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Sphk1 mediates neuroinflammation and neuronal injury via TRAF2/NF-κB pathways in activated microglia in cerebral ischemia reperfusion

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ABSTRACT

Sphingosine kinase 1 (Sphk1), a key enzyme responsible for phosphorylating sphingosine into sphingosine1-phosphate (S1P), plays an important role in mediating post-stroke neuroinflammation. However, the pathway and mechanism of the Sphk1-mediated inflammatory response remains unknown. In this study, we found that suppression of Sphk1 decreased IL17 production and relieved neuronal damage induced by microglia in cerebral ischemia reperfusion (IR) or in an in vitro oxygen-glucose deprivation reperfusion (OGDR) system. Inhibition of Sphk1 with an inhibitor or siRNA decreased tumor necrosis factor receptor-associated factor 2 (TRAF2) and nuclear factor-kappa B (NF-κB) sequentially in microglia in response to IR or OGDR. Moreover, we also found that after suppression of TRAF2 or NF-κB by siRNA in microglia, reductions in the downstream molecules NF-κB and IL-17 and in neuronal apoptosis were observed in response to OGDR. Taken together, we hypothesize that Sphk1, TRAF2 and NF-κB form an axis that leads to increased IL-17 and neuronal apoptosis. This axis may be a potential therapeutic target to control neuroinflammation in brain IR.

1. Introduction

Stroke serves as a major cause of morbidity and mortality in the world today. Over the past several decades, substantial progress has been made in the understanding and management of ischemic stroke, but there remains much to be learned about mechanisms of cerebral ischemic reperfusion injury (IRI) and the associated neuroimmune responses (Petrovic-Djergovic et al., 2016). Recent evidence has shown that cells of the immune system are intimate participants in all stages of the ischemic stroke cascade. Microglia constitute the innate immune cells of the central nervous system, rapidly activated after ischemic stroke (Kettenmann et al., 2011). Microglia participate in neuronal damage in the acute stage via the production of excessive pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and other pro-inflammatory mediators (Block et al., 2007; Mabuchi et al., 2000). Thus, the suppression of microglial activation and microglia-mediated excessive inflammatory responses may prove to be a valid therapeutic strategy for ischemic stroke.

Sphingosine kinases (Sphks) are the rate-limiting kinases that phosphorylate sphingosine to sphingosine-1-phosphate (S1P), which regulates cell proliferation, apoptosis, adhesion and migration (Hofmann et al., 2008; Neubauer and Pitson, 2013; Shida et al., 2008). Sphk includes two isoforms, SphK1 and SphK2, that have different properties and different subcellular localizations (Spiegel and Milstien, 2007; Igarashi et al., 2003). Sphk2 has been reported to be protective following experimental stroke (Pfeilschifter et al., 2011). Sphk1 and its product S1P have been reported to mediate the inflammatory responses induced by various inflammatory stimuli (Dixon et al., 2012; Herrera et al., 2007; van Leyen et al., 2005). Recent research confirmed that microglia were one of the major cellular sources of Sphk1 in cerebral ischemia. Inhibition of Sphk1 decreased microglial induction of pro-inflammatory mediators by ischemic neurons (Zheng et al., 2015). In our previously study, we confirmed that Sphk1/S1P regulates the expression of interleukin-17 (IL17) in activated microglia, inducing neuronal apoptosis in cerebral ischemia/reperfusion (Lv et al., 2016). However the pathway and mechanism of Sphk1/S1P modulating IL17 production is not clear.

It has been reported that intracellular Sphk1-derived S1P was required for NFκB induction during inflammatory cytokine action. S1P was proposed to bind to the tumor necrosis factor receptor-associated factor 2 (TRAF2) and stimulate its E3 ubiquitin ligase activity as a key mechanism for Nuclear factor-kappa B (NFκB) signaling (Alvarez et al.,

Abbreviations: Sphk1, Sphingosine kinase 1; S1P, Sphingosine-1-phosphate; IL17, Interleukin-17; IRI, Ischemia reperfusion injury; OGDR, Oxygen-glucose deprivation reperfusion; TNFR2, Tumor necrosis factor receptor2; TRAF2, Tumor necrosis factor receptor-associated factor 2; NF-κB, Nuclear factor-kappa B.

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2010). TRAF2 is a critical intermediate in the signal transduction of inflammatory cytokines (Vallabhapurapu et al., 2008; Devin et al., 2001; Liu and Malik, 2006), which suggested an essential role of intracellular S1P in cytokine-induced inflammatory pathways. Since NFκB signaling activation is a well-recognized mechanism underlying stroke-induced neuroinflammation, we speculate that Sphk1 induces IL17 production in the brain post-ischemia and neuronal damage via the TRAF2–NFκB axis.

In the present study, we first demonstrated that inhibition of Sphk1 in activated microglia decreased inflammatory immune responses and neuronal injury in the in vivo or in vitro OGDR models. We then studied the effect of the TRAF2–NFκB pathway on the release of the pro-inflammatory cytokine IL17 and the induction of neuronal apoptosis in activated microglia under OGDR conditions.

