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

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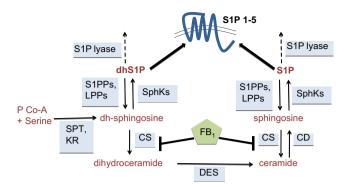[b] Toxicology and Mycotoxin Research Unit, USDA-ARS-SAA, Athens, GA 30604-5677, USA
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ABSTRACT

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.

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$_2$O 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

Dowex AG-50-W4 cationic exchange resin (BioRad, Hercules, CA) followed by neutral alumina columns. The resulting eluate was subjected to scintillation counting after the addition of scintillation cocktail. Data were analyzed using GraphPad Prism software using a nonlinear regression dose–response model. Data shown are representative of at least 3 experiments performed in triplicate. Calculated S1P and dhS1P EC$_{50}$ values from three independent experiments were compared using a t-test, with p-value less than 0.05 considered significant.

2.6. Inositol phosphate assay

Production of inositol phosphates (IP) was measured using established protocols (Hepler et al., 1987). Briefly, hES-NEP or CHO-K1 cells were plated in 24-well plates and grown to ~80% confluency. Cells were incubated with 1 μCi/well [^{3}H] myoinositol (American Radiolabeled Chemical, St. Louis, MO) for 18 h to label the cellular pool of phosphatidyl inositol. The cells were treated with varying concentrations of S1P or dhS1P in the presence of 10 mM lithium chloride, to inhibit the degradation of inositol phosphates, for 30 min at 37 °C. Following aspiration, cells were lysed in cold 50 mM formic acid and neutralized with ammonium hydroxide. The lysates were loaded onto columns of AG 1-X8 anion exchange resin (BioRad, Hercules, California). The columns were washed with water and dilute ammonium formate to remove unhydrolyzed lipids. The [^{3}H] IPs were then eluted with 1.2 M ammonium formate/0.1 M formic acid, and added to scintillation cocktail for counting. In some experiments, cells were treated with 100 ng/mL pertussis toxin for 18 h prior to assay. Data were analyzed using GraphPad Prism software using a nonlinear regression dose–response model. Data shown are representative of at least 3 experiments performed in triplicate. Calculated S1P and dhS1P EC$_{50}$ values from three independent experiments were compared using a t-test, with p-value less than 0.05 considered significant.

2.7. Smad2 phosphorylation

hES-NEP cells were plated in 24-well plates. dhS1P or S1P was added to the cells for 45 min at 37 °C. The reaction was terminated by aspirating the media and adding 100 μL SDS-PAGE sample buffer. Cells lysates were boiled for 5 min in protein sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using a primary antibody targeted against phospho-Smad2 (Cell Signaling Technologies, #3108) and peroxidase conjugate secondary antibody (Bethyl Laboratories, Montgomery, TX). Bands were visualized using SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL). Densitometry analysis was performed using Total Lab 1D Gel Analysis software. Background bands were not subtracted out and all lanes and bandwidths were of equal size. Densitometry results for phospho-Smad2 were normalized to GAPDH to control for loading. Each figure is representative of at least 3 experiments performed in duplicate. Statistical increases in Smad phosphorylation over basal levels were determined using an unpaired, two-tailed t-test using mean and SEM. p-values less than 0.05 were considered significant.
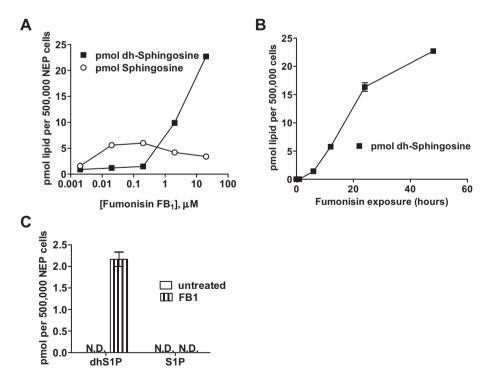
2.8. GTPγS binding

Membrane fractions were prepared from CHO cells stably transfected to over-express the human S1P4 receptor as described (Hooks et al., 2001). These membrane fractions were incubated in 96-well format in 100 μL of binding buffer [50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10 mM MgCl$_2$, and 100 mM NaCl at pH 7.5 containing 0.1% fatty acid-free bovine serum albumin] with 5 μg saponin, 11.5 μM GDP, 0.3 nM [γ-^{35}S] GTP (1200 Ci/mmol) and a concentration range of S1P or dhS1P for 30 min at 30 °C. Membranes were recovered on GF/C filters using a 96-well Brandel Cell Harvester (Gaithersburg, MD). Filter plates containing membranes were analyzed for bound radionuclide using a TopCount beta scintillation counter (Packard). Binding data (in Counts Per Minute) were analyzed in GraphPad Prism software using a nonlinear regression sigmoidal dose–response model. Data shown are representative of quadruplicate samples with error bars representing standard error of the mean.

