Hypoxic Preconditioning-Induced Cerebral Ischemic Tolerance
Role of Microvascular Sphingosine Kinase 2

Bradley K. Wacker, DSc; Tae Sung Park, MD; Jeffrey M. Gidday, PhD

Background and Purpose—The importance of bioactive lipid signaling under physiological and pathophysiological conditions is progressively becoming recognized. The disparate distribution of sphingosine kinase (SphK) isoform activity in normal and ischemic brain, particularly the large excess of SphK2 in cerebral microvascular endothelial cells, suggests potentially unique cell- and region-specific signaling by its product sphingosine-1-phosphate. The present study sought to test the isoform-specific role of SphK as a trigger of hypoxic preconditioning (HPC)-induced ischemic tolerance.

Methods—Temporal changes in microvascular SphK activity and expression were measured after HPC. The SphK inhibitor dimethylsphingosine or sphingosine analog FTY720 was administered to adult male Swiss-Webster ND4 mice before HPC. Two days later, mice underwent a 60-minute transient middle cerebral artery occlusion and at 24 hours of reperfusion, infarct volume, neurological deficit, and hemispheric edema were measured.

Results—HPC rapidly increased microvascular SphK2 protein expression (1.7±0.2-fold) and activity (2.5±0.6-fold), peaking at 2 hours, whereas SphK1 was unchanged. SphK inhibition during HPC abrogated reductions in infarct volume, neurological deficit, and ipsilateral edema in HPC-treated mice. FTY720 given 48 hours before stroke also promoted ischemic tolerance; when combined with HPC, even greater (and dimethylsphingosine-reversible) protection was noted.

Conclusions—These findings indicate hypoxia-sensitive increases in SphK2 activity may serve as a proximal trigger that ultimately leads to sphingosine-1-phosphate-mediated alterations in gene expression that promote the ischemia-tolerant phenotype. Thus, components of this bioactive lipid signaling pathway may be suitable therapeutic targets for protecting the neurovascular unit in stroke. (Stroke. 2009;40:3342-3348.)

Key Words: bioactive lipids ■ endothelium ■ focal stroke ■ neuroprotection ■ neurovascular unit

Cerebral ischemic tolerance is the resistance of cerebral tissue to ischemia/reperfusion injury that is transiently induced after a preconditioning stimulus. Ischemia/reperfusion injury can be characterized by many interconnected pathologies, including inflammation, loss of vascular barrier integrity, reactive oxygen species production, and apoptosis.1 The ischemia-tolerant brain is protected against these injurious mechanisms through numerous adaptive responses triggered by preconditioning stimuli2-4 such as hypoxia; hence, in vivo preconditioning models can be used to identify endogenous factors responsible for these protective effects.2-6 Although the mechanisms underlying preconditioning and ischemic tolerance are recognized as potential therapeutic targets for the treatment of stroke,2,3,5,6 the induction and expression phases of cerebral ischemic tolerance still require considerable elucidation. Targeting the induction phase may be particularly advantageous to stimulate multiple protective pathways with a single trigger.

Given the lack of successful stroke treatments resulting from neuronally oriented protection efforts, an expanded focus on the role of vascular mechanisms and the protection of the neurovascular unit after stroke has been advocated.7-10 Sphingosine kinase (SphK) and its product sphingosine 1-phosphate (S1P) are vascular, particularly endothelial cell, mediators that regulate calcium mobility, migration, proliferation, and permeability.11-13 S1P also activates signaling molecules implicated in the induction of cerebral ischemic tolerance such as Akt and endothelial nitric oxide synthase,14-16 suggesting a potential role for S1P signaling as an early inducer of the gene expression changes promoted by preconditioning. Although delayed preconditioning in the isolated mouse heart depends on SphK1 activation,17 divergent amino acid sequences, substrate specificities, and expression patterns of the two main SphK isoforms indicate unique, tissue-specific roles for each.18

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Although SphK1 has greater expression and activity than SphK2 in many tissues, in the brain—and particularly in cerebral microvascular endothelial cells—SphK2 expression levels are greater than SphK1, pointing to more prominent physiological roles for SphK2 in the brain and brain vasculature.

Therefore, we sought to ascertain the dependence of hypoxic preconditioning (HPC)-induced cerebral ischemic protection on microvascular SphK activity in an adult mouse model of transient focal stroke. To test this hypothesis, we assessed temporal protein expression and activity patterns for each SphK isoform in microvascular isolates early after HPC, during the induction phase of preconditioning. A causal role for SphK signaling in establishing an ischemia-tolerant phenotype was studied by inhibiting SphK activity during HPC and determining the ability of a SphK2-specific sphingosine analog to mimic HPC. Our results indicate a role for microvascular SphK2 signaling inducing cerebral ischemic tolerance, thereby providing a definitive therapeutic target for neurovascular unit protection in stroke.

