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Changes in the Metabolism of Sphingolipids after Subarachnoid Hemorrhage

Fernando D Testai, MD, PhD¹, Hao-Liang Xu, MD, PhD², John Kilkus³, Vidyani Suryadevara, MS⁴, Irina Gorshkova, PhD⁵, Evgeny Berdyshev, PhD⁵, Dale A Pelligrino, PhD², and Dawson Dawson, PhD³

¹Department of Neurology and Rehabilitation, University of Illinois at Chicago, Chicago, IL, USA

²Department of Anesthesiology, University of Illinois at Chicago, Chicago, IL, USA

³Department of Pediatrics, University of Chicago, Chicago, IL, USA

⁴Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA

⁵Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Abstract

Background—We previously described that ceramide (Cer), a mediator of cell death, increases in the cerebrospinal fluid (CSF) of subarachnoid hemorrhage (SAH) patients. This study investigated the alterations of biochemical pathways involved in Cer homeostasis in SAH.

Methods—Cer, dihydroceramide (DHC), sphingosine-1-phosphate (S1P) and the activities of acid sphingomyelinase (ASMase), neutral sphingomyelinase (NSMase), sphingomyelinase synthase (SMS), S1P-lyase, and glucosylceramide synthase (GCS) were determined in the CSF of SAH subjects and in brain homogenate of SAH rats.

Results—Compared to controls (n=8), SAH patients (n=26) had higher ASMase activity (10.0±3.5 IF/μl.min vs. 15.0±4.6 IF/μl.min; p=0.009) and elevated levels of Cer (11.4±8.8 pmol/ml vs. 33.3±48.3 pmol/ml; p=0.001) and DHC (1.3±1.1 pmol/ml vs. 3.8±3.4 pmol/ml; p=0.001) in the CSF. The activities of GCS, NSMase, and SMS in the CSF were undetectable. Brain homogenates from SAH animals had increased ASMase activity (control: 9.7±1.2 IF/μg.min; SAH: 16.8±1.6 IF/μg.min; p<0.05) and Cer levels (control: 3422±26 fmol/nmol of total lipid P; SAH: 7073±2467 fmol/nmol of total lipid P; p<0.05) compared to controls. In addition, SAH was associated with a reduction of 60% in S1P levels, a 40% increase in S1P-lyase activity, and a 2-fold increase in the activity of GCS but similar NSMase and SMS activities than controls.

Conclusions—Our results show an activation of ASMase, S1P-lyase, and GCS resulting in a shift in the production of protective (S1P) in favor of deleterious (Cer) sphingolipids after SAH. Additional studies are needed to determine the effect of modulators of the pathways here described in the outcome of SAH.

Keywords

Cerebrovascular disorders; Subarachnoid hemorrhage; Sphingolipids

Introduction

Subarachnoid hemorrhage (SAH) is responsible for a small proportion of all strokes but carries a significant morbidity and mortality. The pathophysiology of brain injury after SAH is complex and not completely understood. Vasospasm, a frequently encountered complication of SAH, has been considered a major determinant of outcome (Crowley et al., 2011). The use of drugs that effectively reverse vasospasm, however, failed to show an improvement in SAH-associated morbidity and mortality (Macdonald et al., 2011). In this context, the attention was centered on the study of alternative mechanisms of brain injury that take place in this condition with the hope of identifying new therapeutic targets that can ultimately improve neurological outcome.

Sphingolipids constitute a family of endogenous bioactive membrane components that regulate vital cellular processes. Ceramide (Cer), in particular, participates actively in neural and oligodendroglial cell death and post stroke inflammation (Farooqui et al., 2007; Kilkus et al., 2003; Qin et al., 2009; Testai et al., 2004a; Yu et al., 2007). We have previously shown that Cer levels increase in the cerebrospinal fluid (CSF) of SAH survivors, particularly in those with poor neurological outcome (Testai et al., 2012), an observation that suggests that this deleterious mediator could mediate brain injury in this condition. The detrimental effects of Cer are balanced by sphingosine-1-phosphate (S1P) which is a proangiogenic and prosurvival sphingolipid that affects vascular diameter after SAH (Pyne and Pyne, 2010; Tosaka et al., 2001). The interest in S1P has been recently sparked by numerous reports indicating that the S1P analog FTY720 may be protective in both ischemic and parenchymal hemorrhage models (Altay et al., 2014; Brunkhorst et al., 2013; Kawabori et al., 2013; Wei et al., 2011). Despite the increasing evidence supporting the active participation of Cer and S1P in the pathogenesis of brain injury in cerebrovascular diseases, the role of these two bioactive lipids in SAH is virtually unknown. The goal of this study is expand on our previous observations by describing the effect of SAH on the biochemical pathways that participate in sphingolipid homeostasis.

