Up-regulation of Sphingosine-1-phosphate Receptors and Sphingosine Kinase 1 in the Peri-ischemic Area after Transient Middle Cerebral Artery Occlusion in Mice

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Abbreviations: BBB, blood-brain barrier; CD31, cluster of differentiation 31; d, day; GFAP, glial fibrillary acidic protein; MCA, middle cerebral artery; NG2, neural/glial antigen 2; PBS, phosphate-buffered saline; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; SphK, sphingosine kinase; tMCAO, transient middle cerebral artery occlusion.

Abstract

There is thought to be a strong relationship between sphingosine-1-phosphate (S1P) signaling and pathophysiolosy of cerebral ischemia. We examined the change of expression and distribution of S1P receptors (S1PRs) and sphingosine kinases (SphKs) after cerebral ischemia in male C57BL6/J mice using immunohistochemical analysis at 1, 5, 14, and 28 days after 30 min of transient middle cerebral artery occlusion (tMCAO). S1PR1, 3, and 5 were transiently induced in the cells, which were morphologically similar to neurons in the peri-infarct lesion with a peak seen at 1 day after tMCAO ($p < 0.01$ vs. sham control). S1PR2 appeared in the inner layer of vessels in the ischemic core ($p < 0.01$ vs. sham control) and the peri-infarct lesion ($p < 0.01$ vs. sham control) at the acute phase after tMCAO. However, SphK1 was strongly induced at 1 and 5 days after tMCAO ($p < 0.01$ vs. sham control) in the peri-infarct lesion, whereas SphK2 expression did not change. Western blot analysis at 1 and 5 days after 30 min of tMCAO revealed that the expression of S1PRs and SphK1 were transiently enhanced at the acute phase, which was consistent with the immunohistochemical results. Double immunofluorescent analysis revealed S1PR2/NG2- and S1PR2/CD31-, S1PR3/CD31-, and S1PR5/CD31-double positive cells in the peri-infarct lesion 1 day after tMCAO. The present results suggest that S1PRs and SphK1 may be important therapeutic targets for rescuing the peri-infarct lesion.

Keywords: cerebral ischemia, sphingosine-1-phosphate, sphingosine-1-phosphate receptor, sphingosine kinase

1. Introduction

Ischemic stroke is a major neurologic disorder and a leading cause of disability and death worldwide. Recent studies have reported that sphingosine-1-phospate (S1P) receptor 1 (S1PR1)-selective antagonism (Gaire et al., 2018) and S1PR2 knockout (Kim et al., 2015) ameliorated the ischemic insult, which indicates that there is a strong relationship exists between S1P signaling and the pathophysiology of cerebral ischemia.

S1P is formed by the phosphorylation of sphingosine by sphingosine kinase (SphK) 1 or 2. S1P acts as a bioactive lipid mediator via its binding to sphingosine-1-phosphate receptors (S1PRs); these include S1PR1, 2, 3, and 5, which are expressed in the central nervous system (Strub et al., 2010; Groves et al., 2013). Previous studies have shown that S1PR1 and S1PR3 are preferentially expressed in neurons (Kajimoto et al., 2007), astrocytes (Rao et al., 2003), microglia (Tham et al., 2003), and endothelial cells (Lee et al., 1999), whereas S1PR2 is ubiquitously expressed in these cells at lower levels. S1PR5 is preferentially expressed in mature oligodendrocytes (Jaillard et al., 2005). S1P signaling via S1PR1 is involved in up-regulation of pro-inflammatory mediators such as tumor necrosis factor-alpha and interleukin-1 beta, blood-brain barrier (BBB) permeability (Gaire et al., 2018), and angiogenesis (Iwasawa et al., 2018). S1P signaling via S1PR2 is also related to BBB permeability (Kim et al., 2015; Wan et al., 2018). In the ischemic brain, SphK1 expression and S1P concentration are elevated (Nielsen et al., 2016; Sun et al., 2017). Thus, S1P signaling may be involved in the inflammatory reaction and the enhancement of BBB permeability after cerebral ischemia.

However, the spatial distribution and temporal profile of SphKs and S1PRs in cerebral ischemia have not been fully investigated. In the present study, therefore, we evaluated the time-dependent changes in the expression and distribution of S1PRs and SphKs after cerebral ischemia.

