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Sphingosine kinase inhibition ameliorates chronic hypoperfusion-induced white matter lesions

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ABSTRACT

White matter lesions (WML) are thought to contribute to vascular cognitive impairment in elderly patients. Growing evidence show that failure of myelin formation arising from the disruption of oligodendrocyte progenitor cell (OPC) differentiation is a cause of chronic vascular white matter damage. The sphingosine kinase (SphK)/sphingosine-1-phosphate (S1P) signaling pathway regulates oligodendroglia differentiation and function, and is known to be altered in hypoxia. In this study, we measured SphK, S1P as well as markers of WML, hypoxia and OPC (NG2) in a mouse bilateral carotid artery stenosis (BCAS) model of chronic cerebral hypoperfusion. Our results indicated that BCAS induced hypoxia inducible factor (HIF)-1α, Sphk2, S1P, and NG2 up-regulation together with accumulation of WML. In contrast, BCAS mice treated with the SphK inhibitor, SKI-II, showed partial reversal of SphK2, S1P and NG2 elevation and amelioration of WML. In an *in vitro* model of hypoxia, SKI-II reversed the suppression of OPC differentiation. Our study suggests a mechanism for hypoperfusion-associated WML involving HIF-1α-SphK2-S1P-mediated disruption of OPC differentiation, and proposes the SphK signaling pathway as a potential therapeutic target for white matter disease.

1. Introduction

Dementia and cognitive impairment have become prominent healthcare issues in aging populations. Besides Alzheimer's disease, cerebrovascular diseases are now recognized as a major contributor of cognitive impairment (Kalaria et al., 2008; Raz et al., 2016). In particular, white matter lesions (WML), characterized by oligodendrocyte (OLG) loss, myelin rarefaction and axonal damage are frequently observed in patients with vascular cognitive impairment (Englund, 2002; Schmidt et al., 2007), and are believed to contribute to the rapid decline of global functioning in elderly patients (Inzitari et al., 2007). Furthermore, research in animal models showed that WML induced by hypoperfusion led to working memory deficits (Shibata et al., 2007), and treatments which ameliorated WML also restored working memory function (Dong et al., 2011; Miyamoto et al., 2013a).

OLGs form sail-like extensions of their cytoplasmic membrane which wrap around the axon up to 150 layers thick to form myelin sheaths. This process is dynamic even in the mature CNS where oligodendrocyte progenitor cells (OPCs) still persist (Nishiyama et al., 2009). When white matter damage occurs, OPCs respond quickly to proliferate, migrate and differentiate into myelin sheath-forming, mature OLGs (Miyamoto et al., 2010). However, the extent and outcome of endogenous repair is limited in diseases with chronic WML (Alizadeh et al., 2015). There is growing evidence that the failure to form new myelin due to disruption of OPC differentiation is one factor underlying chronic white matter damage (Alizadeh et al., 2015; Miyamoto et al., 2013a, 2013b).

Myelin is a major constituent of white matter in the CNS

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(Baumann and Pham-Dinh, 2001). The composition of compact myelin is unique with a high lipid content of 70–85% (Morell, 1999), about 30% of which are sphingolipids (O'Brien and Sampson, 1965), a class of lipids defined by a backbone of sphingoid bases. Beside the structural role in white matter, sphingolipids including ceramide, sphingosine, and sphingosine-1-phosphate (S1P) have been recognized as important regulators of cellular functions (Hannun and Obeid, 2008). Previous reports have demonstrated the involvement of the S1P signaling pathway regulating OLG lineage-cell survival, proliferation, membrane dynamics and differentiation (Cui et al., 2014; Jung et al., 2007; Miron et al., 2008). Moreover, sphingosine kinase (SphK), the enzyme that produces S1P by ATP-dependent phosphorylation of sphingosine, is known to be activated in hypoxia or ischemia (Schnitzer et al., 2009; Wacker et al., 2012), suggesting S1P is elevated under such conditions. However, direct evidence of S1P changes in hypoxia is lacking, and little is known about the involvement of S1P signaling in hypoxia- or hypoperfusion-associated WML. In this study, we measured S1P and associated signaling pathways in relation to the development of WML using an established mouse model of chronic hypoperfusion (Shibata et al., 2004).