3. Results

3.1. Analysis of sphingolipid metabolites following FB$_1$

To determine the effects of FB$_1$ on sphingolipid metabolism in a human neuroepithelial cell, we treated hES-NEP cells with increasing concentrations of FB$_1$ for 48 h and measured cellular sphingosine and dh-sphingosine levels using LC–MS. We observed a concentration- and time- dependent increase in dh-sphingosine accumulation, with maximal effects observed between 2 μM and 20 μM FB$_1$ treatment and after 48 h treatment (Fig. 2A, B). Sphingosine levels also increased, but to a much smaller extent than dh-sphingosine levels, and the effect was not consistently observed. We also measured sphingosine and dh-sphingosine levels in media, and detected an increase in extracellular dh-sphingosine, but not sphingosine (data not shown).

The accumulation of dh-sphingosine could result in increases in the S1P receptor agonist dhS1P through the action of sphingosine

Fig. 2. Analysis of sphingolipid metabolites following FB$_1$ exposure. hES-NEP cells were treated with the indicated amount of FB$_1$. Cells were harvested and sphingolipid metabolites were quantified using LC–MS as described in Methods. Results are reported as pmol of metabolite per cells. (A) dh-sphingosine and sphingosine accumulation was determined after incubation with various concentrations of FB$_1$ for 48 h (B) dh-sphingosine accumulation was determined after incubation with 20 μM FB$_1$ for various time-points. (C) dhS1P and S1P accumulation was determined after incubation with 20 μM FB$_1$ for 48 h (N.D. = not detected).

kinases. Indeed, we observed detectable levels of dhS1P in cells treated with 20 μM FB$_1$, but not in untreated control cells (Fig. 2C). S1P was not detectable in FB$_1$ treated or untreated cells under these conditions (limit of detection of dhS1P and S1P per well: 0.625 pmol; details in Methods.) These data show that 48 h exposure of hES-NEP cells to FB$_1$ results in accumulation of the sphingoid base dh-sphingosine and the bioactive lysophosphosphingolipid dhS1P, but not sphingosine or S1P.

3.2. Pharmacology of S1P and dhS1P-stimulated second messenger production in hES-NEP cells

The observation that dhS1P, but not S1P, increases after exposure to FB$_1$ raises questions about their relative biological roles. dhS1P is expressed in most biologic systems at much lower levels than S1P and is an agonist at S1P receptors, but its activity has not been well defined. Surprisingly, Trojanowska and colleagues have reported markedly different activities of dhS1P and S1P in fibroblasts (Bu et al., 2008, 2006). To define the relative pharmacologic activity of dhS1P and S1P in hES-NEP cells, we determined the potency and efficacy of S1P and dhS1P in two major second messenger pathways downstream of S1P receptors. Inositol triphosphate (IP$_3$) is generated by phospholipase C, which is typically activated downstream of activated Gαq, and also by Gβγ following activation of Gi coupled receptors. cAMP is generated by adenylyl cyclase, which is inhibited by activation of Gαi. Given that all S1P receptors couple to either Gi or Gq (or both), the use of these two assays allows complete characterization of endogenous S1P receptor activation.

There was no significant difference in the potency or efficacy of S1P and dhS1P stimulation of phospholipase C activity in hES-NEP cells, as measured by IP$_3$ accumulation (Fig. 3A). Both lipids resulted in robust accumulation of IP$_3$, with an EC$_{50}$ of approximately 200 nM. However, in cAMP accumulation assays, dhS1P was consistently and significantly more potent than S1P at inhibiting adenylyl cyclase activity (pEC$_{50}$ S1P: 6.9, pEC$_{50}$ dhS1P: 8.15, $p = 0.045$) (Fig. 3B). Thus, not only is dhS1P accumulation much higher than S1P accumulation following FB$_1$ exposure, dhS1P also has greater apparent potency in Gi coupled signaling than S1P in hES-NEP cells.