2. Results

2.1. S1PR1 expression

In the immunohistochemical analysis, S1PR1 was predominantly labeled in the cytoplasm of the cells, which were morphologically similar to neurons, in both sham control brains and ischemic brains (Fig. 1A). Although S1PR1 staining disappeared after tMCAO in the ischemic core, S1PR1 staining transiently increased at 1 d and 5 d ($p < 0.01$ and $p < 0.05$ vs. sham control, Mann-Whitney U-test with Bonferroni correction) in the peri-infarct lesion, which then gradually returned to the sham control level at 28 d ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 1B). Western blot showed that S1PR1 expression was higher in the peri-infarct lesion group at 1 d after tMCAO compared with the sham control group, which was consistent with the immunohistochemical analysis results (Fig. 1C).

2.2. S1PR2 expression

S1PR2-positive cells were scarcely observed in sham control brains, whereas S1PR2-positive cells were observed in the inner layer of vessels in ischemic brains at 1 and 5 d after tMCAO (Fig. 2A) in the ischemic core ($p < 0.01$ vs. sham control), and at 1 d in the peri-infarct lesion ($p < 0.01$ vs. sham control) ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 2B). Western blot showed that S1PR2 expression was increased in the core at 5 d after tMCAO and peaked in the peri-infarct lesion at 1 d, which was consistent with the immunohistochemical analysis results (Fig. 2C).

2.3. S1PR3 expression

S1PR3-positive cells were scarcely observed in sham control brains. S1PR3 was strongly induced both in the cytoplasm and in the nuclei of the morphologically neuronal cells at 1, 5, and 14 d after tMCAO (Fig. 3A). The number of S1PR3-positive cells peaked at 1 d in the peri-infarct lesion ($p < 0.01$ vs. sham control), then gradually decreased until 28 d ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 3B). Western blot showed that S1PR3 expression was increased both in the core and in the peri-infarct lesion at 1 d and 5 d (Fig. 3C).

2.4. S1PR5 expression

S1PR5 expression was stronger in the nuclei than the cytoplasm of the cells in sham control brains and ischemic brains. S1PR5 was only minimally observed in the ischemic core, while much strongly induced in the peri-infarct lesion at 1 and 5 d after tMCAO (Fig. 4A). The number of S1PR5-positive cells peaked at 1 d in the peri-infarct lesion ($p < 0.01$ vs. sham control), then gradually decreased until 28 d ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 4B). Western blot also showed that S1PR5 expression was increased in the peri-infarct lesion at 1 d and 5 d after tMCAO, which was consistent with the immunohistochemical analysis results (Fig. 4C).

2.5. SphK1 immunohistochemistry

SphK1 was predominantly labeled in the cytoplasm of the cells in sham control brains, while it was labeled more strongly in the nuclei than the cytoplasm of the cells in the peri-infarct lesion. The SphK1-positive cells were morphologically similar to neurons. Although SphK1-positive cells decreased in the ischemic core, SphK1 was strongly induced in the peri-infarct lesion at 1 and 5 d after tMCAO (Fig. 5A). The number of SphK1-positive cells peaked at 1 d in the peri-infarct lesion ($p < 0.01$, vs.

sham control), then gradually decreased until 14 d ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 5B).

2.6. SphK2 immunohistochemistry

SphK2 was labeled both in the cytoplasm and in the nuclei of the cells (Fig. 6A). Although SphK2-positive cells were observed in sham control brains as well as the ischemic core and peri-infarct lesion (Fig. 6A), a mild but statistically significant increase in the number of SphK2-positive cell number was observed in the ischemic core at 1 d. There was no significant difference in SphK2-positive cell number between the groups in the peri-infarct lesion. ($n = 23$; $p < 0.01$, two-tailed Kruskal Wallis H-test; sham control ($n = 3$), 1 d ($n = 5$), 5 d ($n = 5$), 14 d ($n = 5$), and 28 d ($n = 5$); Fig. 6B).

2.7. Double immunofluorescence

S1PR2, S1PR3, and S1PR5 expression was observed around the vessels in the peri-infarct lesion at 1 d after tMCAO, while S1PR1, SphK1, and SphK2 expression was scarcely observed around the vessels (Fig. 7A, B, and C).

