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Hypoxic preconditioning induces stroke tolerance in mice via a cascading HIF, sphingosine kinase, and CCL2 signaling pathway

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Abstract

The induction of ischemic tolerance by preconditioning provides a platform to elucidate endogenous mechanisms of stroke protection. In these studies, we characterize the relationship between hypoxia-inducible factor (HIF), sphingosine kinase 2 (SphK2), and CCL2 in models of hypoxic or pharmacological preconditioning-induced ischemic tolerance. A genetics-based approach using SphK2- and CCL2-null mice showed both SphK2 and CCL2 to be necessary for the induction of ischemic tolerance following preconditioning with hypoxia, the hypoxia-mimetic cobalt chloride, or the sphingosine-1-phosphate (S1P) agonist FTY720. A pharmacologic approach confirmed the necessity of HIF signaling for all three preconditioning stimuli, and showed the SphK/S1P pathway to transduce tolerance via the S1P₁ receptor. Additionally, our data suggest significant cross-talk between HIF and SphK2-produced S1P signaling, which together act to upregulate CCL2 expression. Overall, HIF, SphK, S1P, and CCL2 participate in a signaling cascade to induce the gene expression responsible for the stroke-tolerant phenotype established by hypoxic and FTY720 preconditioning. The identification of these common molecular mediators involved in signaling the genomic response to multiple preconditioning stimuli provides several targets for therapeutic manipulation.

Keywords

bioactive lipids; sphingosine-1-phosphate; neuroprotection; chemokines

Introduction

The brain possesses endogenous mechanisms capable of defending itself against ischemic damage. These innate survival pathways can be activated by a paradigm known as preconditioning, whereby exposure to noninjurious stressors trigger changes in gene and protein expression that lead to an ‘ischemia-tolerant’ phenotype. Experimentally, the preconditioning model represents an elegant tool for increasing our understanding of the pathways and mechanisms of endogenous neuroprotection. Hypoxic preconditioning (HPC) is a well-established approach for inducing tolerance to focal and global cerebral ischemia in both neonates and adults. Moreover, HPC not only protects the brain and other tissues from ischemic insults, it also increases resistance to other types of injury as well. Thus, protective mechanisms induced by HPC may provide therapeutic targets for disease intervention beyond just stroke.

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The HPC stimulus is typically considered to be propagated by the accumulation/activation of the transcription factor hypoxia-inducible factor (HIF), which binds to hypoxia-responsive elements (HREs) in the promoters of genes. In normoxic conditions, proline residues on the alpha subunits of HIF are continuously hydroxylated by an oxygen-dependent prolyl hydroxylase, and then ubiquitinated by pVHL, which labels it for ongoing rapid degradation by the proteasome. However, inhibition of the HIF prolyl hydroxylase under hypoxic conditions, or by metal chelating “HIF mimetics” like cobalt chloride, stabilize HIF and, in turn, promote the transcription of dozens of genes, the protein products of which enhance cell survival in severely hypoxic or ischemic conditions. Prominent HIF-regulated cytoprotective pathways include vascular endothelial growth factor (VEGF), erythropoietin, and glucose metabolism (Aragones *et al.* 2009, Correia & Moreira 2010, Semenza 2009).

One molecule that accumulating evidence suggests is regulated by HIF is sphingosine kinase (SphK) (Anelli *et al.* 2008, Ralph *et al.* 2004, Schnitzer *et al.* 2009, Schwalm *et al.* 2008). Recently, in an adult mouse model of focal stroke, we identified the sphingosine kinase 2 (SphK2) isoform as a necessary mediator of HPC-induced tolerance, in part because of its early upregulation after HPC (Wacker *et al.* 2009). SphK2 is one of two SphK isoforms that catalyze the phosphorylation of sphingosine to form sphingosine 1-phosphate (S1P), a potent signaling lipid. S1P can then act intracellularly, or extracellularly via autocrine or paracrine activation of five S1P receptors (S1P₁₋₅), to regulate a diverse set of cellular metabolic processes. Each S1P receptor induces unique signaling effects, and thus the cell- and tissue-dependent expression of S1P receptors typically determines the effect S1P will exhibit. Using SphK2-null mice, we have demonstrated that SphK2/S1P signaling is critical to inducing ischemic tolerance in response to HPC (Wacker *et al.* 2012), a finding recently corroborated by others (Yung *et al.* 2011). We also recently demonstrated that FTY720, an S1P receptor agonist that is phosphorylated by SphK2, serves as an effective preconditioning stimulus (Wacker *et al.* 2009), further underscoring a role for S1P signaling in stroke tolerance. However, the intermediate mediators of S1P signaling, the S1P receptor involved, and the regulation of additional cellular processes by S1P related to preconditioning-induced ischemic tolerance, remain to be elucidated.