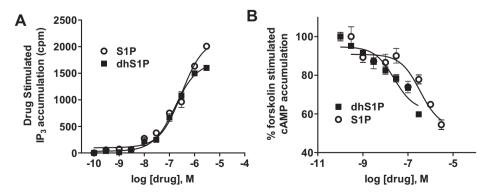## 3.3. Pharmacology of S1P and dhS1P in Smad phosphorylation

Trojanowska and colleagues reported that in dermal fibroblasts, dhS1P and S1P showed opposing effects on transforming growth factor-β (TGFβ)/Smad signaling. In these cells, S1P activated Smad phosphorylation while dhS1P inhibited TGFβ induced Smad phosphorylation (Bu et al., 2008). Smads are key regulators of differentiation and pluripotency pathways in stem cells. If dhS1P and S1P have opposing effects on Smad signaling in neural progenitor cells as reported in fibroblasts, then the selective upregulation of dhS1P versus S1P following FB$_1$ exposure would strongly inhibit Smad activity and tightly control proliferation and differentiation of these cells. However, in hES-NEP cells, we found that both S1P and dhS1P induced Smad2 phosphorylation (Fig. 4A), and neither sphingolipid inhibited activation of Smad2 by the TGFβ ligand, activin (Fig. 4B). Thus, dhS1P and S1P do not show opposing effects on Smad signaling in hES-NEP cells. We did observe differences in activity of dhS1P and S1P. dhS1P resulted in higher levels of Smad activity at 1 μM and 10 μM than equal concentrations of S1P, and the effect of dhS1P was significant at both concentrations, while the effect of S1P was only statistically significant at 10 μM (Fig. 4A). These data suggest that dhS1P is more efficacious than S1P at Smad2 phosphorylation in hES-NEP cells, and these lipids regulate Smad signaling though different mechanisms or pathways in neural progenitors versus fibroblasts.

3.4. Pharmacology of S1P and dhS1P-stimulated signaling in stable S1P receptor-expressing CHO-K1 cell lines

Our results in hES-NEP cells document that dhS1P is more potent than S1P at a subset of S1P/dhS1P-stimulated responses, suggesting that dhS1P may have higher activity at one or more of the S1P receptors expressed in hES-NEP cells. We have previously reported that hES-NEP cells express S1P receptors 1-3, 5 (Hurst et al., 2008). Further, we have previously shown that dhS1P can activate S1P receptors in a broken cell assay with overexpressed Gi heterotrimers (Im et al., 2001). To determine the relative activity of dhS1P and S1P at each of the S1P receptors in an intact cell system with endogenous G-proteins, we generated stable CHO-K1 cell lines expressing individual human S1P receptors. The potency of both ligands was assessed in IP$_3$ and cAMP assays in parental cells and each receptor-expressing cell line.

No S1P-stimulated or dhS1P-stimulated activation of IP$_3$ accumulation or inhibition of cAMP accumulation was observed in the parental CHO-K1 cell line (Fig. 5A). Due to distinct G-protein and effector system coupling of the different receptors, some receptor-expressing cell lines were able to activate only one of the pathways, for example cells expressing S1P1 showed potent Gi-coupled inhibition of cAMP accumulation but not IP$_3$ accumulation in response to S1P, while S1P2 expressing cells showed no cAMP response, but robust IP$_3$ accumulation in response to S1P.

Fig. 3. Pharmacology of S1P and dhS1P in hES-NEP cells. (A) hES-NEP cells were treated with S1P or dhS1P for 30 min and assayed for IP levels as described in Methods. Results are reported as percent of basal inositol phosphate accumulation (CPM) with counts for drug treated wells divided by values for vehicle treated wells. (B) hES-NEP cells were treated with 50 μM forskolin and S1P or dhS1P for 20 min then assayed for cAMP levels as described in Methods. Results are reported as percent of forskolin stimulated cAMP accumulation with forskolin, +vehicle treated well representing 100%.
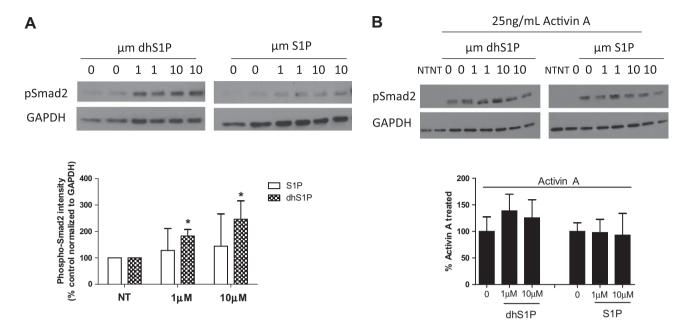
A