There were no GFAP and S1PR2, S1PR3, or S1PR5 double-positive cells (Fig. 7A). Conversely, S1PR2 immunofluorescent signals colocalized with NG2 presenting pericytes (Fig. 7B and C) as well as endothelial CD31. Several cells expressing S1PR3 and S1PR5 were only positive for endothelial CD31 (Fig. 7C).

3. Discussion

The present study is the first to demonstrate the change of expression and distribution of S1PRs and SphKs after cerebral ischemia. S1PR1 has been reported to be involved in inflammation (Kraft et al., 2013; Gaire et al., 2018), blood-brain barrier

(BBB) permeability (Gaire et al., 2018), and angiogenesis (Iwasawa et al., 2018) in the ischemic brain, and an S1PR1-selective functional antagonist was found to ameliorate the ischemic insult (Gaire et al., 2018). Therefore, the upregulation of S1PR1 in the peri-infarct lesion may account for the exacerbation of ischemic insult. Thus, S1PR1 antagonism may be an important therapeutic target for rescuing the penumbra.

Positive staining of S1PR2 in inner layer of vessels suggests the induction of S1PR2 in pericytes and endothelial cells, as previously reported (Kim et al., 2015; Wan et al., 2018). S1PR3 expression was elevated both in the cytoplasm and in the nuclei at the peri-infarct lesion until the subacute phase of cerebral infarction, which is consistent with a previous report (Moon et al., 2015). Our results also support those of a previous study that found that S1PR5 was preferentially expressed in endothelial cells, and that this was related to the suppression of inflammatory cell invasion by enhancing BBB integrity (Doorn et al., 2012). This suggests that the upregulation of S1PR5 in cerebral ischemia may account for the protection from the ischemic insult.

For the first time, we also found an upregulation of SphK1 both in the cytoplasm and in nuclei following cerebral ischemia. A previous study reported an upregulation of SphK1 expression after cerebral ischemia (Sun et al., 2017). Given that S1P concentration has also been found to be elevated in the ischemic brain (Nielsen et al., 2016), the upregulation of SphK1 in the peri-infarct lesion may account for the exacerbation of ischemic insult. SphK1 may also be an important therapeutic target in cerebral ischemia. In contrast, the SphK2 expression level did not change after brain ischemia, which is consistent with previous report (Zheng et al., 2015).

The S1PR3/CD31- and S1PR5/CD31-double-positive cells in the peri-infarct lesion found in the present study indicates that cerebral ischemia induced S1PR3 and S1PR5 expression in endothelial cells. As previously reported (Kim et al., 2015; Wan et al., 2018), we also observed S1PR2/NG2- and S1PR2/CD31-double-positive cells. Considering that upregulated S1PR2, S1PR3, and S1PR5 could inhibit cell death (An et al., 2000; Herr et al., 2015), these receptors may contribute to resistance against cerebral ischemia.

The limitation of this study was that the function of each S1PR and SphK was not studied. However, it is anticipated that the findings described herein will be of fundamental importance to further understanding of the pathology of cerebral ischemia.

Taken together, the present findings suggest that the cerebral ischemia changes not only the expression level, but also the spatial distribution of S1PRs and SphK1. Upregulation of S1PR1 and SphK1 was evident, which suggests that these may be important therapeutic targets for rescuing the penumbra.

4. Experimental procedure

4.1. Animals and experimental groups

All experiments were approved by the Animal Committee of the Graduate School of Medicine and Dentistry, Okayama University (OKU-2018672), and performed in accordance with the guidelines of Okayama University on animal experiments. Experiments were performed on male C57BL6/J mice at 8 weeks of age (body weight 23–26 g; Japan SLC Inc., Shizuoka, Japan). Animals were placed on a basal diet and maintained for at least 7 days before the experiment in a temperature-regulated room (23°C-25°C) on a 12-hour light/dark cycle under specific pathogen-free conditions. The mice were fasted but allowed free access to water overnight before surgery. To detect an increase of S1PRs- and SphKs- expression at 1, 5, 14, and 28 days (d) after the surgery to induce cerebral ischemia in comparison to the sham control group with a two-sided 5% significance level and a power of 80%, a sample size of 5 mice was necessary. We used 32 mice, including 6 mice in the sham control group (mean body weight: 23.06 g) and 26 mice in the transient middle cerebral artery occlusion (tMCAO) group. The tMCAO group was divided into 4 groups based on the time course after tMCAO, which included the 1 d group (n = 8, mean body weight: 23.50 g; n = 5 for immunohistochemistry, n = 3 for western blot analysis), 5 d group (n = 8, mean body weight: 23.92 g; n = 5 for immunohistochemistry, n = 3 for

