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Sphingosine kinase 1 mediates neuroinflammation following cerebral ischemia

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ABSTRACT

Sphingosine kinases (Sphks) are the rate-limiting kinases in the generation of sphingosine-1-phosphate, which is a well-established intracellular pro-survival lipid mediator. Sphk2 has been reported to be protective following experimental stroke. We investigated the role of Sphk1 in cerebral ischemia using a mouse middle cerebral artery occlusion (MCAO) model and an in vitro glucose–oxygen deprivation (OGD) model. Sphk expression and activity were assessed in the ischemic brain with quantitative PCR (qPCR), Western blot, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Pharmacological and gene knockdown approaches were utilized to investigate the effects of Sphk1 on stroke outcomes. The expression of Sphk1 but not that of Sphk2 was rapidly induced in the cortical penumbra over 96 h after MCAO, and the microglia were one of the major cellular sources of Sphk1 induction. Consistently, Sphk activity was enhanced in the cortical penumbra. In contrast to the protective role of Sphk2, pharmacological inhibition and cortical knockdown of Sphk1 reduced infarction at 24 and 96 h after reperfusion. Additionally, the Sphk1 inhibitor improved the neurological deficits at 96 h after reperfusion. Mechanistically, Sphk1 inhibition and knockdown significantly attenuated MCAO-induced expression of inflammatory mediators in the cortical penumbra. Moreover, using a conditioned medium transfer approach, we demonstrated that OGD-treated neurons induced the expression of Sphk1 and pro-inflammatory mediators in primary microglia, and the microglial induction of pro-inflammatory mediators by ischemic neurons was blunted by Sphk1 inhibition. Taken together, our results indicate that Sphk1 plays an essential role in mediating post-stroke neuroinflammation.

Introduction

Sphingosine kinases 1 and 2 (Sphk1 and Sphk2) convert sphingosine into sphingosine-1-phosphate (S1P) and are the rate-limiting enzymes in the process of endogenous S1P generation (Bryan et al., 2008). S1P, both as an extracellular signaling molecule via G-protein coupled receptors and as an intracellular signaling molecule, plays essential roles in regulating cell proliferation, apoptosis, adhesion and migration (Hofmann et al., 2008; Neubauer and Pitson, 2013; Shida et al., 2008). Specifically, the Sphk2/S1P axis has emerged as an important player in cerebral ischemia (Pfeilschifter et al., 2011). The expression of Sphk2 but not that of Sphk1 is enhanced by a short-duration of ischemia that induces preconditioning protection (Blondeau et al., 2007; Wacker et al., 2009). Consequently, the induction of Sphk2 by isoflurane or hypoxic preconditioning acts through neuronal and microvascular mechanisms to mediate ischemic tolerance (Wacker et al., 2009, 2012a, b; Yung et al., 2012). Moreover, the sphingosine analog fingolimod (FTY720), which is specifically phosphorylated by Sphk2, confers protection in rodent stroke models in a Sphk2-dependent manner (Wei et al., 2011; Yung et al., 2012). Additionally, Sphk2 knockout mice exhibit larger infarcts compared to wild-type littermates (Pfeilschifter et al., 2011).

Although accumulating evidence suggests that Sphk2 is essential for brain protection following stroke, we currently know very little about the role of Sphk1 following ischemic stroke. Sphk1 and Sphk2 might not play redundant roles as suggested by their tissue-specific expression patterns and differential sub-cellular localizations (Blondeau et al., 2007). Specifically, increasing evidence suggests that Sphk1 and its product S1P are essential mediators of the inflammatory responses induced by various inflammatory stimuli (Dixon et al., 2012; Herrera et al., 2007; van Leyen et al., 2005). Thus, in contrast to the protective role of Sphk2, Sphk1 possibly exacerbates stroke outcomes via a pro-inflammatory mechanism. We tested the hypothesis in this study and provided evidence suggesting that Sphk1 plays a deleterious role

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following cerebral ischemia by acting as an important mediator of post-stroke neuroinflammation.

Methods

Mouse model of transient focal cerebral ischemia

All animal protocols were conducted according to the regulations of the Laboratory Animal Care of Soochow University. Male CD-1 mice (8- to 9-week-old) were purchased from the SLAC Company (Shanghai, China). Middle cerebral artery occlusion (MCAO) was induced in the mice using an intraluminal monofilament technique as described previously (Wang et al., 2014; Zhu et al., 2010). Briefly, the mice were anesthetized with isoflurane. A laser probe was affixed to the mouse skull to monitor cortical perfusion (Periflux System 5000, PERIMED Inc., Stockholm, Sweden). For the MCA occlusion, the tip of a nylon thread (6-0) was heat-blunted and further coated with silicon. The thread was introduced into the right internal carotid artery through the right external carotid artery. The thread was advanced until the tip of the thread reached the origin of the middle cerebral artery as evidenced by an abrupt drop in cortical perfusion. After 60 min of occlusion, the thread was withdrawn to allow reperfusion. The mice that displayed cortical perfusions below 25% of the baseline value during the occlusion and cortical reperfusion exceeding 75% of the baseline were included. Sham surgeries were performed exactly as the MCAO procedures were conducted with the exception of the thread insertion. Body temperatures were maintained at 37 ± 0.5 °C with a heating lamp during the MCAO surgeries.