B

Fig. 4. Role of S1P and dhS1P in Smad2 phosphorylation. (A) hES-NEP cells were untreated (NT) or treated with S1P or dhS1P for 45 min and then assayed for phosphorylated Smad2 as described in Methods. (B) hES-NEP cells were also treated with 25 ng/mL Activin A for 45 min in the presence or absence of S1P or dhS1P. Densitometry results are reported as the ratio of pSmad2 to GAPDH.

dhS1P and S1P exhibited similar potencies at S1P receptors S1P1 (cAMP assay), S1P3 (cAMP and IP$_3$ assay), and S1P5 (cAMP). However, dhS1P was significantly less potent than S1P at receptor S1P2 (IP$_3$ assay, Fig. 5C). Even though S1P4 is expressed at very low to undetectable levels in hES-NEP cells, we also compared the potency of dhS1P and S1P at S1P4 since the relative activity of dhS1P and S1P has not been defined at this receptor subtype. As observed at SIP2, dhS1P was markedly less potent than S1P at S1P4 receptors in a GTPγS binding assay (Fig. 5D). Thus, dhS1P did not show significantly higher pharmacologic activity at any of the individual S1P receptors, suggesting that receptor selectivity does not account for the increased potency of dhS1P in cAMP inhibition or Smad phosphorylation in hES-NEP cells.

3.5. Clearance of extracellular S1P and dhS1P in hES-NEP cell cultures

Given that receptor selectivity does not account for the observed differences in activity, we next predicted that S1P and dhS1P have different half-lives in extracellular media of hES-NEP cells to account for the difference in their apparent potency in these cells. To test this hypothesis, we treated hES-NEP cell cultures with 1 μM S1P or dhS1P in complete media for 2, 5, 10 and 30 min at 37 °C, consistent with the conditions and duration of the cAMP, IP$_3$, and Smad phosphorylation assays. We then isolated the media and cell monolayers, and performed lipid extraction and LC–MS as above to quantify the percent of extracellularly applied dhS1P and S1P in the cellular and media compartments at the end of each incubation period. We found that a significantly higher percent of the original level of dhS1P remained in the media during this time course. At 10 min, 70% of the original dhS1P remained in media, while only 48% of S1P remained (Fig. 6). Under these conditions, endogenous levels of cellular S1P and dhS1P were below the limit of detection. However, following addition of extracellular lipid, cell-associated S1P or dhS1P was detected at each time point. Inverse to the trend observed in media, cellular dhS1P levels were significantly lower than S1P levels in cells. dhS1P uptake peaks after 2 min and

does not exceed 25% of exogenously applied lipid, while S1P levels continue to increase until 5 min and reach levels as much as 80% of exogenously applied lipid.