western blot analysis), 14 d group (n = 5, mean body weight: 24.34 g), and 28 d group (n = 5, mean body weight: 23.17 g).

4.2. Ischemia/reperfusion model

Transient focal ischemia was induced by right middle cerebral artery (MCA) occlusion (MCAO) according to our previous reports (Abe et al., 1992; Yamashita et al., 2006). Briefly, the mice were anesthetized with a mixture of nitrous oxide, oxygen, and isoflurane (69/30/1%) using a face mask. A midline neck incision was made and the right common carotid artery was exposed. At the same time, an incision was made in the head skin to expose the right parietal skull. Regional cerebral blood flow of the right frontoparietal cortex was measured before, during and after tMCAO through the surface of the right parietal skull (2 mm posterior and 5 mm lateral from the bregma) using a laser blood flow meter (Flo-C1; Omegawave, Tokyo, Japan) The right MCA was occluded by insertion of 6-0 surgical nylon thread with a silicone coating through the common carotid artery. Meanwhile, a drop of more than 70% drop in cerebral blood flow confirmed the MCAO was occluded. During these procedures, body temperature was monitored with a rectal probe, and was maintained at 37 ± 0.3°C using a heating pad (model BMT-100; Bio Research Center, Tokyo, Japan). The surgical incision was then closed and the mice were allowed to recover at room temperature. After 30 min of occlusion, the thread was removed to restore blood flow (reperfusion). Cerebral blood flow quickly recovered to the basal levels. After that, the incisions were sutured. Sham control animals (n = 6) underwent cervical surgery without the insertion of a thread. The animals were kept at ambient temperature until sampling, and had free access to food and water.

4.3. Immunohistochemistry

At the time of sampling after tMCAO or 1 d after the sham operation, animals were deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg), and

then perfused with chilled phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 mol/l phosphate buffer. Brains were removed and immersed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer overnight, and were subsequently incubated in 10%, 20%, and then 30% sucrose in 0.1 mol/l phosphate buffer for 24 hours at 4°C. The tissues were embedded in OCT compound (Sakura Fine Technical; Tokyo, Japan), frozen in liquid nitrogen, and stored at -80°C. Coronal brain sections (20-μm thick) were prepared using a cryostat at -20°C and mounted on silane-coated glass slides. Five serial brain slices, at 0.5-mm intervals between 1.0 mm anterior and 1.5 mm posterior to the bregma were stained with Cresyl violet as Nissl staining to detect the infarct area. For immunohistochemistry, frozen sections were incubated in 0.3% hydrogen peroxide/PBS for 20 min to prevent endogenous peroxidase activity. After washing in PBS, brain sections were blocked in 5% bovine serum albumin for 2 hours. Then, they were incubated 4°C overnight with the following primary antibodies: rabbit anti-S1PR1 polyclonal antibody (1:100; Abcam, ab11424), rabbit anti-S1PR2 polyclonal antibody (1:100; Proteintech, 21180-1-AP), rabbit anti-S1PR3 polyclonal antibody (1:500; Bioss, bs-7541R), rabbit anti-S1PR5 polyclonal antibody (1:500; Bioss, bs-11457R), rabbit anti-SphK1 polyclonal antibody (1:100; Abgent, AP7237c), rabbit anti-SphK2 polyclonal antibody (1:500; Abgent, AP7238), mouse anti-GFAP antibody for astrocytes (1:500; Chemicon International, MAB3402, AB_10627989), mouse anti-NG2 antibody for pericytes (1:100; Millipore, 05-710, AB_309925), and rat anti-mouse CD31 antibody (1:100; BD Pharmingen, 553371). The sections were washed in PBS and incubated with biotinylated secondary antibody (1:500; Vector Laboratories) for 2.5 hours. The sections were then incubated with the ABC Elite complex (Vector Laboratories), and visualized with diaminobenzidine substrate dissolved in PBS. For double immunofluorescent staining, each primary antibody was detected by appropriate secondary antibodies conjugated with Alexa Fluor 488 or 594TM (1:500; Invitrogen). A set of sections was also stained in a similar way but without the primary antibody and served as the negative control.