Materials and Methods

Standards and Reagents

NBD-C₆-Erythro-ceramide was purchased from Matreya Inc. (Pleasant Gap, PA), hexamethylumbelliferyl phosphorylcholine was from Moscerdam Substrates (Amsterdam, Holland), and 2S-ammonio-3R-hydroxy-5-((2-oxo-2H-chromen-7-yl)oxy)pentyl hydrogen phosphate was from Cayman Chemical (Seattle, WA, USA). Silica gel HPTLC plates were from Whatman (Clifton, NJ) and the protein assay kit was from BioRad (Hercules, CA). Solvents used for HPTLC were ACS grade from Fisher Scientific (Pittsburgh, PA). Standards used in mass spectrometry analysis were obtained from Avanti Polar Lipids (Alabaster, AL). The antibody for S1P-lyase was from Santa Cruz Biotechnology Inc. (Santa

Cruz, CA) and the antibodies for sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2) were from Abcam Inc. (Cambridge, MA).

Participants

Human subjects were recruited at the University of Illinois Hospital. Institutional Review Board approval was obtained before study initiation and written informed consent was required to participate in this study. The study design, including inclusion and exclusion criteria for both cases and controls, was previously described (Testai et al., 2012). Indicators of stroke severity commonly used in clinical practice, including Glasgow Coma Scale and World Federation of Neurological Surgeons Scale, were determined at admission. Samples of CSF were obtained within 48 hours of symptom onset, centrifuged at $270 \times g$ for 15 minutes at 5°C, and the supernatant stored at -80°C until analysis.

Subarachnoid hemorrhage model

Experimental protocols were approved by the institutional Animal Care Committee of the University of Illinois at Chicago. We utilized the endovascular perforation of the terminal internal carotid artery (ICA) model. Adult Sprague–Dawley male rats (250–300 g) were randomly assigned to the SAH or sham-operated group (n=10 per group). Animals were anesthetized with 2% isoflurane and mechanically ventilated. Physiological variables, including blood pressure, blood gases, and body temperature were continuously monitored and kept within normal range. Regional cerebral blood flow (rCBF) was monitored before, during, and after SAH induction by Laser Doppler flowmetry (LDF) attached to the skull over the right middle cerebral artery (MCA) territory. After an anterior midline incision was made, the right ICA and external carotid artery (ECA) were isolated to its origin at the common carotid artery (CCA) bifurcation. The ECA was ligated and shaped into a short stump. The CCA was temporarily clipped, and a hollow polytetrafluoroethylene (PTFE) tube was advanced rostrally into the ICA from the ECA stump until resistance was felt. Then a tungsten wire was partially advanced through the PTFE tube to perforate the bifurcation of the anterior and middle cerebral arteries. Immediately after puncture, the PTFE tube and tungsten wire were retracted into the ECA stump and the ICA was reperused. The incision was then closed with nylon monofilament sutures and rats were extubated and returned to their cages. SAH was confirmed by a transient drop in cerebral blood flow of >85% and post-mortem examination. Rats were sacrificed at 48 hours post SAH by decapitation. Brains were isolated and homogenized in a lysis buffer (25 mM 2-[N-morpholino] ethanesulfonic acid, 150 mM NaCl, 1.0% Triton X-100, 1 mM Na₃VO₄, pH 6.5 supplemented with a protease inhibitor cocktail [leupeptin, phenylmethylsulfonyl fluoride, and aprotinin]) at 4°C using a loose-fit Dounce homogenizer.

Lipid Analysis

Lipids from brains were homogenized in 1 ml PBS and samples were standardized by protein content. Lipids were extracted by adding methanol (1 ml) to brain homogenate (0.6 ml) followed by chloroform (2 ml). The lower phase was evaporated to dryness under nitrogen and subjected to alkaline methanolysis for 1h with 1 ml of 0.6N NaOH. Samples were neutralized with 70 µl of concentrated HCl and salts were pelleted. Chloroform (2 ml)

and water (0.6 ml) were added to the supernatant. The upper phase was discarded and the lower phase was evaporated to dryness under nitrogen. The lipids were reconstituted in 300 μ l of chloroform:methanol (2:1 v/v) and 10 μ l were applied to HPTLC plates. Cer and cholesterol were resolved using chloroform:methanol:glacial acetic acid (94:1:5 v/v). For sphingomyelin (SM) TLCs were run up to 2/3 of the top in solvent 1 (chloroform:methanol:30% OHNH_4 65:25:5 v/v), evaporated to dryness, and then further resolved in solvent 2 (chloroform:acetone:methanol:glacial acetic acid:water 50:20:10:10:5 v/v). Lipids were visualized by charring with 10% CuSO_4 – 8% H_2SO_4 . The relative intensities of lipid bands were quantified by densitometry using ImageJ software (Molecular Dynamics, Sunnyvale, CA). SM and Cer levels were standardized by total cholesterol.