2. Methods

2.1. Animals and chronic hypoperfusion model

Mice were housed in ventilated cages in the vivarium at National University of Singapore (NUS) on a 12 h light/12 h dark cycle, with *ad libitum* access to water and standard chow. All procedures were approved by the Institutional Animal Care and Use Committee at NUS, and followed the ARRIVE guidelines of the National Centre for the Replacement Refinement and Reduction of Animals in Research. All chemicals and reagents were of analytical grade and purchased from Sigma Aldrich Co. (USA) unless otherwise specified. Chronic hypoperfusion was induced by bilateral common carotid artery stenosis (BCAS) as previously described (Shibata et al., 2004). Briefly, 10-week-old male C57BL/6 mice (25–29 g) were anaesthetized with ketamine (100 mg/kg)/medetomidine (10 mg/kg, both i.p.). Not all assays were performed for all animals. For optimization and validation of the BCAS procedure, an initial set of 22 mice (11 each for the sham-operated and BCAS groups) were subjected to cerebral blood flow (CBF) measurements with Laser-Doppler flowmetry (LDF) by fixing a plastic guide cannula (outer diameter 3 mm, inner diameter 2 mm, and length 4 mm) for the LDF probe perpendicularly to the skull at 1 mm posterior and 2.5 mm lateral to the bregma using dental resin. CBF values were recorded for 15 min before surgery, and set as baseline (100%). Both common carotid arteries (CCAs) were exposed and freed from their sheaths through a midline cervical incision. Two 7–0 silk sutures were placed around the distal and proximal parts of the right CCA. Then, the artery was gently lifted and placed between the loops of a microcoil (Inner diameter 0.18 mm, purchased from Sawane Spring Co., Japan) just below the carotid bifurcation. The microcoil was twined by rotating it around the CCA. The same procedures are applied to left CCA. CBF values were recorded continuously during and after the procedure until the CBF became stable. Sham-operated mice underwent the same procedure without microcoil implantation. After validation of the BCAS procedure (see Fig. 1A), a second set of 27 mice were used (9 sham operated, 18 BCAS). The BCAS mice were then randomly assigned to two groups ($N = 9$ per group): one group received SphK inhibitor 4-[[4-(4-Chlorophenyl)-2-thiazolyl]amino]phenol (SKI-II, 50 mg/kg, i.p., Selleck Chemicals, USA) (French et al., 2003) immediately after the surgery and every other day thereafter up to day 15, while the other group received vehicle (DMSO) on the same regimen. Of the 27 animals, $N = 3$ in each of the sham-operated, BCAS-vehicle and BCAS-SKI-II groups were used for histological studies (see Methods Section 2.2), while the remaining six in each group were used for S1P and MBP measurements, of which five in each group also had immunoblot data for the other markers under study (see Methods Sections 2.3 and 2.4).

2.2. Histological assessment

Fifteen days after surgery, the mice were anesthesized with ketamine/medetomidine before undergoing intra-cardiac perfusion with 0.1 M PBS at a rate of 2.5 mL/min until the perfusate exiting the right atrium was clear, followed by 4% paraformaldehyde in 0.1 M PBS (25 ml). The brains were post-fixed in the same paraformaldehyde solution overnight and dehydrated in 20% sucrose in 0.1 M PBS. Coronal sections (12 μm) were cut with a cryostat at −20 °C, then subject to Klüver-Barrera (KB) staining for myelin and nerve cells (Klüver and Barrera, 1953).

2.3. Immunoblotting

White matter tissues (WMT, including corpus callosum, internal and external capsules) were dissected and homogenised in RIPA buffer. Tissue lysates (20–100 μg of total protein per lane) were immunoblotted using standard protocols (Mahmood and Yang, 2012). Primary antibodies used were MBP (1:1000), SphK1 (1:1000) and β-actin (1:1000) from Cell Signaling Technology (USA); NG2 (1:500) and GAPDH (1:10000) from Merck-Millipore (USA); SphK2 (1:1000) from Santa Cruz (USA) and HIF-1α (1:1000) from GeneTex (USA).

2.4. Profiling of sphingosine-1-phosphate

WMT were dissected, minced and then sonicated at 4 °C for 1 h in lipid extraction solvent (1:1 butanol: methanol) containing S1P internal standard (S1P d18:1 13C2D2, 50 μl, 20 ng/ml, Toronto Chemicals, Canada). Sample lysates were then centrifuged for 10 min at 14,000 g. The total lipid extract (supernatant) was collected and measured after derivatization as previously described (Narayanaswamy et al., 2014). The lipid extract, dried completely and resuspended in methanol (100 μl), was then mixed with TMS-diazomethane (10 μl, 2 M in hexane, Acros Organics, USA) and incubated for 20 min at room temperature under thorough mixing (750 rpm). The derivatization reaction was stopped by the addition of acetic acid followed by centrifugation for 10 min at 14,000 g. The supernatants were collected, dried and resuspended in mobile phase B and samples (1 μl) were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis with an Agilent system (1260 HILIC-chip system connected to Agilent 6490 QQQ mass spectrometer). Solvents used for HILIC HPLC: 50% acetonitrile in water containing 25 mM ammonium formate pH 4.6 (solvent A), 95% acetonitrile containing 25 mM ammonium formate pH 4.6. The pH value was adjusted with formic acid. The Agilent 6490 was operated in positive mode for MRM (electrospray voltage was set to 1580 V (Vcap), temperature 185 °C, drying gas 12 l/min and collision energy of 25 V). In positive ion MRM mode, two product ions were monitored after CID of the S1P precursors. M/z 60 was used as a 'quantifier' (due to its high intensity) and m/z 113 was used as a 'qualifier'.

2.5. Primary oligodendrocyte precursor cell cultures

Cultured OPCs were prepared as previously described (Chen et al., 2007). Briefly, cortices from P1–2 Sprague Dawley rats were dissected and minced into single cell suspensions. Cells were