Animal groups

To investigate post-MCAO induction of Sphk1, mice were subjected to 1 h of MCAO and brain tissue was harvested at 6, 12, 24, 48, 72 and 96 h after reperfusion for qPCR assessment of Sphk1 or Sphk2 mRNA levels (n = 4 for each point). Brain tissue was also harvested at 6, 12, 24 and 48 h after reperfusion for Western blot analysis of post-ischemic induction of Sphk1 protein (n = 5 for each time point). To investigate the effects of post-ischemic induction of Sphk1 on stroke outcomes, mice were randomly divided into the following groups: the mice receiving vehicle or the Sphk1 inhibitor 5C at 3 h after reperfusion (n = 8 for infarction analysis at 24 h and n = 13 for infarction and behavioral analysis at 96 h after reperfusion) and mice receiving intracerebroventricular (ICV) injection of nonsense (NS) siRNA or Sphk1 siRNA and subsequent MCAO at 2 days after ICV injection (n = 10 for infarction analysis at 24 h after reperfusion). To investigate the effects of Sphk1 inhibition or knockdown on post-MCAO neuroinflammation, qPCR assessment of pro-inflammatory mediators was performed on brain tissue harvested at 24 h after reperfusion from the cortexes of the mice receiving vehicle or the Sphk1 inhibitor 5C and the mice receiving ICV injection of NS or Sphk1 siRNA (n = 3–4).

Drug administration

At 3, 12, 24, 48 and 72 h after reperfusion, the mice were injected with 2 mg/kg of the Sphk1 inhibitor 5C (CAY10621, Cayman Chemical, Ann Arbor, MI, USA) via the tail vein. 5C is a selective inhibitor of Sphk1 (IC_{50} = 3.3 μM) that does not have inhibitory effects on Sphk2 (Park et al., 2012; Wong et al., 2009). Specifically, the inhibitory effects of 5C on Sphk1 have been validated in an in vivo mouse model at the dose of 2 mg/kg (Tu et al., 2010). The inhibitor 5C was supplied as a methyl acetate solution. To prepare the 5C solution for intravenous injection, a vial containing 5C was exposed to a gentle stream of nitrogen until all of the methyl acetate solvent evaporated. Next, the 5C was dissolved in DMSO and further diluted with saline. Mice that received intravenous injections of vehicle (0.1% DMSO in saline) served as control animals.

Intracerebroventricular injection of siRNA

The siRNA knockdown technique was used to reduce target gene expression in the cortical penumbra in the mouse MCAO model, as previously reported (Hayakawa et al., 2012). Briefly, the mice were intracerebroventricularly injected with Sphk1 siRNA or NS siRNA at 2 days before MCAO. The coordinates for the right lateral ventricle were 0.5 mm posterior to, 0.8 mm lateral to and 2.5 mm below bregma. The siRNA complexes were prepared according to the in vivo siRNA transfection protocol for brain delivery outlined in the PolyPlus Transfection reagent instructions (PolyPlus Transfection, IIIkirch, France). Each mouse was administered 2 μg of siRNA in a 4-μL mixture. The sequences of the siRNAs were as follows: Sphk1 sense strand (5′-GGGC AAGGCUCUGCAGCUCdTT-3′), and NS sense strand (5′-UAGGCGCAGC UCCGGAUCGdTT-3′).

Quantitative PCR (qPCR)

At the indicated time-points, samples were harvested from the cortical penumbral areas as described in our previous publication (Cheng et al., 2011) or from primary microglial cultures. Total RNA was isolated with TRIzol reagent (Invitrogen, Camarillo, CA, USA). cDNA was reverse transcribed from 1 μg of total RNA using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with the cDNA on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green technique. The thermal cycling parameters for the qPCR were as follows: incubation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The mRNA levels of the target genes were normalized to the 18S mRNA levels. The qPCR primers were as follows:

Sphk1 forward primer: 5′-ACAGTGGGCACCTTCTTTC-3′
Sphk1 reverse primer: 5′-CTTCTGCACCAGTGTAGAGGC-3′
Sphk2 forward primer: 5′-CGGATGCCCATTGGTGTCCTC-3′
Sphk2 reverse primer: 5′-TGAGCAACAGGTCAACACCGAC-3′
iNOS forward primer: 5′-CAGGAGGAGAGAGATCCGATTTA-3′
iNOS reverse primer: 5′-GCATTAGCATGGAAGCAAAGA-3′
TNF-α forward primer: 5′-CTGAGGTCAATCTGCCCAAGTAC-3′
TNF-α reverse primer: 5′-CTTCACAGAGCAATGACTCCAAAG-3′
IL-1β forward primer: 5′-TGGAAAAGCGGTTTGTCTTC-3′
IL-1β reverse primer: 5′-TACCAGTTGGGGAACTCTGC-3′
IL-6 forward primer: 5′-GAGGATACCACTCCCAACAGACC-3′
IL-6 reverse primer: 5′-AAGTGCATCATCGTTGTTCATACA-3′
18S forward primer: 5′-TCAACACGGGAAACCTCAC-3′
18S reverse primer: 5′-CGCTCCACCAACTAAGAAC-3′

Western blot analysis

The mice were decapitated at 6, 12, 24 and 48 h after reperfusion. The cortical tissues were harvested and homogenized on ice in RIPA lysis buffer (Millipore, Billerica, MA, USA) containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 13,200 rpm for 20 min at 4 °C, and the supernatants were collected. The protein concentrations of the supernatants were determined using a BCA protein assay kit (Thermo Scientific, MA, USA). Equal amounts of denatured proteins (denatured at 95 °C for 5 min) were electrophoresed on 10% Tris-glycine gels and transferred to a PVDF membrane. Next, the membrane was blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 5% non-fat milk for 2 h at room temperature. The membranes were incubated with primary antibodies against Sphk1 (1:300, Abgent, San Diego, CA, USA) or β-actin (1:2000, Huaan Biotechnology Company, Hangzhou, China) overnight at 4 °C. Then, the membranes were washed and incubated with horseradish peroxidase-labeled secondary antibodies (1:2000,