4. Discussion

Numerous studies have demonstrated an important role for sphingolipid mediators in the regulation of neural development and in the developmental neural toxicity of FB$_1$ exposure in mice, but a human model system to study the mechanism of FB$_1$ toxicity has been lacking. This study was initiated to define the ability of human neuroepithelial progenitor cells to generate sphingolipid mediators in response to FB$_1$ exposure and to respond to extracellular S1P and dhS1P. Our findings indicate that hES-NEP cells generate dihydrosphingosine and dhS1P in response to FB$_1$ exposure, but not sphingosine and S1P. Further, we document 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 powerful human *in vitro* model system to study the mechanism of FB$_1$ toxicity and the molecular pharmacology of sphingolipid signaling cascades. Multiple studies have established a clear role for phosphosphingolipids in the regulation of many neural cell types; however, results from these studies vary because of the range of model systems differing in species of origin, stage of development, and origin/derivation of cell lines. There are multiple differences in the regulation of differentiation and pluripotency signaling pathways between human and rodent stem cells, making results from rodent systems difficult to apply to human developmental toxicity. hES-NEP cells are advantageous because they are human cells that grow as an adherent, homogeneous monolayer and can be further differentiated to neurons and glia in culture. Additionally, hES-NEP cells are a stable, renewable *in vitro* model of the neuroepithelial
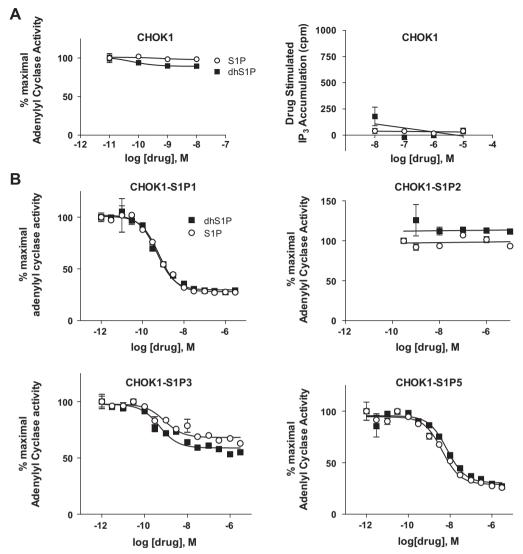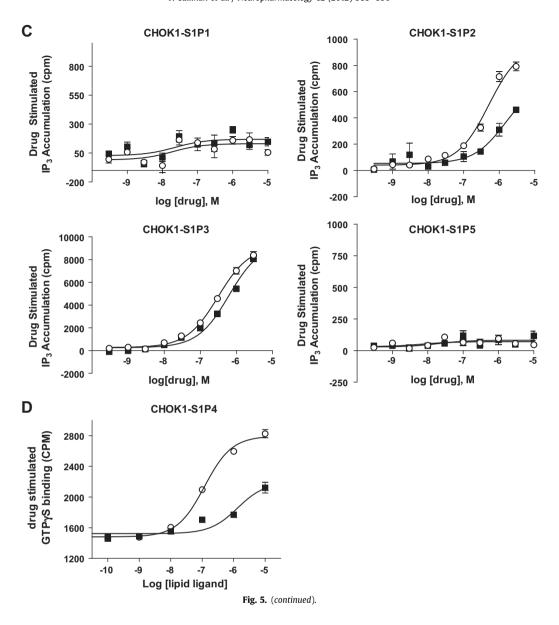
Fig. 5. Pharmacology of S1P and dhS1P-stimulated signaling in stable S1P receptor-expressing CHO-K1 cell lines. CHO-K1 cells stably expressing individual human S1P receptors were generated as described in Methods. (A) Parental CHO-K1 cells were assayed for S1P- or dhS1P-stimulated inhibition of cAMP and activation of PLC as described. (B) Stable receptor-expressing CHO-K1 cells were treated with 50 μM forskolin and the indicated drugs for 20 min then assayed for cAMP levels as described in Methods. Results are reported as percent of forskolin stimulated cAMP accumulation with forskolin, +vehicle treated well representing 100%. (C) Stable receptor-expressing CHO-K1 cells were treated with each of the indicated drugs for 30 min and assayed for IP levels as described in Methods. Results are reported as percent of basal inositol phosphate accumulation (CPM) with counts for drug treated wells divided by values for vehicle treated wells. (D) GTPγS binding assay using membranes from CHO-K1 S1P4 cells.

cell layer responsible for neural tube closure, a critical step in nervous system development.

Closure of the neural tube occurs when neuroepithelial progenitor cells undergo rapid proliferation and migration resulting in the fusion of ectodermal folds at the embryonic midline (Copp and Greene, 2010). Disruption of this process leads to neural tube defects (NTDs) such as spina bifida and anencephaly. NTDs are among the most common birth defects worldwide and their causes are not well understood. The incidence of NTDs is often high in populations where corn is a dietary staple and the diets are likely to be deficient in folic acid and other B vitamins (Marasas et al., 2004). In many of these areas the corn is frequently contaminated with high levels of fumonisin FB_1 (Torres et al., 2007) which has been shown to disrupt sphingolipid metabolism and induce NTD in mouse models (Gelineau-van Waes et al., 2009).

