Changes in the Cerebrospinal Fluid Ceramide Profile After Subarachnoid Hemorrhage

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Background and Purpose—The purpose of this study was to investigate changes in the cerebrospinal fluid sphingolipid profile in patients with subarachnoid hemorrhage in relation to the occurrence of symptomatic vasospasm and outcome at hospital discharge.

Methods—The ceramide profile in the cerebrospinal fluid was determined by mass spectrometry in control subjects and patients with Fisher 3 grade subarachnoid hemorrhage within 48 hours of the bleed. Patients were prospectively followed and subcategorized based on the occurrence of symptomatic vasospasm and modified Rankin Scale at discharge.

Results—Compared to control subjects, patients with subarachnoid hemorrhage had higher cerebrospinal fluid levels of total ceramide (12.4±8.8 versus 54.6±49.3 pmol/mL; P<0.001). In the subgroup analysis, total ceramide levels in individuals with symptomatic vasospasm (104.2±57.0 pmol/mL) were higher than in those with asymptomatic vasospasm (32.4±25.7 pmol/mL; P=0.006) and no vasospasm (30.9±15.7 pmol/mL; P=0.003). In addition, compared to patients with a good outcome (modified Rankin Scale ≤3), individuals with poor outcome (modified Rankin Scale ≥4) had higher cerebrospinal fluid levels of total ceramide (79±25 versus 23±6 pmol/mL; P=0.008). When the relative contributions of the different ceramide species were calculated, a higher relative concentration of C18:0 ceramide was observed in individuals with symptomatic vasospasm (P=0.018) and poor outcome (P=0.028).

Conclusions—Ceramide profile changes occur in subarachnoid hemorrhage. In this small case-based series elevation of levels of this sphingolipid, particularly C18:0, was associated with the occurrence of symptomatic vasospasm and poor neurological outcome after subarachnoid hemorrhage. (Stroke. 2012;43:2066-2070.)

Key Words: AVM ■ cerebral aneurysm ■ cerebrovascular disease/stroke ■ subarachnoid hemorrhage

Aneurysmal subarachnoid hemorrhage (SAH) is a neurological emergency that accounts for a small proportion of the strokes but is associated with significant morbidity and mortality. As many as 50% of the patients with vasospasm (VS) develop cerebral infarction and a substantial proportion of these individuals have disabling stroke or die from cerebral ischemia.1 The pathogenesis of brain injury in SAH is complex and different mechanisms other than VS have been implicated including endothelial and blood–brain barrier dysfunction, neuronal apoptosis, microcirculatory thrombi formation, and cortical spreading depression.2 Sphingolipids are important cell membrane components that participate in signaling events that regulate a wide variety of vital cellular processes such as cell differentiation, proliferation, survival, and apoptosis. The role of bioactive sphingolipids such as ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P) in the pathogenesis of stroke has not been completely elucidated. Furthermore, there is evidence to suggest that sphingolipids may have both brain injury and neurovascular protective functions. In animal models of stroke and ischemia–reperfusion, cerebral ceramide content is increased by activation of enzymes that participate in sphingomyelin degradation (sphingomyelinase) and de novo ceramide biosynthesis (ceramide synthase), and inhibition of sphingomyelin synthase (online-only Data Supplement Figure I).3–5 Elevated ceramide levels may participate in neuronal and oligodendroglial cell death and poststroke neuroinflammation.6–10 In addition, some ceramide species have vasoactive properties and may therefore affect cerebral perfusion.11,12 It has been proposed that the detrimental effects of ceramide are, at least in part, counterbalanced by S1P. Several recent reports indicate that the S1P modulator

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FTY720 reduces brain inflammation and improves functional recovery in both ischemic and hemorrhagic stroke. The data suggest that modulation of metabolic pathways that participate in the production of bioactive sphingolipids may provide a strategy to improve stroke outcome.

The sphingolipid profile in humans who have had a stroke has not been yet described. In this study we examine the cerebrospinal fluid (CSF) content of ceramide and S1P in control subjects and patients with Fisher 3 SAH and its relationship to the occurrence of VS and neurological outcome at hospital discharge.

