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Distinct generation, pharmacology, and distribution of sphingosine 1-phosphate and dihydrosphingosine 1-phosphate in human neural progenitor cells

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ABSTRACT

In vivo and *in vitro* studies suggest a crucial role for Sphingosine 1-phosphate (S1P) and its receptors in the development of the nervous system. Dihydrosphingosine 1-phosphate (dhS1P), a reduced form of S1P, is an agonist at S1P receptors, but the pharmacology and physiology of dhS1P has not been widely studied. The mycotoxin fumonisin B1 (FB_1) is a potent inhibitor of ceramide synthases and causes selective accumulation of dihydrosphingosine and dhS1P. Recent studies suggest that maternal exposure to FB_1 correlates with the development of neural tube defects (NTDs) in which the neural epithelial progenitor cell layers of the developing brain fail to fuse. We hypothesize that the altered balance of S1P and dhS1P in neural epithelial cells contributes to the developmental effects of FB_1. The goal of this work was first to define the effect of FB_1 exposure on levels of sphingosine and dh-sphingosine and their receptor-active 1-phosphate metabolites in human embryonic stem cell-derived neural epithelial progenitor (hES-NEP) cells; and second, to define the relative activity of dhS1P and S1P in hES-NEP cells. We found that dhS1P is a more potent stimulator of inhibition of cAMP and Smad phosphorylation than is S1P in neural progenitors, and this difference in apparent potency may be due, in part, to more persistent presence of extracellular dhS1P applied to human neural progenitors rather than a higher activity at S1P receptors. This study establishes hES-NEP cells as a useful human *in vitro* model system to study the mechanism of FB_1 toxicity and the molecular pharmacology of sphingolipid signaling.

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1. Introduction

Sphingosine 1-phosphate (S1P[1]) is a bioactive signaling lipid that is generated in cells from sphingosine by the action of sphingosine kinases (SphKs). S1P activates a family of five G-protein coupled S1P receptors (S1P 1-5) to regulate diverse physiologic and pathologic processes (Fyrst and Saba, 2010). Dihydrosphingosine, the reduced form that is a biosynthetic precursor of ceramide and thus sphingosine, is also a substrate for SphKs, giving rise to dihydrosphingosine 1-phosphate (dhS1P) (see Fig. 1). dhS1P is also a ligand for S1P receptors (Im et al., 2001), but the pharmacology and physiology of dhS1P is less well studied, in part due to its presence at much lower concentrations than S1P in cells.

In vivo and *in vitro* studies suggest a crucial role for S1P and its receptors in the development of the nervous system (McGiffert et al., 2002; Mizugishi et al., 2005; Pitson and Pebay, 2009). For example, mouse embryos with depleted S1P levels resulting from deletion of Sphk alleles display increased apoptosis and decreased mitosis in neuroepithelial cells of the developing nervous system. In this system, the loss of SphK1/2 activity also resulted in failed neural tube closure in the mouse embryos (Mizugishi et al., 2005). *In vitro* studies also suggest important effects of S1P signaling in neural stem cells, for example, S1P regulates proliferation of rat neural progenitors (Harada et al., 2004), and we have recently shown that S1P promotes proliferation of human embryonic stem cell-derived neural epithelial progenitor (hES-NEP) cells through a Gi dependent mechanism (Hurst et al., 2008). The role of dhS1P in these effects has not been defined.

Recent studies suggest that maternal exposure to corn crops contaminated with the fungus *Fusarium verticilloides* that produces

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[1] S1P – sphingosine 1-phosphate; SphK – sphingosine kinases; S1P 1-5 – S1P receptors 1-5; dhS1P – dihydrosphingosine 1-phosphate; fumonisin B1 – FB_1; neural tube defects – NTDs; ceramide synthase – CS; human embryonic stem cell-derived neural epithelial progenitor – hES-NEP.

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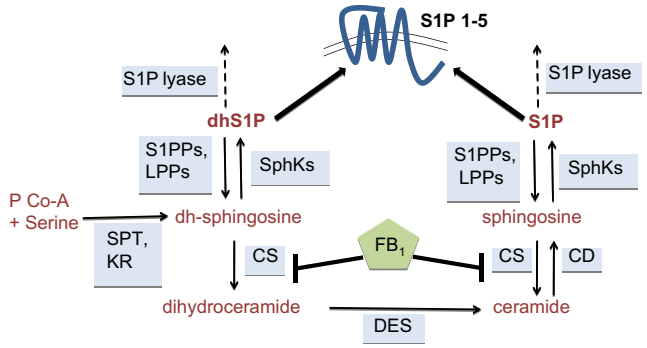