Mass Spectrometric Analysis of Ceramide and Dihydroceramide

Lipids from mouse brain tissues were extracted using modified Bligh and Dyer procedure as described earlier with the use of C17-sphingosine, N17:0-Cer, and C17-sphingosine-1-phosphate as internal standards (Bligh and Dyer, 1959). Total lipid extract was subjected to total lipid phosphorus analysis and then divided in two portions (Vaskovsky et al., 1975). The first part was directly analyzed for sphingoid bases by the LC-MS/MS and then per-acetylated for LC-MS/MS analysis of sphingoid base-1-phosphates as *bis*-acetate derivatives (Berdyshev et al., 2005). The second portion was subjected to solid phase extraction using silica SPE cartridges to partially purify Cer prior to LC-MS/MS analysis. Total lipids in chloroform were loaded on chloroform-equilibrated silica cartridge and Cer-containing fraction was eluted with 3 ml chloroform/methanol (95:5 v/v). Then, partially purified Cer were analyzed by LC-MS/MS. The analyses of Cer and dihydroceramide (DHC) in the CSF were performed by combined HPLC–tandem mass spectrometry with the use of C17-sphingosine and N17:0-Cer as internal standards as previously described (Testai et al., 2012). The instrumentation used was an AB Sciex 5500 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source interfaced with an automated Agilent 1200 series HPLC and autosampler (Agilent Technologies, Wilmington, DE). Analysis of the molecular species of Cer and DHC used positive ion electrospray mass spectrometry with multiple reaction monitoring analysis. Cer and DHC levels were standardized by total phosphorus in brain homogenate or ml of CSF.

Sphingomyelinase and α -Fucosidase assays

Sphingomyelinase (SMase) activity was measured as previously described (Qin et al., 2009). Briefly, 5 μ L of rat brain homogenate (50 μ g protein) or 30 μ L of human CSF were mixed with the fluorogenic substrate hexamethylumbelliferyl phosphorylcholine (0.5 mM). For neutral SMase (NSMase) the incubation was at 37°C in PBS containing 0.5 mM MgCl_2 , pH 7.4. The presence of phosphate was previously shown to inhibit any acid SMase (ASMase) activity (Testai et al., 2004b). The activity of ASMase was determined in 150 mM sodium acetate buffer containing 0.1 mM ZnCl_2 , pH 5.0. α -Fucosidase activity was determined by using the fluorescent substrates 4-methylumbelliferyl- α -l-fucoside. Samples were buffered in 100 μ L 150 mM sodium acetate, pH 5.0, and incubated at 37°C for up to 1 h with the substrate (0.5 mM). The fluorescence was monitored at different times using 370 nm excitation and 460 nm emission in a Biotech microplate reader. The enzyme activity was

calculated from the slope of the graph of intrinsic fluorescence plotted against time and standardized by μg of protein (homogenate) or volume (CSF).

Sphingosine-1-phosphate lyase activity

The S1P-lyase activity was determined using the fluorogenic substrate 2S-ammonio-3R-hydroxy-5-((2-oxo-2H-chromen-7-yl)oxy)pentyl hydrogen phosphate as previously described (Bedia et al., 2009). Triton X-100 has been previously shown to inhibit S1P-lyase. Thus, brains were homogenized in lysis buffer as indicated above without Triton X-100. Approximately 250 μg of brain homogenate were incubated at 37°C with 125 μM of the fluorogenic substrate in K_3PO_4 , pH 7.5, containing 25 μM Na_2VO_4 and 0.25 mM pyridoxal phosphate. The reaction was followed in a 96-well microplate fluorescence reader for 6h using 370 nm excitation and 460 nm emission. The enzyme activity was calculated from the slope of the graph of intrinsic fluorescence plotted against time and standardized by mg of protein.