FB_1 inhibits ceramide synthase, thereby disrupting *de novo* sphingolipid biosynthesis (Fig. 1). In most systems, this inhibition results in the rapid accumulation of dh-sphingosine, rather than

sphingosine (Riley et al., 2001). In hES-NEP cells, endogenous cellular levels of sphingosine are approximately two-fold higher than sphinganine levels (endogenous S1P and dhS1P were below detection limits under the conditions used in our experiments.) Upon FB_1 exposure, dh-sphingosine levels increase sharply, with no significant increase in sphingosine, resulting in markedly higher cellular levels of dh-sphingosine compared to sphingosine. Our results also show that FB_1 exposure causes increases in cellular levels of dhS1P, but not S1P. However, the magnitude of the increase in dhS1P in hES-NEP cells is much lower than in other cell types and tissues such as mouse red blood cells, liver, kidney and placenta (Gelineau-van Waes, personal communication). Indeed, conditioned media removed from FB_1 treated hES-NEP cells did not contain sufficient phosphosphingolipids to activate S1P receptors in CHO-K1 cells expressing the S1P1 receptor (data not shown), and both S1P and dhS1P were below the detection limit by LC–MS in media samples before or after FB_1 exposure. The low level of dhS1P production in hES-NEP cells is consistent with observations in pregnant mice, in

C

CHOK1-S1P1

CHOK1-S1P2

CHOK1-S1P3

CHOK1-S1P5

D

CHOK1-S1P4

Fig. 5. (*continued*).

which the embryo itself has only modest increases in dhS1P following FB$_1$ exposure, while maternal tissues and blood experience greater increases ((Voss et al., 2009) and unpublished data).

The relatively low level of dhS1P production by hES-NEP cells may be due to cell-specific levels of kinase, lyase, or phosphatase activities. hES-NEP cells apparently express sphingosine kinase activity, as the sphingosine analog FTY720 and dihydrosphingosine are converted to FTY720-P and dhS1P, respectively (data not shown). FTY720 is primarily phosphorylated by SphK2, and both sphingosine and dh-sphingosine are higher affinity substrates for SphK2 than is FTY720. Thus, there is likely sufficient SphK2 activity to generate S1P and dhS1P from the sphingoid bases. Further, the relatively low magnitude of dhS1P accumulation by neural progenitors in response to FB$_1$ may reflect rapid metabolism of the 1-phosphates by lipid phosphate phosphatase activity or lyase activity. Regardless, if FB$_1$-induced increases in dhS1P activity in neural progenitor cells are contributing to the developmental toxicity associated with this toxin, it is likely that the dhS1P derived from maternal tissue and distributed to the embryo by the maternal blood could also serve as a source of extracellular dhS1P.

We also explored the relative ability of extracellular dhS1P and S1P to stimulate receptor signaling cascades in hES-NEP cells. As described above, Bu et al. reported that dhS1P and S1P differentially regulate Smad phosphorylation (Bu et al., 2008). Smad signaling pathways are critical determinants of pluripotency and differentiation in stem cells, and therefore may be relevant in regulating hES-NEP cell biology and regulating neural tube closure. Our results indicate that dhS1P was more active than S1P in activating Smad phosphorylation in hES-NEP cells. This result suggests that dhS1P regulation of Smad2 occurs through different mechanisms in human neural progenitors and fibroblasts. We further found that dhS1P is significantly more potent than S1P in inhibiting adenylyl cyclase activity, while the two lipids had equal potency in activating phospholipase C. As described above, S1P is an important regulator of neural progenitor function, and loss of S1P (and dhS1P) synthesis via deletion of the sphingosine kinase enzymes leads to neural tube defects in mice {Mizugishi et al., 2005 #3430}. This is seemingly at odds with the observation that FB$_1$, which stimulates accumulation of a potent S1P receptor agonist, also causes neural tube defects in mice. It is likely that *in vivo*, perturbations of agonist concentrations trigger
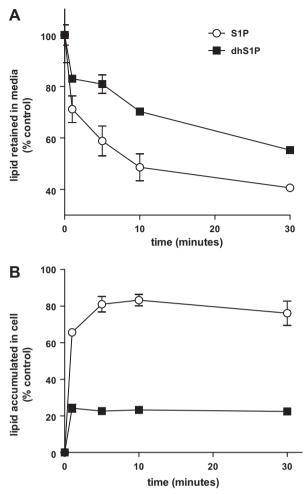
Fig. 6. Analysis of S1P and dhS1P in hES-NEP media and cells. hES-NEP cells (∼500,000 per well) were untreated (NT) or treated with 1 μM S1P or dhS1P for 2, 5, 10, and 30 min. Cells and media were harvested and sphingolipid metabolites were quantified using LC–MS as described in Methods. Results are reported as % of total exogenous applied lipid. (A) S1P and dhS1P in hES-NEP media. (B) S1P and dhS1P in hES-NEP cells.

complex feedback mechanisms to regulate the strength of receptor signaling. Indeed, FTY720-P is a high potency agonist of S1P receptors *in vitro* but is a functional antagonist *in vivo* due to its ability to induce receptor internalization and down regulation. Additional studies are required to reconcile these *in vitro* and *in vivo* observations.