Fig. 1. Model of sphingolipid metabolism. This schematic depicts the *de novo* and salvage pathways of sphingolipid metabolism. Sphingosine 1-phosphate phosphatases (S1PPs), lipid phosphate phosphatases (LPPs), sphingosine kinases (SphKs), S1P receptors 1-5 (S1P 1-5), serine palmitoyl transferase (SPT), ketosphingosine reductase (KR), ceramide synthase (CS), ceramidase (CD), sphingosine 1-phosphate (S1P), dihydrosphingosine 1-phosphate (dhS1P), dihydroceramide desaturase (DES), Fumonisin B1 (FB_1), Palmitoyl Co-A (P Co-A).

the mycotoxin fumonisin B1 (FB_1) has been associated with the development of neural tube defects (NTDs) in areas where corn is a dietary staple and diets are likely to be deficient in folate (Marasas et al., 2004; Missmer et al., 2006). FB_1 exposure also causes NTDs in a mouse model, in which maternal exposure to FB_1 leads to NTD formation in nearly 80% of pups (Gelineau-van Waes et al., 2005). FB_1 is structurally related to sphingoid bases and is a potent inhibitor of ceramide synthase (CS) enzymes, key mediators of *de novo* sphingolipid biosynthesis (Wang et al., 1991). Thus, FB_1 inhibits multiple sphingolipid dependent cellular processes (Merrill et al., 2001; Stevens and Tang, 1997). The sphingoid base analog FTY720 also inhibits CS (Berdyshev et al., 2009) and induces NTDs in a mouse model (Gelineau-van Waes et al., 2008).

Given that FB_1 or FTY720 exposure and SphK deletion lead to similar NTDs in mouse models, it has been proposed that the mechanism of FB_1 developmental neurotoxicity is related to its effects on sphingolipid metabolism. In *de novo* sphingolipid biosynthesis, ceramide synthase enzymes are required to convert dihydrosphingosine to dihydroceramide, which is then converted first to ceramide by a desaturase and then to sphingosine by ceramidase (Fig. 1). Thus, FB_1 inhibition of ceramide synthase typically causes accumulation of dihydrosphingosine, increasing the dh-sphingosine:sphingosine ratio in cells (Enongene et al., 2002; Yoo et al., 1996). Given that both sphingoid bases are substrates for SphKs, FB_1 exposure may also alter the pools of S1P and dhS1P. FB_1-induced accumulation of S1P and dhS1P has been demonstrated in maternal mouse tissue of pregnant dams (Voss et al., 2009) and dhS1P has been detected in fetal mouse liver from dams fed diets contaminated with FB_1 (Riley et al., 2006).

As described, human epidemiology studies suggest that FB_1 may be a developmental neurotoxin causing NTDs, and mouse studies suggest the mechanism may be in part related to disruptions in S1P receptor signaling. However, a human cell system in which mechanistic studies can be carried out is critical to bridge these two systems, given the well-documented differences between mouse and human progenitor cell signaling pathways (Callihan et al., 2011). We have previously shown that hES-NEP cells express functional S1P receptors that regulate second messenger production, proliferation, and cellular morphology (Hurst et al., 2008). Therefore, hES-NEP cells represent a potential model system to study the effects of FB_1 exposure on S1P receptor pharmacology and possible mechanisms of NTD development. The twin goals of this work were to define the effect of FB_1 exposure on levels of sphingosine and dh-sphingosine and their receptor-active 1-phosphate metabolites in hES-NEP cells, and the relative activity of dhS1P and S1P in human neural progenitor cells. We report selective elevation of dhS1P following FB_1 exposure and distinct pharmacologic activity and distribution of dhS1P and S1P in hES-NEP cells. Our results suggest that FB_1 may specifically induce dhS1P mediated signaling effects in this developmentally important cell type.

2. Methods and materials

2.1. Materials

Fumonisin B1 (Cayman Chemical), S1P, dhS1P, and C17 S1P (Avanti Polar Lipids), C20-sphinganine (Matreva), IBMX and forskolin (Sigma-Aldrich), [^{3}H]-adenine and [^{3}H]-myoinositol (American Radiolabeled Chemicals), [^{14}C]-cAMP (GE Healthcare), pSmad2 antibody (Cell Signaling Technologies). Activin A was generously provided by Dr. Steven Dalton, University of Georgia. S1P stocks were prepared in H_2O containing 1% fatty acid-free BSA, and dhS1P stocks were prepared in DMSO. Both stocks were diluted into culture media containing 0.1% fatty acid-free BSA. Vehicle controls were included for each experiment.