2. Methods

2.1. Preparation of cerebral ischemia model by MCAO

Sprague Dawley (SD) rats (250– 350 g, male) were used from the Experimental Animal Center of Harbin Medical University, Harbin, China. All rats were kept at the animal facility with adaptive temperature and humidity. All animal protocols were conducted according to the Experimental Animal Ethics Committee of Harbin Medical University, China. Chloral hydrate (300 mg/kg) was used to anesthetize the rats. The method for occluding the middle cerebral artery (MCA) using an intraluminal suture was described in detail previously (Lv et al., 2016). Briefly, the right common carotid artery, internal carotid artery, and external carotid artery were exposed surgically. A paraffin wax-coated fishing thread (overall diameter 0.28 mm) was introduced into the right internal carotid artery through the right carotid artery and the carotid bifurcation. It was then advanced distally up to the right internal carotid artery in a distance of 17.5 ± 0.5 mm from the carotid bifurcation to occlude the origin of the MCA for 2 h, then the fishing thread was gently removed (reperfusion starts). For sham-operated rats, the thread was immediately removed as soon as the origin of the MCA was reached. The sham groups and experimental groups were each divided into three subgroups to assess different time points after surgical manipulation: 12 h ($n = 6$), 24 h ($n = 6$) and 48 h ($n = 6$). An additional six animals were used as controls.

2.2. Injection of PF-543

Animals were given an intraperitoneal (i.p.) injection of PF-543 (1.2 mg/kg) 6 h before MCAO. PF-543 is a specific inhibitor of SphK1 and can suppress the function of Sphk1 (Schnute et al., 2012). PF-543 was dissolved in dimethyl sulphoxide (DMSO) and further diluted with saline. Control animals received intravenous injections of vehicle (0.1% DMSO in saline).

2.3. Reagents and antibodies

Goat anti-rat Sphk1, rabbit anti-rat IL-17 and goat anti-rat GAPDH antibodies were obtained from Santa Cruz Biotechnologies, USA. Rabbit polyclonal anti-rat TRAF2, rabbit polyclonal to anti-rat NFkB p65 and mouse anti-rat Iba1 monoclonal antibodies (Ab) were purchased from Abcam, USA. AlexaFluor 488 conjugated anti-goat IgG Ab, AlexaFluor 488 conjugated anti-rabbit IgG Ab, and Alexa 594 conjugated anti-mouse IgG Ab were purchased from Invitrogen, USA. Exogenous S1P was obtained from Abcam, USA. Sphk1 siRNA was obtained from Applied Biosystems (ABI, USA). NFκB siRNA was obtained from Santa Cruz Biotechnologies, USA. PF-543 was purchased from Millipore.

2.4. Preparation of cerebral tissue sections

At each time point after the IR or sham operation, some of the animals were sacrificed and the brains were immediately removed and frozen in liquid nitrogen. The other animals were anesthetized and transaortically sequentially perfused with normal saline and buffered paraformaldehyde. The brains were removed, dehydrated, and frozen in OCT. Then, the brains were cut into 5-μm sections and mounted on glass slides before being stored at −80 °C.

2.5. Primary culture

Primary microglia were prepared from 1- or 2-day-old rat pups as previously described (Lv et al., 2016). Briefly, Cortical brain tissue was removed from the meninges and blood vessels and digested by trypsin. The cell suspension was then passed through a 200-μm pore filter. The cells were incubated in high-glucose-Dulbecco"'s modified Eagle's minimum essential medium (DMEM 4.5 g/l) with 5% fetal calf serum and 1% penicillin/streptomycin solution (HyClone USA) at 37 °C. After 6 or 7 d of primary culture, the microglia were separated from other cell types by shaking the flasks overnight at 300 rpm on a rotary shaker at 37 °C. The primary microglia were removed from the flask, resuspended in DMEM complete microglia medium containing 1% Microglia Growth Supplement (ScienCell, USA) for 2 to 3 days. The purity of the cultures was 98%, as identified by staining with a specific microglial marker, ionized calcium-binding adapter molecule-1 (Iba1).

Primary neurons were prepared from embryonic day 17 SD rats as previously described (Lv et al., 2016). Briefly, cortical brain tissue was dissected and digested for 15 min at 37 °C with 0.25% trypsin. Then, the cell suspension was passed through a 200-μm pore filter and resuspended in Neurobasal™ Medium containing 2% B27 Supplement (both Invitrogen) and 1% penicillin/streptomycin solution. Experiments were performed using cells cultured for 7 to 9 days. The purity of the cultures was >96%, as identified by NeuN-specific immunostaining.

2.6. Immunohistochemistry

Tissue sections were rinsed with phosphate-buffered saline (PBS) and then incubated in goat serum diluted in PBS plus Triton X-100 at room temperature for 1 h. Tissue sections were incubated overnight at room temperature with a mixture of primary antibodies followed by removal of serum. The corresponding secondary antibodies were incubated after washing with PBS. Images of the tissue sections were detected under a confocal microscope (Olympus Fluoview 1000, Tokyo, Japan) at a magnification of 200×. The mean optical density (OD) of the proteins, which reflected the amount of the labeled protein, was quantified by Image-Pro Plus 6.0 software. In each section analyzed, 4 to 5 random field samples throughout the cortex were measured. Density values from the sampled area were averaged for each section.

2.7. Oxygen–glucose deprivation reperfusion (OGDR) treatment

Primary microglia and neurons were induced by oxygen-glucose deprivation (OGD). Briefly, the culture medium was replaced by glucose-free Earle's balanced salt solution, and cells were maintained in anoxic incubator filled with 95% N_2 and 3% CO_2 and 2% O_2 at 37 °C for 2 h. Control cells were incubated in normoxic conditions (95% O_2/ 5% CO_2) for the same time period. To terminate OGD, cells were returned to normoxic conditions with regular medium.

2.8. Western blotting analysis

Western blotting analysis was used to estimate the total Sphk1, TRAF2 and NFκB protein levels under different conditions after MCAO or OGDR. Protein concentrations were determined using a BCA kit (Universal Microplate Spectrophotometer; Bio-Tek Instruments, Winooski,