Sphingomyelin synthase assay and glucosylceramide synthase assay

Sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS) activities were determined *in vitro* by utilizing fluorescent Cer (NBD-Cer) and exogenous phosphatidylcholine (20 μg) or UDP-glucose (1 mM) as phosphatidylcholine or glucose donors, respectively, as previously described (Kilkus et al., 2008; Qin et al., 2009). Briefly, samples (100 μg protein of rat brain homogenate or 100 μl of human CSF) were incubated overnight at 37°C with NBD-Cer (1 μg) and phosphatidylcholine or UDP-glucose. Lipids were extracted and the NBD-sphingolipids isolated by HPTLC using the solvent system chloroform:methanol:acetic acid:water (70:25:8.8:4.5 v/v). NBD-sphingomyelin, NBD-Cer and NBD-glucosylceramide bands were visualized by fluorography and quantified with a BioRad Chemi-Doc XRS scanner over the linear range using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). SMS and GCS activities were expressed as the ratios NBD-sphingomyelin/NBD-Cer and NBD-glucosylceramide/NBD-ceramide, respectively.

Western blotting of enzymes

Approximately 10 mg of brain tissue from control and SAH animals were suspended in 200 μl of lysis buffer along with protease and phosphatase inhibitors. Samples were homogenized with an electric homogenizer, sonicated, and centrifuged at 10,000 $\times g$ at 4°C in a microcentrifuge for 10 minutes. The supernatants were collected and the protein concentration determined using the BCA protein assay (Pierce Chemical, Rockford, IL). Cell lysates were boiled with 6 $\times$ lamelli buffer for 5 min. Samples (30 μg) were then subjected to SDS-gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in transfer buffer Novex (Life Technologies, Grand Island, NY). Membranes were incubated for 1h at room temperature in blocking buffer (Tris-buffered saline with 0.05% Tween-20, TBST) supplemented with 1% bovine serum albumin and then incubated with the respective primary antibodies for S1P-lyase, SK1, and SK2 overnight at 4°C according to the manufacturer's instructions. After four 10-min washes with TBST, the membranes were incubated for 1h with the secondary antibody in TBST with 1% bovine

serum albumin. The membranes were rinsed four times with TBST and the bands were detected using Supersignal luminol enhancer (Perbio Science UK Ltd., Cheshire, UK) followed by exposure to blue-light-sensitive film Hyperfilm (Amersham Biosciences UK Limited, Little Chalfont, UK). Equal protein loading was verified by re-probing of membranes with anti- β -actin antibody. The relative intensities of protein bands were quantified by densitometry using ImageJ software. Results were expressed as a ratio of specific protein signal to β -actin signal.

Statistical Analysis

Sphingolipid levels and enzyme activities in the CSF of SAH patients were expressed as medians $\pm$ SD and compared using the nonparametric Mann-Whitney U test. The correlation between Cer and DHC levels in the CSF was assessed using Spearman correlation coefficients. Sphingolipid levels, enzyme activities, and enzyme expression in rat brains homogenates were expressed as means $\pm$ SD and analyzed by Student's t test. Results were considered statistically significant when $p < 0.05$.

Results

Metabolism of sphingolipids in SAH subjects

The baseline characteristics are depicted in Table 1. Compared to the control group, SAH patients were older and more likely to be women. SAH patients had an approximate 4-fold increase in total Cer and DHC levels (Table 2). The concentration of different DHC subtypes is shown in Fig. 1. After SAH there was an increase in the concentration of all the acyl-chain DHC measured. In the SAH group, the CSF levels of Cer had a high correlation with DHC (Fig. 2; $\rho = 0.91$, $p < 0.001$). The correlation remained highly significant even after censoring extreme values ($\rho = 0.88$, $p < 0.001$). Also, the activity of ASMase was higher in the CSF of patients with SAH than in controls (15.0 ± 4.6 vs. 10.0 ± 3.5 IF/ μ l.min; $p = 0.009$) (Fig. 3). The activities of NSMase, SMS and GCS in the CSF were below the limit of detection of our methods.

