyl cyclase signaling also connects ET_BR to S1PR1 in the regulation of P-glycoprotein activity in killifish tubules. However, it is clear that sphingolipid signaling is not involved in the regulation of basal Mrp2 activity in renal proximal tubules (present results) and in brain capillaries (Cannon et al., 2012).

One can ask what events connect S1PR1 to the loss of P-glycoprotein transport activity? This question can be addressed at two levels: one concerned with signaling and the second concerned with the mechanism by which transporter activity decreases. With regard to downstream signaling, S1PR1 is a G-protein-coupled receptor that can potentially signal through multiple discrete pathways, including, PI3K/protein kinase B (Akt), phospholipase C/PKC, Ras/extracellular signal-related kinase (ERK), and adenylyl cyclase/cAMP. In initial experiments with rat brain capillaries, we found S1P effects on P-glycoprotein activity were abolished by inhibitors

Fig. 4. Lack of effect of S1P on transport mediated by Mrp2 or Oat. Tubules were incubated to steady state (60 minutes) in MTS containing 2 μ M FL-MTX (A) or 1 μ M FL (B). MK-571, an inhibitor of Mrps, and *p*-aminohippurate (PAH), an inhibitor of Oat, are positive controls for inhibition of transport. Shown are mean $\pm$ S.E. for 10–28 tubules collected from three fish. Statistical comparisons: ***Significantly lower than controls, $P < 0.001$.

Fig. 5. Dependence of sphingolipid signaling on sphingosine kinase and S1PR1. Tubules were incubated to steady state (60 minutes) in MTS containing 2 μ M NBD-CSA (control) or 2 μ M NBD-CSA plus the indicated additions. W146 is a S1PR1 antagonist; SK1 is a SK inhibitor; FTY720 is a prodrug that is phosphorylated to form FTY720P, a S1PR agonist. W146 is a S1PR1 antagonist (A–D); sphingosine (Sphingo) is a S1P precursor (B); SK1 is an SK inhibitor (B–C); FTY720 is a prodrug that is phosphorylated to form FTY720P, an S1PR agonist (D). Shown are mean $\pm$ S.E. for 10–20 tubules collected from three to four fish. Statistical comparisons: **Significantly lower than controls, $P < 0.01$; ***Significantly lower than controls, $P < 0.001$.

of PI3K or Akt; S1P also increased specific phosphorylation of Akt, an indicator of Akt activation (R.E. Cannon and D.S. Miller, unpublished data). These findings are consistent with signaling through the PI3K/Akt pathway in brain capillaries. Whether the same is true for renal proximal tubule remains to be determined.

With regard to loss of transporter activity, two general types of mechanism have been proposed to underlie reductions in the activity of plasma membrane ABC transporters at the blood-brain barrier: 1) trafficking between the plasma membrane and intracellular compartments, i.e., transporter internalization; 2) altered microenvironment within the plasma membrane; and 3) covalent modifications of the transporter or accessory proteins (Hawkins et al., 2010). Recent experiments in rat using an in vivo protease K protection assay in which the protease was infused into the brain's vasculature indicated that although PKC β 1 activation reduced P-glycoprotein transport activity it did not cause movement of the transporter away from the luminal plasma membrane surface of the endothelium; in these capillaries, PKC β 1 signals upstream of S1PR1 (Hawkins et al., 2010). Thus, Hawkins et al. (2010) argued that loss of activity could be the result of altered microenvironment within the endothelial cell plasma membrane. In renal proximal tubule, events upstream and downstream of S1PR1 remain to be elucidated.

Irrespective of downstream and upstream events, the present data for renal proximal tubule, along with previous studies in blood-brain and blood-spinal cord barriers (Cannon et al., 2012; Cartwright et al., 2013), indicate that sphingolipid signaling regulates basal P-glycoprotein transport activity in multiple tissues that are important determinants of drug distribution and excretion. In addition, a recent publication

shows that exposure to S1P improves delivery of chemotherapeutics to MCF-7 tumor cells, although the mechanisms affected were not explored (Sultan et al., 2013). Given these findings, one would expect that targeting S1PR systemically could have complex effects on drug pharmacokinetics, reducing drug excretion and increasing delivery to the brain through both mass action (increased plasma retention) and reduced blood-brain barrier P-glycoprotein activity. One way to do this, at least experimentally, would be through the use of S1PR agonists, such as FTY720/FTY720P, which are currently in use in the clinic.

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Authorship Contributions

Participated in research design: Miller.

Conducted experiments: Miller.

Performed data analysis: Miller.

Wrote or contributed to the writing of the manuscript: Miller.

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