2.2. hES-NEP cell culture

Commercially available stocks of hES-NEP cells derived from WA09 human embryonic stem cells were used (available as STEMEZ™ hNP1™, Aruna Biomedical). Tissue culture plates were first coated with matrigel (BD Biosciences) diluted 1:200 in NEUROBASAL™ medium (GIBCO) for 1.5 h at room temperature, and washed with HyClone® DPBS/MODIFIED (1X) with calcium & magnesium (Thermo Scientific) prior to application of media containing cells. Cells were grown in AB2™ media with ANS™ (Aruna Biomedical) supplemented with 2 mM L-glutamine (Sigma) and 20 ng/mL b-FGF (R&D Systems). Cells were passaged approximately every 48 h and split 1:2 following manual dissociation using a 25 cm cell scraper (Sarstedt).

2.3. Liquid chromatography/tandem mass spectrometry LC–MS

hES-NEP cells were plated in 6-well plates (BD Falcon) at 300,000 cells/well and allowed to grow to confluency (~500,000 cells/well). Cells were treated as described and collected using a 25 cm cell scraper and pelleted at 200 × g for 5 min at 4 °C in a 15 mL conical culture tube. Cell pellets and media were separated and stored at −80 °C until extraction. Samples were extracted and analyzed using a modification of the method described previously (Zitomer et al., 2008). Briefly, cell pellets were thawed on ice and 1.0 mL of cell extraction mixture [1:1 acetonitrile:water containing 5% formic acid and 60 pmol/mL of C20-sphinganine and C17-sphingosine 1-P internal standards] is added to the tube and vortexed gently. Samples were then placed in a sonicator at 50 °C for 1 h. Following sonication, samples were rocked gently for 2 h at room temperature. Extracted cell samples were then centrifuged, and 0.5 mL of supernatant was clarified using 1.5 mL 0.45 μm nylon centrifuge tube filters (COSTAR®, Corning Inc., Corning. NY, USA). Samples were transferred to LC–MS vials and analyzed via LC–MS as described previously (Zitomer et al., 2008). Media samples (0.5 mL) were extracted and analyzed using a similar protocol except that the extraction mixture was 0.5 mL of acetonitrile containing 10% formic acid and 120 pmol/mL of C20-sphinganine and C17-sphingosine 1-P internal standards.

The limits of detection in cells were 0.25 pmol/500,000 cells (dh-sphingosine), 0.625 pmol/500,000 cells (sphingosine), 0.625 pmol/500,000 cells (dh-sphingosine 1-P), and 0.625 pmol/500,000 cells (sphingosine 1-P). The limits of detection in media samples were 0.5 pmol/mL (dh-sphingosine), 1.25 pmol/mL (sphingosine), 1.25 pmol/mL (dh-sphingosine 1-P), and 1.25 pmol/mL (sphingosine 1-P). Data shown are representative of at least 2 experiments performed in duplicate.

2.4. Generation of S1P receptor-expressing CHO cells

SIP receptor-expressing CHO cells were generated as described previously (Kennedy et al., 2011). Briefly, CHO-K1 cells were transfected with pcDNA containing DNA sequences for HA-tagged S1P1, 2, 3, 4, or 5. Cells expressing the desired receptor were sorted using anti-hemagglutinin-phycoerythrin fluorescent antibody (Milentyi Biotec Inc, Auburn, CA) and a FACSVantage SE Turbo Sorter (BD Biosciences, Franklin Lakes, NJ). Cell populations were maintained in F-12K medium supplemented with 10% fetal bovine serum and 1 mg/mL geneticin (G418).

2.5. Adenylyl cyclase activity

We used a modified version of established protocols (Hettinger-Smith et al., 1996). hES-NEP or CHO-K1 cells were plated in 24-well plates (BD Falcon) and labeled with 0.6 μCi [^{3}H]-adenine for 3 h in the presence or absence of 200 ng/mL pertussis toxin (US Biologics). Assay buffer containing 1 mM isobutylmethylxanthine (IBMX), 50 μM forskolin (FSK), and varying concentrations of S1P or dhS1P (Avanti Polar Lipids) were added to the cells for 20 min at 37 °C. Reactions were terminated by aspiration followed by addition of stop solution containing 1.3 mM cAMP and 2% sodium dodecyl sulfate. [^{14}C]-cAMP stock was added to each well to control for recovery of cAMP, followed by perchloric acid to lyse cells. Lysates were neutralized with KOH and cAMP was isolated using sequential column chromatography over