Metabolism of sphingolipids in SAH animals

Two animals died in the SAH group and none in the control group. Blood pressure, body temperature, and blood gas parameters were similar in both groups. CBF decreased abruptly by 85–90% after SAH reaching its minimum in the first min of SAH, and recovered gradually to 60% of baseline values within 20 min. SAH was confirmed by post mortem visual inspection. Due to sensitivity issues, the measurement of sphingolipid levels and enzyme activities in animals had to be performed in brain homogenates. SAH resulted in a 2-fold increase in Cer (control: $3,422 \pm 26$; SAH: $7,073 \pm 2,467$ fmol of Cer/nmol of total lipid P; $p < 0.05$) and DHC levels (control: 70 ± 8 ; SAH: 187 ± 12 fmol of DHC/nmol of total lipid P). Similar to what we observed in human samples, the percentages of DHC relative to Cer in both groups were comparable (control: 2.0 ± 0.3 ; SAH: 2.0 ± 0.2 ; $p = 0.3$). Results obtained by TLC analysis confirmed that SAH is associated with an increased production of Cer and revealed a 2-fold increase in the Cer/SM ratio after brain hemorrhage (Fig. 4). In addition, compared to control brains, SAH animals had higher ASMase activity (19.3 ± 1.6 vs. 12.2 ± 1.2 IF/ μ g.min; $p < 0.05$) but similar NSMase activity (1.1 ± 0.3 vs. 0.9 ± 0.1 IF/ μ g.min;

$p>0.05$). Cer can be converted back into sphingomyelin via SMS or into less toxic glycosphingolipids via GCS. Compared to controls, SAH animals had a 2-fold increase in GCS activity (1.95 ± 0.37 vs. $0.73\pm0.04\%$; $p<0.05$) but similar SMS activity (Fig. 5). α -Fucosidase, a lysosomal hydrolase that is unrelated to sphingolipid metabolism or cerebrovascular disease, was used as an internal control. The activity of this enzyme was similar in both the control and SAH groups (0.29 ± 0.03 vs. 0.32 ± 0.08 IF/ μ g.min; $p>0.05$). Cer can also be metabolized into sphingosine (Sph) which is subsequently converted to S1P by SK1 and SK2. The exit point from sphingolipids is regulated by S1P-lyase which irreversibly cleaves S1P to hexadecenal and phosphoethanolamine. We observed a trend for Sph to be higher in the SAH group but this did not reach statistical significance. In comparison, S1P levels were decreased after SAH by approximately 50% (control: 318 ± 106 ; SAH 152 ± 29 fmol/nmol of total lipid P; $p=0.01$) (Table 3). We also measured the expression of the enzymes that regulate S1P homeostasis. SAH was associated with a 70% increased expression of S1P-lyase but similar SK1 and SK2 levels compared with controls (Fig. 6). The effect of SAH on S1P-lyase was confirmed by measuring the enzyme activity. Using a specific fluorogenic substrate, we determined that the activity of S1P-lyase in SAH brains was 40% higher than in controls (Fig. 5).

Discussion

We have demonstrated that SAH is associated with profound changes in the metabolism of sphingolipids which mainly result in the increased production of Cer and DHC and decreased S1P. Sphingolipids are a family of membrane-associated lipids that participate in multiple cellular signaling pathways and have become increasingly associated with brain pathologies from cognition to ischemia and hypoxia (Herr et al., 1999; Mielke et al., 2011; Mielke et al., 2013; Takahashi et al., 2004; Testai et al., 2014). We sought to test this in a rat model of SAH and to compare the results with findings in human patients. The *de novo* synthesis of sphingolipids begins with the condensation of serine and palmitoyl CoA to form dihydrosphingosine followed by acylation to form DHC which is converted into Cer by the action of an oxygen-dependent desaturase, making this step especially vulnerable to oxygenation in the brain. Cer can also be generated from sphingomyelin by both secretory and lysosomal isoforms of ASMase or the membrane-bound NSMase. Cells, especially tumor cells, also regulate Cer by the Golgi-associated SMS and GCS (Fig. 7) (Hannun and Obeid, 2008). Cer regulates apoptosis and senescence in cells of different lineages including neurons and glial cells (Kilkus et al., 2003; Testai et al., 2004a). In addition, this sphingolipid has been shown to cause vasoconstriction in cerebral vessels and mediates mitochondrial dysfunction, vascular dysregulation and autophagy (Altura et al., 2002; Sentelle et al., 2012; Zhang et al., 2012). We have recently shown that Cer levels increase in the CSF shortly after SAH, particularly in subjects with poor neurological outcome (Testai et al., 2012). This observation and the known deleterious effects of Cer in the CNS support the notion that this sphingolipid participates in the pathogenesis of brain injury after SAH. In this study we investigated the metabolism of sphingolipids in SAH. Unexpectedly we found that both Cer and its direct precursor, DHC, were significantly elevated in SAH subjects. Furthermore, the levels of both sphingolipids had a strong linear correlation suggesting a relationship of dependence between both variables. Sphinganine (or dihydrosphingolipids)