4.4. Western blot analysis

Western blot analysis was performed using brain tissues from the ischemic core, the peri-infarct lesion, and the contralateral cortex, which were obtained from the sham control group (n = 3), 1 d group (n = 3), and 5 d group (n = 3). After decapitation under deep anesthesia at each time point after reperfusion, the brain was removed and the brain tissues (about 0.01 mg) were quickly frozen in liquid nitrogen. The brain samples were gently homogenized and sonicated in ice-cold cell lysis buffer (Thermo ScientificTM, 78510) containing protease inhibitor (Roche, 11836170001). The samples were centrifuged at 15,000 rpm at 4°C for 30 min. The protein concentrations were determined by Takara BCA Protein Assay Kit (Takara, T9300A).

To evaluate the expression levels of the proteins S1PR1, S1PR2, S1PR3, S1PR5, and SphK1, 20 μg of total protein extract was loaded onto an 12% polyacrylamide gel, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes (Millipore). After washing with PBS containing 5% skim milk and 0.1% Tween 20, the membranes were incubated with the following primary antibodies: rabbit anti-S1PR1 polyclonal antibody (1:1000), rabbit anti-S1PR2 polyclonal antibody (1:1000), rabbit anti-S1PR3 polyclonal antibody (1:1000), rabbit anti-S1PR5 polyclonal antibody (1:1000), and rabbit anti-SphK1 polyclonal antibody (1:1000) at 4°C overnight. After washing with PBS, the membranes were probed with a horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (1:2000; Amersham Biosciences), and then the signal was visualized using an enhanced chemiluminescence substrate (Thermo Fisher Scientific, 32106).

4.5. Quantitative and Statistical Analysis

For the quantitative evaluation of histochemical staining, stained sections were selected from three levels of the caudate putamen (1.0, 0.5, and 0 mm rostral to the bregma) in each animal. Three areas were randomly selected from the sections at ischemic core, peri-infarct lesion, and contralateral cortex, and captured at 200×magnification with a light microscope (OlympusBX51). The number of S1PR1-,

S1PR2-, S1PR3-, S1PR5-, SphK1-, and SphK2-positive cells per 1 mm^2 in each field was counted, and the same procedure was repeated for each section. We confirmed the border between the infarcted and peri-ischemic region using Nissl staining of the adjacent sections based on a previous report (Omori et al., 2002). Data are expressed as the mean ± standard deviation. The Kruskal Wallis H-test was used to test for within-group differences in the expression of S1PR1, S1PR2, S1PR3, S1PR5, SphK1, and SphK2 expression in the ischemic core, peri-infarct lesion, and contralateral cortex. The differences between the sham control group and ischemia group were evaluated using the Mann-Whitney U-test with Bonferroni correction. A P-value less than 0.05 was considered significant. All statistical analyses were performed using Excel ystat 2002 (ystat 2002.xls; Shinya Yamazaki, Japan).

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Conflicts of interest

The authors declare no conflicts of interest.