Materials and Methods

Animals

Two hundred twenty-four adult male Swiss-Webster ND4 mice (Harlan Laboratories, Indianapolis, Ind) were used in this study as follows: 45 for SphK protein expression, 68 for SphK activity analyses, 8 for verification of in vivo SphK2 inhibition with dimethylsphingosine (DMS), and 103 for in vivo ischemia experiments, as detailed in Table 1. Animals were housed on a 12-hour light/dark cycle with water and food ad libitum. Efforts were made to reduce the number of mice used and minimize stress to the animals. All experimental procedures were approved by the Washington University Animal Studies Committee.

In Vivo Hypoxic Preconditioning

Mice were preconditioned with 4-hour systemic hypoxia by transferring them to new cages continually flushed with 8% oxygen (balance nitrogen). Control mice underwent sham preconditioning (sham HPC) by transferring the mice to a new cage flushed with normoxic air for 4 hours. HPC preceded transient ischemia by 48 hours.

Pharmacological Treatments

The nonselective SphK inhibitor DMS (0.33 mg/kg, intravenously) was administered by retro-orbital injection alone or immediately before the injection of the SphK2-specific substrate, FTY720 (0.24 or 1.0 mg/kg, intraperitoneally), 30 minutes before HPC. For mice not subjected to HPC, pharmacological treatment preceded transient ischemia by 48 hours.

Transient Focal Cerebral Ischemia

A 60-minute transient middle cerebral artery occlusion (tMCAO) was induced in mice anesthetized with halothane as previously described. Blood flow through the middle cerebral artery was measured by laser Doppler flowmetry. Mice retaining >15% of baseline perfusion during ischemia and mice that did not reach at least 50% of baseline by 5 minutes postreperfusion or 70% after 24 hours were excluded. In addition, animals showing evidence of intracerebral bleeding or subarachnoid hemorrhage on brain extraction were excluded from the study. The exclusion criteria used to eliminate mice from further consideration are tabulated in Table 1. Experimental conditions were intermixed to prevent batch effects from affecting a single experimental condition.

Infarct and Edema Quantification

Infarct volume was delineated 24 hours after tMCAO with 2,3,5-triphenyl tetrazolium chloride staining using an intensity threshold in Sigma Scan Pro 4. Total infarct volume was corrected for edema. Edema was quantified as the increase in ipsilateral hemispheric volume relative to the contralateral hemisphere.

Neurological Deficit Scoring

Neurological deficit was scored 24 hours after reperfusion, immediately before euthanasia, as described previously.

Microvessel Isolation

Cortical samples collected at 0, 1, 2, 4, and 24 hours after HPC were snap-frozen and microvessel-rich homogenates were prepared as described previously with a few modifications: 5% phosphatase inhibitor buffer (Active Motif, Carlsbad, Calif) was added to the sucrose buffer; the initial 1000 g spin was not repeated; and the final 200 g spin was 2 minutes. The microvessel-enriched pellet was washed with 0.01 mol/L phosphate-buffered saline, spun 2 minutes at 8000 g, the supernatant discarded, and the pellet frozen at −80°C.

Immunoblotting

Microvessel-rich homogenates were sonicated in lysis buffer (Cell Lysis Buffer [Cell Signaling, Danvers, Mass], Pefabloc [Roche, Indianapolis, Ind], Pefabloc Plus [Roche], EDTA-free protease inhibitor solution [Roche], and phosphatase inhibitor [Active Motif]), and SphK1 and SphK2 protein expression was detected using commercially available antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif).

SphK Activity Assay

Microvessel-rich homogenates were sonicated in lysis buffer (50 mmol/L HEPES, pH 7.4, 10 mmol/L KCl, 20% glycerol, 2 mmol/L dithiothreitol, 15 mmol/L NaF, 2 mmol/L semicarbazide, 0.1 mmol/L leupeptin, 0.1 mmol/L pepstatin, 0.1 mmol/L aprotinin) and assayed for SphK activity using the commercial SphK2 assay kit (Cayman, Ann Arbor, Mich) using 600 μg protein and 60 μmol/L FTY720. Total protein in the homogenates was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif).
Table 2. Physiological Variables (Mean±SEM) of Mice Subjected to tMCAO