**Materials and Methods**

**Participants**

Subjects were recruited at the University of Illinois Medical Center at Chicago. Institutional Review Board approval was obtained before study initiation. Cases were eligible to participate if they were ≥18 years of age and had nontraumatic aneurysmal SAH, and age visualization by either digital subtraction catheter angiography or noninvasive cerebral angiography (either CT or MR angiography), SAH Fisher Grade 3, and presence of an external ventricular drain. Exclusion criteria were SAH due to causes other than a ruptured saccular aneurysm, pregnancy, previous neurological disability (defined as preadmission modified Rankin Scale score >2), and history of stroke, brain tumor, intracranial surgery, traumatic brain injury, previously treated cerebral aneurysm, or other vascular malformation. Control subjects were nonpregnant persons ≥18 years of age with no previous neurological disorders who underwent lumbar puncture or CSF drainage for evaluation of headache or for congenital hydrocephalus and suspected shunt malfunction and had normal CSF analysis including chemistry, cell count and differential, and cultures. Samples of CSF and serum were obtained within 48 hours of symptoms onset, centrifuged at 270°C for 15 minutes at 5°C, and the supernatant stored at −80°C until analysis.

**Assessments**

Subjects with SAH were prospectively followed during hospitalization and subcategorized into those with VS or without VS (no-VS). Other prespecified subgroups included symptomatic VS (sVS), asymptomatic VS, and no symptomatic VS (no sVS). Vasospasm was defined as ≥30% arterial narrowing on digital subtraction angiography not attributable to atherosclerosis, catheter-induced spasm, or vessel hypoplasia as determined by an independent neuroradiologist. Symptomatic VS was defined as development of new focal neurological signs, deterioration in level of consciousness, or both when the cause was felt to be ischemia attributable to VS after other possible causes of worsening had been excluded or the appearance of new cerebral infarction on CT or MR thought to be attributable to VS. Outcome at discharge was assessed by using the modified Rankin Scale and patients with SAH were dichotomized into those with good outcome (modified Rankin Scale ≤3) and poor outcome (modified Rankin Scale ≥4).

**Standards and Reagents**

Sphingosine (Sph), dihydro-sphingosine, a 17-carbon analog of Sph (C17), S1P, dihydro-sphingosine-1-phosphate, a 17-carbon analog of S1P (C17-S1P), N-myristoyl (14:0), N-palmitoyl (16:0), N-oleyl (18:1), N-stearoyl (18:0), N-arachidonyl (20:0), N-lignoceryl (24:0) sphingosines (camerides [Cer]), N-palmitoyl (16:0), N-oleyl (18:1), N-stearoyl (18:0), N-arachidonyl (20:0), N-lignoceryl (24:0), dihydro-sphingosine (dihydrosphingosines), N-heptadecanoyl sphingosine (17:0-Cer), and N-palmitoyl (16:0), N-stearoyl (18:0), dihydro-sphingosine (C24:0) coenzyme A substrates were obtained from Avanti Polar Lipids (Alabaster, AL). The sphingolipid profile in humans who have had a stroke has not been yet described. In this study we examine the cerebrospinal fluid (CSF) content of ceramide and S1P in control subjects and patients with Fisher 3 SAH and its relationship to the occurrence of VS and neurological outcome at hospital discharge.

**Lipid Extraction and Analysis of Sphingoid Bases, Sphingoid Base 1-Phosphates, and Ceramides**

CSF lipid extraction and analyses of the sphingolipids were performed by combined liquid chromatography–tandem mass spectrometry as previously described. 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 liquid chromatograph and autosampler (Agilent Technologies, Wilmington, DE). The sphingolipids were ionized through electrospray ionization with detection through multiple reaction monitoring. Analysis of sphingoid bases and the molecular species of ceramides used electrospray ionization in positive ions with multiple reaction monitoring analysis as previously described.

**Statistical Analysis**

Differences between groups were determined by using the Student t test for continuous variables or χ² test for categorical variables. Ceramide levels in different groups were compared using the nonparametric Mann-Whitney U test. The nonparametric Kruskal–Wallis method was used when analyzing ≥3 subgroups. The relative concentrations of ceramide species were compared using analysis of variance with Bonferroni correction. The Pearson coefficient was used to assess the correlation between total CSF protein and ceramide levels. The CSF levels of ceramide in patients with SAH were divided into quartiles (Q) and the rate of sVS calculated for each Q.

**Results**

**Characteristics of SAH Cases and Control Subjects**

Twenty-three SAH cases and 8 control subjects were recruited. Seven control subjects (87%) underwent lumbar puncture for evaluation of headache of sudden onset and only 1 of them had a history of migraine headaches. The eighth control subject was a patient with congenital hydrocephalus who underwent lumbar drainage placement for malfunction of a ventriculoperitoneal shunt.