were until recently thought to be metabolically inactive biosynthetic intermediaries but recent studies have illustrated that DHC and other sphinganine accumulate in hypoxic conditions and may regulate cell survival, cerebral microendothelial cell barrier function, and autophagy (Breen et al., 2013; Siddique et al., 2013; Stiban et al., 2006; Testai et al., 2014). Desaturases efficiently convert DHC into Cer but the addition of exogenous C₂- or C₆-Cer to cell cultures does not modify the levels of DHC, indicating that the metabolism of DHC into Cer is irreversible (Qin et al., 2010). The increase of all the acyl-DHC subtypes observed in SAH subjects suggests a decreased conversion into Cer (Fig. 2). Using an *in vitro* model of ischemia, we have recently demonstrated that desaturases are indeed inhibited in hypoxic conditions (Testai et al., 2014). Our findings are not surprising since SAH is characterized by a rapid increase in intracranial pressure leading to global cerebral hypoperfusion (Sehba et al., 2013). Thus, we hypothesize that the elevated levels of DHC in SAH relate to reduced tissue perfusion leading to tissue hypoxia. The contribution of newly synthesized DHC and Cer to CSF levels has not been investigated but our data suggest a catabolic origin. Ceramide-transfer protein (CERT) mediates the non-vesicular intracellular trafficking of ceramides with C14-C20 fatty acids (Lev, 2010). Cer and DHC, in addition, are neutral lipids that are expected to flip-flop across biological membranes. Cer, in particular, has a rapid transmembrane diffusion rate (Lopez-Montero et al., 2005) and has been shown to traffic from the intracellular to the extracellular compartment via exosome formation (Trajkovic et al., 2008).

Our results also demonstrate that the activity of ASMase, an enzyme that participates in the catabolism of sphingomyelin into Cer, is increased in the CSF of SAH patients suggesting that the increase in Cer observed in the CSF of humans is likely to result from the activation of the Zn²⁺-dependent secretory form of ASMase. Since the limited volume of CSF obtained from rats prevented us from measuring sphingolipid levels and enzyme activity in this biological fluid, we examined whole brain homogenates and confirmed that both Cer and DHC levels and ASMase activity increased after brain hemorrhage. In addition, we observed that the Cer/SM ratio was elevated after SAH supporting an increased turnover of SM into Cer. In comparison, the activity of other enzymes that regulate Cer homeostasis (NSMase and SMS) and the representative lysosomal hydrolase α -fucosidase remained unchanged. SAH animals also had an increase in GCS, an enzyme which converts Cer into glycosylceramide for subsequent use in the synthesis of gangliosides (Fig. 7). In both cerebral ischemia and ischemia-reperfusion models, increased GCS activity has been linked to improved outcome and this may be linked to attempts to reduce ceramide levels (Hisaki et al., 2008; Kwak et al., 2005; Liu et al., 2005; Takahashi et al., 2004; Testai et al., 2014; Whitehead et al., 2011; Yamagishi et al., 2003). There is, in addition, ample evidence in the cancer literature showing that Cer glycosylation increases cell survival and that downregulation of GCS leads to Cer accumulation and apoptosis (Gupta et al., 2012; Haynes et al., 2012). These observations allow us to speculate that the increase in GCS observed after SAH may represent an endogenous detoxification mechanism of the deleterious Cer. It is worth noting that the activities of NSMase, SMS, and GCS in the CSF of SAH patients were below the level of detection of the methods used in this project, most likely because they are membrane-associated and therefore largely absent from CSF.

Despite our increasing understanding of the biological effects of sphingolipids in the brain, the effect of SAH on the metabolism of these bioactive molecules and the consequences of such changes have not been previously investigated. This is the first study to describe the effect of SAH on pathways that regulate Cer homeostasis. Previous studies done in ischemic models suggest that Cer mediates brain damage after stroke. In ischemia-reperfusion injury, for example, Cer increases in the reperfusion phase and this has been attributed to the activation of ASMase (Tian et al., 2009). In addition, upregulation of ASMase and downregulation of SMS have been reported in association with focal cerebral ischemia (Dmitrieva et al., 2008; Ohtani et al., 2004). Downstream, Cer has been associated with mitochondrial dysfunction and mediates neuronal and glial cell death via inhibition of prosurvival pathways, such as PtdIns 3-kinase and Akt, and activation of pro-apoptotic mechanisms, including caspases and cathepsin D (Hannun and Obeid, 2008; Novgorodov and Gudz, 2009). In addition, Cer has been shown to regulate post stroke inflammation and to inhibit endothelial nitric oxide synthase which is a key enzyme in the regulation of cerebrovascular reactivity in SAH (Xiao-Yun et al., 2009; Yu et al., 2000).