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Age, weeks</th>
<th>Body Mass, g</th>
<th>Ischemic CBF, %</th>
<th>5 Minutes Reperfusion CBF, %</th>
<th>Infarct Volume, mm³</th>
<th>Neurological Deficit (0–4)</th>
<th>Ipsilateral Edema, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham/HPC</td>
<td>13±2</td>
<td>31±2</td>
<td>8±1</td>
<td>89±3</td>
<td>86±6</td>
<td>70±6</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>HPC</td>
<td>11±2</td>
<td>32±1</td>
<td>9±2</td>
<td>89±4</td>
<td>97±5</td>
<td>29±4</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>DMS/HPC</td>
<td>14±1</td>
<td>30±1</td>
<td>7±1</td>
<td>95±10</td>
<td>99±10</td>
<td>70±14</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>DMS</td>
<td>16±1</td>
<td>33±1</td>
<td>8±1</td>
<td>77±7</td>
<td>93±7</td>
<td>61±11</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Low FTY720</td>
<td>15±2</td>
<td>32±1</td>
<td>8±2</td>
<td>82±6</td>
<td>101±7</td>
<td>57±8</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>FTY720/HPC</td>
<td>11±0</td>
<td>31±1</td>
<td>8±1</td>
<td>77±9</td>
<td>90±7</td>
<td>38±8</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>DMS/FTY720/HPC</td>
<td>14±1</td>
<td>31±1</td>
<td>8±3</td>
<td>94±8</td>
<td>103±7</td>
<td>13±4</td>
<td>0.9±0.2</td>
</tr>
</tbody>
</table>

HPC Uregulates SphK2, but Not SphK1, Protein Expression and Activity

SphK1 and SphK2 protein expression in microvessels in vivo was measured at various times over the first 24 hours after HPC. No change in microvascular SphK1 was revealed (Figure 1A), whereas SphK2 increased after HPC, exhibiting a 1.7±0.2-fold increase peaking at 2 hours (Figure 1B). Because protein expression does not necessarily reflect the activity of the enzymes, isoform-specific SphK activity was measured. Like with protein expression, microvascular SphK1 activity was unchanged after HPC (Figure 1C), but SphK2 activity changes after HPC showed the same trend seen with SphK2 protein expression, albeit with a slightly higher increase in peak activity (2.5±0.6-fold over sham HPC) than protein expression (Figure 1D).

HPC-Induced Ischemic Protection Depends on SphK

To test if the increase in SphK2 protein expression and activity is vital to HPC-induced ischemic protection, we used our adult mouse model of tMCAO and inhibited the activity of SphK during HPC with DMS. First, we confirmed that DMS given intravenously would inhibit the increase in
SphK2 activity seen after HPC (shown in Figure 1D). DMS given 30 minutes before HPC reduced peak SphK2 activity 2 hours after HPC by approximately 50% (n=4, data not shown).

We then documented that DMS before HPC completely abolished (P<0.05) the reduction in infarct volume provided by this preconditioning stimulus (Figure 2A). Improvements in neurological deficit scores (45% reduction relative to sham HPC) obtained with HPC (Figure 2B) were also fully blocked (P<0.05) by SphK inhibition during HPC, consistent with the hypothesis that HPC-induced protection requires SphK activity. Finally, the significant reduction in postischemic ipsilateral edema by HPC (71% reduction; Figure 2C) was partially lost when SphK was inhibited during HPC by DMS (48% reduction).

FTY720 Mimics Preconditioning and Provides Synergistic Protection With HPC

As a pharmacological mimic for HPC-induced SphK activation, we administered the SphK2 substrate and sphingosine analog FTY720—which, when activated by SphK2-mediated phosphorylation, then serves as an S1P analog—48 hours before ischemia in the absence of HPC. FTY720 provided dose-dependent protection across all 3 end points when used as a preconditioning mimic (Figure 3). When the low dose of FTY720 was administered concomitant with HPC, robust, synergistic tolerance was evidenced. DMS blocked (P<0.05) this protection, consistent with our contention that an HPC-induced increase in SphK2 activity mediates the induction of ischemia-protective effects. Similarly, postischemic neurological deficits and ipsilateral edema were also reduced by FTY720/HPC.
Several studies have measured indices of cerebrovascular protection induced by preconditioning. Lipopolysaccharide preconditioning abrogated the ischemia-induced impairment of endothelium relaxation in arterioles,28 possibly through increased endothelial nitric oxide synthase expression,15 thereby contributing to the preservation of microvascular perfusion after ischemia.29 Preconditioning was also documented to reduce posts ischemic ipsilateral edema.30 Our observation that inhibiting SphK activity during preconditioning reversed the reduction in edema normally afforded by HPC, and the ability of the SphK2 substrate FTY720 to reduce posts ischemic edema when given as a preconditioning mimic, implicates SphK—most likely the SphK2 isoform—as playing a vasculoprotective as well as a neuroprotective role.