Compared with SAH cases, control subjects were more likely to be younger and male but equally likely to have hypertension (Table). Within the SAH group, the mean time to obtain CSF samples was 37 ± 18 hours. Angiographic evidence of VS occurred in 15 (65%) SAH cases and 7 of these (47%) were symptomatic (sVS). Compared with sVS...
Figure 1. Ceramide profiles in the CSF. A, Bars represent the mean±SD observed in controls (black) and patients with SAH (white; *P<0.05; **P<0.01). The inset shows the total ceramide concentration in the CSF of control and patients with SAH (P<0.001). B, Bars represent the mean value±SD for patients with SAH with symptomatic VS (white), asymptomatic VS (gray), and no VS (black; *P<0.05; **P<0.01). The inset shows the total ceramide concentration in the different subgroups; the total ceramide content in individuals with symptomatic VS (sVS) was higher than in those with asymptomatic VS and those without VS (no-VS; P=0.006). CSF indicates cerebrospinal fluid; SAH, subarachnoid hemorrhage; VS, vasospasm.

Ceramide Content and Outcomes

The rates of sVS in SAH cases increased with the level of total CSF ceramide and were 0% (Q1 and Q2), 33% (Q3), and 100% (Q4). Patients with poor outcome had higher levels of total CSF ceramide (79±25 versus 23±6 pmol/mL; P=0.008). Different ceramide species contributed to the excess of ceramide including C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, and C26:0 (Figure 3). When the relative contributions of the different ceramide species were calculated, cases with poor outcome had a higher relative concentration of C18:0 ceramide than those with a good outcome (21% versus 14%; P=0.028).

Discussion

Biologically active sphingolipids are increasingly recognized as signaling molecules that participate in cell survival, apoptosis, differentiation, inflammation, immunity, and oxidative stress. Ceramide, a key sphingolipid, was found to be elevated in the CSF of patients with SAH. This increase in ceramide concentration was associated with worse clinical outcomes, particularly in cases of symptomatic VS. The specific role of ceramide in the pathophysiology of SAH remains to be fully elucidated, but these findings suggest that ceramide could be a potential therapeutic target for improving outcomes in patients with SAH.
stress. The role of sphingolipids in the pathogenesis of brain injury after stroke, particularly SAH, has not been completely elucidated. There is, however, evidence to suggest that ceramide plays an active role in cerebrovascular disease pathogenesis.

Shortly after SAH, there may be rapid increase in intracranial pressure (ICP) leading to a global reduction of cerebral blood flow and neuronal dysfunction.\textsuperscript{2} Hypoperfusion and consequent hypoxia, as occurs in the early phase of SAH, may have profound effects in the homeostasis of sphingolipids. In rodent models, for example, cerebral ischemia is associated with a 5-fold increase in ceramide resulting from activation of glial sphingomyelinase, decreased mRNA expression of the SMS-1 gene, and downregulation of glucosylceramide synthase in astroglia.\textsuperscript{3,4,17,18} As the ICP normalizes in SAH, a second phase characterized by reperfusion injury takes place. Furthermore, in middle cerebral artery ischemia–reperfusion animal models, increased ceramide production through activation of ceramide synthase has been observed in the reperfusion phase.\textsuperscript{5}

In this study we show a significant elevation of CSF ceramide in the first 48 hours of SAH, particularly in those cases who subsequently developed cerebral VS and had poor neurological outcome. The relationship between ceramide and the occurrence of VS, as observed in this study, has not been investigated previously. There is evidence, however, to support the notion that sphingolipids have vasoactive properties. Ceramide, in particular, has been shown to reduce the diameter of small bovine coronary arteries in a dose-dependent manner and to mediate vasoconstriction in rat and human pulmonary arteries.\textsuperscript{19,20} In addition, in a canine animal model, Zheng et al\textsuperscript{21} showed that both ceramide analogs and neutral–sphingomyelinase cause vasoconstriction of cerebral arterial rings and increase intracellular Ca\textsuperscript{2+} concentration in cerebral vascular smooth muscle cells. Mechanistically, ceramide regulates the activity of enzymes that control vasomotor response such as endothelial- and inducible-nitric oxide synthase.\textsuperscript{22,23} These 2 enzymes are involved in nitric oxide homeostasis and may have a pivotal role in the occurrence of VS in SAH.