References

1. Gaire BP, Lee CH, Sapkota A, et al. Identification of Sphingosine 1-Phosphate Receptor Subtype 1 (S1P1) as a Pathogenic Factor in Transient Focal Cerebral Ischemia. Mol Neurobiol. 2018;55(3):2320–2332. https://doi.org/10.1007/s12035-017-0468-8
2. Kim GS, Yang L, Zhang G, et al. Critical role of sphingosine-1-phosphate receptor-2 in the disruption of cerebrovascular integrity in experimental stroke. Nat Commun. 2015;6:7893. Published 2015 Aug 5. doi:10.1038/ncomms8893
3. Strub GM, Maceyka M, Hait NC, Milstien S, Spiegel S. Extracellular and intracellular actions of sphingosine-1-phosphate. Adv Exp Med Biol. 2010;688:141–155. https://doi.org/10.1007/978-1-4419-6741-1_10
4. Groves A, Kihara Y, Chun J. Fingolimod: direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. J Neurol Sci. 2013;328(1-2):9–18. https://doi.org/10.1016/j.jns.2013.02.011
5. Kajimoto T, Okada T, Yu H, Goparaju SK, Jahangeer S, Nakamura S. Involvement of sphingosine-1-phosphate in glutamate secretion in hippocampal neurons. Mol Cell Biol. 2007;27(9):3429–3440. https://doi.org/10.1128/MCB.01465-06
6. Rao TS, Lariosa-Willingham KD, Lin FF, et al. Pharmacological characterization of lysophospholipid receptor signal transduction pathways in rat cerebrocortical astrocytes. Brain Res. 2003;990(1-2):182–194. https://doi.org/10.1016/s0006-8993(03)03527-3
7. Tham CS, Lin FF, Rao TS, Yu N, Webb M. Microglial activation state and lysophospholipid acid receptor expression. Int J Dev Neurosci. 2003;21(8):431–443. https://doi.org/10.1016/j.ijdevneu.2003.09.003
8. Lee MJ, Thangada S, Claffey KP, et al. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell. 1999;99(3):301–312. https://doi.org/10.1016/s0092-8674(00)81661-x
9. Jaillard C, Harrison S, Stankoff B, et al. Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. J Neurosci.

2005;9;25(6):1459-69. https://doi.org/10.1523/JNEUROSCI.4645-04.2005. PMID: 15703400; PMCID: PMC6726002.

10. Iwasawa E, Ishibashi S, Suzuki M, et al. Sphingosine-1-Phosphate Receptor 1 Activation Enhances Leptomeningeal Collateral Development and Improves Outcome after Stroke in Mice. J Stroke Cerebrovasc Dis. 2018;27(5):1237–1251. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.11.040
11. Wan Y, Jin HJ, Zhu YY, et al. MicroRNA-149-5p regulates blood-brain barrier permeability after transient middle cerebral artery occlusion in rats by targeting S1PR2 of pericytes. FASEB J. 2018;32(6):3133–3148. https://doi.org/10.1096/fj.201701121R.
12. Nielsen MM, Lambertsen KL, Clausen BH, et al. Mass spectrometry imaging of biomarker lipids for phagocytosis and signalling during focal cerebral ischaemia. Sci Rep. 2016;6:39571. Published 2016 Dec 22. https://doi.org/10.1038/srep39571
13. Sun W, Ding Z, Xu S, Su Z, Li H. Crosstalk between TLR2 and Sphk1 in microglia in the cerebral ischemia/reperfusion-induced inflammatory response. Int J Mol Med. 2017;40(6):1750–1758. https://doi.org/10.3892/ijmm.2017.3165
14. Kraft P, Göb E, Schuhmann MK, et al. FTY720 ameliorates acute ischemic stroke in mice by reducing thrombo-inflammation but not by direct neuroprotection. Stroke. 2013;44(11):3202–3210. https://doi.org/10.1161/STROKEAHA.113.002880
15. Moon E, Han JE, Jeon S, Ryu JH, Choi JW, Chun J. Exogenous S1P Exposure Potentiates Ischemic Stroke Damage That Is Reduced Possibly by Inhibiting S1P Receptor Signaling. Mediators Inflamm. 2015;2015:492659. https://doi.org/10.1155/2015/492659
16. van Doorn R, Lopes Pinheiro MA, Kooij G, et al. Sphingosine 1-phosphate receptor 5 mediates the immune quiescence of the human brain endothelial barrier. J Neuroinflammation. 2012;9:133. Published 2012 Jun 20. https://doi.org/10.1186/1742-2094-9-133
17. Zheng S, Wei S, Wang X, et al. Sphingosine kinase 1 mediates neuroinflammation following cerebral ischemia. Exp Neurol. 2015;272:160–169. https://doi.org/10.1016/j.expneurol.2015.03.012