Although the mechanisms by which HPC induces ischemic tolerance are not well understood, the prominent role of SphK2 signaling in microvascular endothelium early after HPC suggests a number of S1P-mediated possibilities. S1P may protect the vasculature by reducing leukocyte adhesion secondary to altering endothelial adhesion molecule expression32 and preventing endothelial apoptosis through Bcl2 activation.31 There is also evidence that S1P may act as a proximal trigger of cerebroprotection (both neuronal and vascular) through activation of signaling molecules such as Akt and endothelial nitric oxide synthase.14–16 Our data also demonstrate a SphK2 signaling-dependent decrease in ipsilateral edema resultant from HPC before ischemic insult. Although S1P has not been directly implicated in blood–brain barrier maintenance, it can decrease endothelial permeability32 through multiple mechanisms, including regulating pericyte interactions33 and adherens junction formation.12,34 S1P was also shown to enhance endothelial barrier integrity by stimulating ZO-1/α-catenin interactions at cellular junctions.35 The wealth of previous work showing that S1P signaling improves vascular barrier integrity suggests preservation of the blood–brain barrier mediated by S1P-driven transcriptional or posttranslational modifications may be a primary mechanism contributing to preconditioning-induced ischemic tolerance. Further studies of the mechanistic basis of S1P-mediated ischemic protection in brain, particularly at the level of the blood–brain barrier, are needed to document these vascular-based protective effects.

Our expression and activity analyses suggest that it is the SphK2 isoform that is involved in mediating HPC-induced ischemic tolerance in contrast to the findings in other organs such as the heart in which ischemic protection is mediated by SphK1 signaling.17 The increase in SphK2 protein expression and activity we observed after hypoxia fits with previous studies in the brain that showed, unlike in other tissues, only SphK2 mRNA increased after ischemia, whereas SphK1 mRNA remained unaffected.20 Hypoxia is also documented to increase SphK2 activity in other cells as well.36 At higher doses, the SphK2 substrate FTY720 served as a preconditioning mimic, whereas at a lower dose, FTY720 concomitant with HPC exhibited a synergistic effect, promoting robust ischemic tolerance are not well understood, the prominent role of SphK2 signaling in microvascular endothelium early after HPC suggests a number of S1P-mediated possibilities. S1P may protect the vasculature by reducing leukocyte adhesion secondary to altering endothelial adhesion molecule expression32 and preventing endothelial apoptosis through Bcl2 activation.31 There is also evidence that S1P may act as a proximal trigger of cerebroprotection (both neuronal and vascular) through activation of signaling molecules such as Akt and endothelial nitric oxide synthase.14–16 Our data also demonstrate a SphK2 signaling-dependent decrease in ipsilateral edema resultant from HPC before ischemic insult. Although S1P has not been directly implicated in blood–brain barrier maintenance, it can decrease endothelial permeability32 through multiple mechanisms, including regulating pericyte interactions33 and adherens junction formation.12,34 S1P was also shown to enhance endothelial barrier integrity by stimulating ZO-1/α-catenin interactions at cellular junctions.35 The wealth of previous work showing that S1P signaling improves vascular barrier integrity suggests preservation of the blood–brain barrier mediated by S1P-driven transcriptional or posttranslational modifications may be a primary mechanism contributing to preconditioning-induced ischemic tolerance. Further studies of the mechanistic basis of S1P-mediated ischemic protection in brain, particularly at the level of the blood–brain barrier, are needed to document these vascular-based protective effects.

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supports our conclusion that increased SphK2 activity/expression after HPC is responsible for inducing an ischemia-tolerant phenotype in brain.

Despite these findings, the abrogation of HPC-induced tolerance with the nonspecific inhibitor DMS does not allow us to completely rule out the participation of SphK1, and the lack of an isoform-specific inhibitor precluded such an experiment. Studies in SphK1 and SphK2 knockout mice would confirm with more certainty an isoform-specific role for SphK2 in cerebral ischemic tolerance. Although SphK protein expression and activity were measured specifically in the microvasculature, our results also cannot rule out a role for SphK2 signaling from nonmicrovascular cell types, because SphK inhibition through intravenous delivery of DMS presumably inhibits SphK activity in multiple cell types. Evidence of SphK2 stimulation in neurons and astrocytes after ischemia\(^{20}\) suggests a cooperative or synergistic signaling among the various cell types may also occur in response to HPC. In fact, Blondeau et al showed SphK2 upregulation 24 hours after brief ischemia in cultured neurons and astrocytes, but not in endothelial cells,\(^{20}\) which, together with our data, may suggest the possibility of a temporarily based paracrine effect, in which endothelial cells provide an early increase in SphK2 activity that ultimately leads to later activation of the same enzyme in neurons and astrocytes.

**Summary**

Our findings indicate that, unlike in myocardium, an increase in SphK2 expression and activity after HPC participates in establishing the reductions in infarct size and edema associated with ischemic tolerance. These findings point to a new signaling pathway for the treatment of stroke. Elucidation of the SphK2-associated signal transduction pathways that ultimately promote the ischemia-tolerant phenotype could collectively serve as therapeutic targets for neurovascular unit protection in the patient with stroke.

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**Disclosures**

None.

**References**


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