In our study we observed that higher CSF levels of ceramide were noted not only in individuals with sVS, but also in those with poor outcome at discharge. VS is a frequently encountered complication of SAH and is considered a major cause of delayed cerebral ischemia and thus morbidity and mortality. Interventions to reduce the rate of VS, however, have failed to improve neurological outcome.\textsuperscript{24} The dissociation observed between both variables adds to the growing body of evidence that the pathogenesis of brain injury in SAH is a multifactorial phenomenon mediated by different factors including neuronal apoptosis.\textsuperscript{25} Ceramide is a well-known mediator of neuronal and oligodendroglial cell death. Molecular targets of ceramide include the so-called ceramide-activated protein phosphatases, PP1A and PP2A. During hypoxia/reoxygenation, activated PP2A dephosphorylates and inhibits the prosurvival mediators Bax and Akt. In addition, ceramide has been shown to activate the caspase cascade and suppress the mitochondrial respiratory chain, which results in an increase in the production of reactive oxygen species and neuronal and oligodendroglial cell damage.\textsuperscript{6–8,26}

Ceramide is a family of compounds formed by the addition of a C\textsubscript{14}-C\textsubscript{26} fatty acid to the amino group of a sphingoid base (online-only Data Supplement Figure I). It has been hypothesized that different acyl groups may affect the biophysical and biological properties of sphingolipids.\textsuperscript{27} This N-acylation is mediated by ceramide synthase and a total of 6 different types of ceramide synthase have been described. Each of them has relatively restricted fatty acid specificity and is thus involved in the synthesis of a discrete subset of ceramides. Particular acyl-ceramide profiles have been described in neurological diseases such as Alzheimer disease, neuronal ceroid lipofuscinoses, HIV-associated memory impairment, epilepsy, and ischemia–reperfusion brain injury.\textsuperscript{5,28–31}

In our study, cases with sVS had elevation of the relative content of C\textsubscript{18:0} and low C\textsubscript{22:0} ceramide. In addition, elevation of C\textsubscript{18:0} ceramide production in SAH was associated with a poor outcome at discharge. The role of ceramide species in stroke has not been investigated. C\textsubscript{18:0}-ceramide has been consistently shown to be elevated in neurodegenerative disorders supporting its deleterious effect in the nervous system. In addition, using an ischemia–reperfusion model, Yu et al\textsuperscript{5} showed that mitochondrial dysfunction, as commonly observed in stroke and particularly in SAH, may be caused by excessive accumulation of C\textsubscript{16:0}, C\textsubscript{18:0}, and C\textsubscript{18:1} ceramide.

Our study has several limitations. The distribution of the clinical and radiological characteristics are representative of what has historically been described in patients with SAH.\textsuperscript{32} The sample is small, however, and our results need to be confirmed in a larger study. In addition, the small sample may limit our ability to detect additional changes in the ceramide profile that although may be subtle may have clinical significance. The sample size is, however, similar or larger than those used in other studies designed to investigate sphingolipid profiles in biological fluids in human subjects.\textsuperscript{28,33–35} Lastly, the reported changes in the CSF ceramide profile could have been influenced by the extravasation of circulating ceramide into the subarachnoid space. In our study we observed that ceramide species C\textsubscript{16:0} and C\textsubscript{18:0} were major contributors to the total ceramide pool in the CSF and that S1P is present only in small amounts (<1 nmol/L). This CSF profile is similar to that reported by others and differs considerably from the 1 described in plasma, which is characterized by low C\textsubscript{16:0} and C\textsubscript{18:0} ceramide and significant S1P levels (0.3 μmol/L).\textsuperscript{33,35,36} Additionally, the poor correlation between CSF protein level and total ceramide suggests a low contribution of plasma content to the measured variables.

Conclusion
We have shown that ceramide profile changes occur in the CSF in SAH. In addition, we observed that elevated levels of ceramide, particularly C\textsubscript{18:0}, were associated with sVS and poor neurological outcome in SAH. Collectively, our results and the known deleterious effects of ceramide in the central nervous system suggest that this sphingolipid may be a key mediator of vascular injury and brain damage in SAH. Our findings support the need for further studies of sphingolipids as biomarkers of outcome in stroke and the study of ceramide...
metabolism modulators, including sphingomyelinase inhibitors, for the treatment of cerebrovascular disease.

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