18. An S, Zheng Y, Bleu T. Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. J Biol Chem. 2000;275(1):288–296. https://doi.org/10.1074/jbc.275.1.288
19. Herr DR, Reolo MJ, Peh YX, et al. Sphingosine 1-phosphate receptor 2 (S1P2) attenuates reactive oxygen species formation and inhibits cell death: implications for otoprotective therapy. Sci Rep. 2016;6:24541. Published 2016 Apr 15. https://doi.org/10.1038/srep24541
20. Abe K, Kawagoe J, Araki T, Aoki M, Kogure K. Differential expression of heat shock protein 70 gene between the cortex and caudate after transient focal cerebral ischaemia in rats. Neurol Res. 1992;14(5):381–385. https://doi.org/10.1080/01616412.1992.11740089
21. Yamashita T, Deguchi K, Sawamoto K, Okano H, Kamiya T, Abe K. Neuroprotection and neurosupplementation in ischaemic brain. Biochem Soc Trans. 2006;34(Pt 6):1310–1312. https://doi.org/10.1042/BST0341310
22. Omori N, Jin G, Li F, et al. Enhanced phosphorylation of PTEN in rat brain after transient middle cerebral artery occlusion. Brain Res. 2002;954(2):317–322. https://doi.org/10.1016/s0006-8993(02)03366-8

Figure legends

Figure 1. S1PR1 immunohistochemistry (A) Representative immunostaining of S1PR1 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral cortex (scale bar = 20 μm). (B) Semiquantitative analysis, showing that S1PR1-positive cells transiently increased at 1 and 5 d in the peri-infarct lesion. Data are shown as the mean ± standard deviation; *$p < 0.05$, **$p < 0.01$ vs. sham control. (C) Representative pictures of western blot for S1PR1 and β-actin in the ischemic core, the peri-infarct lesion, and the contralateral cortex from the sham control group, 1 and 5 d after tMCAO.

Figure 2. S1PR2 immunohistochemistry (A) Representative immunostainings of S1PR2 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral cortex (scale bar = 20 μm). (B) Semiquantitative analysis showing that S1PR2-positive cells increased at 1 and 5 d in the ischemic core and in the peri-infarct lesion. **$p < 0.01$ vs. sham control. (C) Representative pictures of western blot for S1PR2 and β-actin in the ischemic core, the peri-infarct lesion, and the contralateral cortex from sham control, 1 and 5 d after tMCAO.

Figure 3. S1PR3 immunohistochemistry (A) Representative immunostainings of S1PR3 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral cortex (scale bar = 20 μm). (B) Semiquantitative analysis showing that S1PR3-positive cells peaked at 1 d in the peri-infarct lesion. **$p < 0.01$ vs. sham control. (C) Representative pictures of western blot for S1PR3 and β-actin in the ischemic core, the peri-infarct lesion, and the contralateral cortex from sham control, 1 and 5 d after tMCAO.

Figure 4. S1PR5 immunohistochemistry (A) Representative immunostainings of S1PR5 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral (scale bar = 20 μm). (B) S1PR5-positive cells peaked at 1 d in the

peri-infarct lesion. **p < 0.01 vs. sham control. (C) Representative pictures of Western blot for S1PR5 and β-actin in the ischemic core, the peri-infarct lesion, and the contralateral cortex from sham control, 1 and 5 d after tMCAO.

Figure 5. SphK1 immunohistochemistry (A) Representative immunostainings of SphK1 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral cortex (scale bar = 20 μm). (B) SphK1-positive cells peaked at 1 d in the peri-infarct lesion. *p < 0.05, **p < 0.01 vs. sham control.

Figure 6. SphK2 immunohistochemistry (A) Representative immunostainings of SphK2 at 1, 5, 14, and 28 d after tMCAO in the ischemic core, the peri-infarct lesion, and the contralateral cortex (scale bar = 20 μm). (B) A mild increment of SphK2-positive cell numbers was seen in the ischemic core at 1 d. **p < 0.01 vs. sham control.

Figure 7. Double immunofluorescence (A) Double immunofluorescent analysis at 1 d after tMCAO. Note that there were no S1PRs- or SphKs- and GFAP-double-positive cells. (B) Arrowheads indicate S1PR2/NG2-double-positive cells. (C) Arrowheads indicate S1PR2/CD31-, S1PR3/CD31-, and S1PR5/CD31-double-positive cells. Scale bar = 10 μm.