Given the higher potency and/or activity of dhS1P versus S1P at specific responses in hES-NEP cells, we predicted that dhS1P would have higher potency than S1P at one or more of the S1P receptors. However, the above data indicate that dhS1P potency is equal to or less than that of S1P at each individual S1P receptor. Importantly, receptor expression is likely much higher in the transfected CHO cells than hES-NEP cells. Such receptor reserve can inflate the apparent potency of drugs, as reflected in the lower EC_{50} values of both ligands in cAMP inhibition assays performed in CHO cells compared to hES-NEP cells. Regardless, the results from these studies did not explain our findings in hES-NEP cells. A possible trivial explanation for the observed differences in activity of dhS1P and S1P is that, due to differences in solubility, dhS1P and S1P stock solutions were prepared differently. However, the same stocks were used throughout the study, so that if differences in stock preparations affected potency, the difference in potency would be consistent in all assays. In contrast, we observed equal potency in some assays, greater dhS1P potency in some assays, and greater S1P potency in some assays. Thus, stock preparation does not account for the observed differences.

We next hypothesized that differences in dhS1P versus S1P metabolism and cellular uptake could account for the signaling differences in hES-NEPs. We found that significantly more dhS1P remains intact in the extracellular media during the time course of the signaling assays than S1P. Mirroring this trend, we found much lower accumulation of extracellularly applied dhS1P in the cellular fraction than S1P. This prolonged presence of dhS1P in media may contribute to its higher activity in cAMP inhibition and Smad phosphorylation. However, it should be noted that the fold difference in concentrations of S1P and dhS1P in media is less than the difference in their potency, suggesting other factors also contribute to the higher apparent potency of dhS1P in NEP cells. For example, dhS1P and S1P may have different intrinsic efficacies, which may not have been observed in CHO cells due to receptor reserve. Finally, the apparent equal potency in IP$_3$ assays may reflect that this effect is likely mediated by the Gq coupled S1P2 receptors, which exhibit a higher potency with S1P than dhS1P, thus canceling out the effect of the prolonged presence of dhS1P in media.

The higher accumulation of cellular S1P than dhS1P suggests that cellular uptake indeed contributes to the loss of S1P in the media, but we cannot discount the potential role of differential rates of metabolism by enzymes such as the LPP ecto-phosphatases. It is tempting to speculate that dhS1P is degraded more rapidly than S1P, given that the combined extracellular and cellular S1P remaining after 30 min is considerably higher than dhS1P;

however, matrix effects between media and cell fraction samples may be quite different, so quantitative comparisons between LC–MS analysis of cellular and media samples are not supported. Regardless, it is clear that within media samples, dhS1P is present at higher concentrations at later time points, and in cellular samples, S1P concentrations are much higher than dhS1P. The subcellular localization of sphingolipid metabolizing enzymes and sphingolipid transporters has been the focus of recent studies. Specifically, the movement of sphingoid base 1-phosphates across cell membranes may be mediated by either ABC transporters (ABCC1 or ABCG2) (Takabe et al., 2010) and/or SPNS2 (Hisano et al., 2010). The relative expression, activity, and selectivity of these transporters for dhS1P and S1P in neural progenitors need to be defined to determine their significance in our observations.

In conclusion, we have shown that exposure of hES-NEP cells to FB_1 results in preferential increases in dh-sphingosine over sphingosine. In turn, higher levels of the receptor-active dhS1P are produced than S1P. In hES-NEP cells dhS1P is more potent than S1P in stimulating adenylyl cyclase inhibition and Smad phosphorylation assays. These effects do not result from differences in potency at individual S1P receptors, rather from differences in cellular uptake and metabolism. Ultimately, this study indicates that dhS1P may play a role in regulating the response of neural progenitor cells to FB_1 exposure.

Acknowledgments

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