It should be noted that the similarities in the changes in sphingolipid metabolism observed in SAH and ischemia-reperfusion may not be coincidental. Thus, shortly after SAH, there is a rapid increase in intracranial pressure leading to a transient no-flow state similar to that seen in ischemia-reperfusion suggesting that both entities may share common pathogenic mechanisms of brain injury (Macdonald et al., 2007; Pluta et al., 2009; Sehba et al., 2013).

Cer can also be a precursor in the synthesis of Sph via ceramidase. Sphingosine is subsequently phosphorylated into S1P by SK1 and SK2. S1P is a proangiogenic sphingolipid that regulates hemodynamic responses, enhances endothelial barrier permeability, increases cell survival, and contributes to recovery in ischemia-reperfusion injury (Fyrst and Saba, 2010). The effect of SAH on the metabolism of S1P has not been previously reported. Although the concentration of S1P in the CSF was below the limit of detection of the HPLC/MS/MS method here utilized, in brain we observed decreased S1P levels after SAH. SK2-mediated S1P synthesis has been shown to be beneficial in treating cerebral ischemia (Pfeilschifter et al., 2011; Wacker et al., 2012; Yung et al., 2012). This, along with the protective effect of the S1P analogs against cerebral ischemia, supports the beneficial effect of S1P in treating stroke (Fu et al., 2014; Hasegawa et al., 2010; Rolland et al., 2011; Wei et al., 2011). The shift in the production of protective (S1P) in favor of deleterious (Cer) sphingolipids here described suggests the participation of these bioactive lipids in brain injury following SAH. The S1P/Cer ratio of 9.0% for control was almost 4 times the 2.5% ($p=0.01$) that we observed in SAH. Further, the S1P/Sph ratio was decreased 50% in SAH. Since S1P can be recycled to ceramide or metabolized to hexadecenal and ethanolamine phosphate by S1P-lyase, we measured the activity of this enzyme and found it to be increased by 40% in brain homogenates of control and SAH animals. In addition, the expression of S1P-lyase was increased by 60% in SAH brains. In contrast, the expression of SK1 and SK2 were unchanged. Therefore, we interpret our findings to suggest that the low S1P levels observed after SAH are driven by a hypermetabolic state rather than a decreased synthesis.

In conclusion, SAH is associated with ASMase activation, elevated levels of Cer and DHC, and a relative deficiency of S1P. The known negative effects of Cer on neurons and glial cells and the association between elevated Cer levels and poor outcome support additional studies looking at the effect of modulators of the pathways herein described in the management of SAH.

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Glossary

ASMase	acid sphingomyelinase
CCA	common carotid artery
Cer	ceramide
CSF	cerebrospinal fluid
DHC	dihydroceramide
ECA	external carotid artery
GCS	glucosylceramide synthase
ICA	internal carotid artery
LDF	laser Doppler flowmetry
MCA	middle cerebral artery
MS	mass spectrometry
NBD	nitrobenzoxadiazole
NSMase	neutral sphingomyelinase
PTFE	polytetrafluoroethylene
rCBF	regional cerebral blood flow
S1P	sphingosine-1-phosphate
S1P-lyase	sphingosine-1-phosphate lyase
SAH	subarachnoid hemorrhage
SK1	sphingosine kinase 1
SK2	sphingosine kinase 2
Sph	sphingosine
SM	sphingomyelin
SMS	sphingomyelin synthase
TBST	Tris-buffered saline with 0.05% Tween-20

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Fig. 1. Dihydroceramide (DHC) profiles in the CSF of human subjects

CSF was obtained from controls (solid dots, n=8) and subarachnoid hemorrhage patients (open dots, n=26) and DHC levels measured by MS/MS. Dots represent individual measures and lines medians. The inset shows the total DHC concentration in controls and patients with subarachnoid hemorrhage. Significance was determined using the nonparametric Mann-Whitney U test (*p<0.01; **p<0.001).

Fig. 2. Correlation of ceramide (Cer) with dihydroceramide (DHC) levels in the CSF of patients with SAH

CSF was obtained from SAH patients and Cer and DHC levels measured by MS/MS.

Significance was determined using Spearman's rank correlation coefficient ($r=0.91$; $p<0.001$). The inset shows the correlation between sphingolipids after censoring the two most extreme values (arrow) ($r=0.88$; $p<0.001$).

Fig. 3. Acid sphingomyelinase (ASMase) activity in the CSF of human subjects

The activity of ASMase in controls (solid dots; n=8) and subarachnoid hemorrhage cases (open dots; n=26) was determined 48h after SAH. ASMase activity is expressed as intrinsic fluorescence (IF) per unit of time and volume. Dots represent individual measures and lines medians. Significance was determined by Mann-Whitney test.

Fig. 4. Ceramide (Cer), sphingomyelin (SM) and cholesterol (Chol) content in brain homogenates from control and SAH animals

Panel A: cerebral lipids were extracted as indicated in methods and resolved by HPTLC.

Panel B: bands were quantified by densitometry using the ImageJ software. SM and Cer levels were standardized by total cholesterol. Data represent means $\pm$ SD. n=4–5 animals per group. * p<0.05. ** p<0.01.

Fig. 5. Enzymatic activity in brain homogenates

Panel A: Acid sphingomyelinase (ASMase), neutral sphingomyelinase (NSMase), sphingosine-1-phosphate lyase (S1P-lyase) and α -fucosidase activity in brain homogenates from control (white, n=8) and subarachnoid hemorrhage (SAH, black) animals were measured using fluorescent substrates as described in methods. Panel B: Sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS) activity in control (white) and SAH (black) animals were measured *in vitro* by using NBD-ceramide as indicated in methods. SMS is expressed as sphingomyelin (SM) to ceramide (Cer) ratio, and GCS as glucosylceramide (GluCer) to Cer ratio. Each bar represents the means $\pm$ SD. n=8 per group.

Fig. 6. Cerebral expression of enzymes involved in the metabolism of sphingosine-1-phosphate Control and subarachnoid hemorrhage (SAH) animals were euthanized and brain homogenates were subjected to SDS-PAGE and Western blot analysis as described in methods. Blots were probed with anti-sphingosine-1-phosphate lyase (A, S1P-lyase), sphingosine kinase 1 (B, SK1), and sphingosine kinase 2 (C, SK2) antibodies. Relative intensity was quantified by densitometry and normalized to total β -actin. The expression of S1P-lyase was significantly increased in the brain of SAH animals. Bars represent means $\pm$ SD. n=4-5 animals per group.

Fig. 7. Ceramide metabolism

The *de novo* pathway involves the condensation of palmitoyl-CoA and serine which after a series of reactions generate dihydroceramide. Ceramide can be synthesized from dihydroceramide via desaturases or by the breakdown of sphingomyelin by either neutral or acid sphingomyelinases. Ceramide can be transformed back into sphingomyelin via sphingomyelin synthase or into glucosylceramide via glucosylceramide synthase or sphingosine-1-phosphate. Glucosylceramide can then be converted into complex glycosphingolipids, such as gangliosides. The dotted arrows show the pathways activated after SAH. DAG: diacylglycerol; DHC: dihydroceramide; PC: phosphatidylcholine; S1P-lyase: sphingosine-1-phosphate; Ser: serine; SK1: sphingosine kinase 1; SK2: sphingosine kinase 2; SMase: sphingomyelinase; UDP: uridine diphosphate.

Table 1

Baseline characteristics.

	Control (n=8)	Subarachnoid hemorrhage (n=26)
Age (years) *	30±17	53±10
Male (%)	50	37
GCS score *		9.5±4.0
WFNS score *		4.0±1.5

* Median±SD

GCS, Glasgow Coma Scale

WFNS, World Federation of Neurological Surgeons

Table 2

Levels of ceramide and dihydroceramide in controls and SAH patients.

	Control* (n=8)	Subarachnoid hemorrhage* (n=26)	p value
Cer (pmol/ml)	11.4±8.8	33.3±48.3	0.001
DHC (pmol/ml)	1.3±1.1	3.8±3.4	0.001
DHC/Cer	0.11±0.02	0.11±0.03	p>0.05

* Data represent medians±SD.

Table 3

Levels of sphingolipids in controls and SAH brain homogenates.

	Control*	Subarachnoid hemorrhage*	P value
Cer (fmol/nmol of lipid P)	3422±26	7073±2467	0.03
DHC (fmol/nmol of lipid P)	70±8	187±12	0.01
DHC/Cer(%)	2.0±0.3	2.0±0.2	0.30
Sph (fmol/nmol of lipid P)	240±63	326±60	0.08
S1P (fmol/nmol of lipid P)	318±16	152±29	0.03
S1P/Cer (%)	9.0±3.1	2.5±1.3	0.01
S1P/Sph (%)	1.0±0.3	0.5±0.1	0.02

* Data represent means±SD. n=3–4 animals per group.