We recently identified the chemokine CCL2 (also known as monocyte chemoattractant protein-1 [MCP-1]) as essential to the establishment of ischemic tolerance by HPC (Stowe *et al.* 2012). It was already known that CCL2 can be produced by central nervous system (CNS) astrocytes, microglia, endothelial cells, and neurons, as well as by immune cells; it is best known for its role in inflammatory cell recruitment. However, we found CCL2 mRNA and protein were rapidly upregulated in mouse cortex following HPC, and immunohistochemistry localized this expression to neurons. The primary CCL2 receptor, CCR2, is found on most CNS cell types. CCL2/CCR2 signaling induces angiogenesis in the vasculature (Niu *et al.* 2008, Stamatovic *et al.* 2006) and protects against ischemia in the myocardium (Martire *et al.* 2003). The promoter region for CCL2 contains several binding sites for HIF, which is believed to account for the increased CCL2 expression in response to either hypoxia- or chemically-induced HIF stabilization (Mojsilovic-Petrovic *et al.* 2007). Furthermore, while studies of cultured endothelium and mast cells suggest CCL2 expression is upregulated by S1P (Chen *et al.* 2004, Oskeritzian *et al.* 2008), whether this same signaling pathway is operative in brain, particularly in the context of cerebral ischemic tolerance, is unknown. However, the necessity for both CCL2 and SphK2 in our model of HPC-induced ischemic tolerance (Yung *et al.* 2011, Wacker *et al.* 2012, Stowe *et al.* 2012) led us to hypothesize that HIF, S1P, and CCL2 are part of a signal transduction cascade responsible for the observed neurovascular protection.

Thus, we undertook the present experiments to characterize the functional interrelationships between HIF, SphK2, and CCL2 in models of preconditioning-induced ischemic tolerance. We hypothesized that following HPC, HIF accumulation causes a downstream increase in SphK2 expression, and the S1P so formed acts through the S1P₁ receptor to increase CCL2 expression, culminating in the establishment of an ischemia-tolerant phenotype. We also explored this pathway in the setting of pharmacologic preconditioning with cobalt and FTY720, and determined causality and the signaling sequence with both a genetic (SphK2 and CCL2 knockout mice) and pharmacologic (inhibitors of HIF, SphK2, and S1P₁) approach. We found that SphK2 and CCL2 signaling is required downstream of hypoxic, cobalt, or FTY720 preconditioning for the induction of stroke tolerance. Additionally, our data suggest cross-talk between HIF and SphK2-generated S1P. Our identification of key mediators participating in the induction of the gene expression changes promoting endogenous neurovascular protection provides several molecular targets for therapeutic preconditioning.

Materials and Methods

Mice

Adult, male mice, 10–15 wks old, were used in all studies. Wildtype C57BL/6 (Jackson Laboratories, SphK2 knockout, and CCL2 knockout mice were used for the genetic knockout experiments in Figure 1. SphK2^{-/-} mice, congenically backcrossed to C57BL/6 mice for at least 7 generations, were provided by Dr. Richard Proia (NIH). CCL2^{-/-}/Cx3CR1^{+GFP} mice on a C57BL/6 background were used for the CCL2-null studies, courtesy of Dr. Keiko Hirose (Washington University). SW-ND4 mice (Harlan) were used for the HIF, SphK, and S1P₁ inhibition experiments shown in Figures 2 and 3. All animals were housed on a 12-hour light/dark cycle with water and food ad libitum. Efforts were made with respect to experimental design to reduce the number of mice used and to minimize stress to the animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University and conformed to the National Institutes of Health guidelines for the care and use of animals in research and the ARRIVE guidelines. Surgical procedures were performed using sterile/aseptic techniques and experimental conditions were intermixed to prevent batch effects from affecting a single experimental condition; all analyses were blinded.

Hypoxic and Pharmacologic Preconditioning

Mice were subjected to HPC (normobaric 8% oxygen for 4 h) in modified home cages with water and food ad libitum (Wacker et al. 2009, Wacker et al. 2012, Stowe et al. 2012). Ambient oxygen concentration was confirmed through monitoring cage outflow air with an oxygen analyzer (Vascular Technology). Pharmacological preconditioning was induced by injection of the HIF stabilizer cobalt chloride (CoCl₂; 60 mg/kg, i.p.; Sigma) or the SphK2-specific substrate FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) (1.0 mg/kg, i.p.; Cayman Chemical). Naïve/control animals were not preconditioned with hypoxia, cobalt, or FTY720. All preconditioning treatments preceded focal ischemia by 48 h.

Pharmacological Inhibition of HIF, SphK, and S1P₁

R59949 (60 µg/kg in 0.1 % DMSO, i.v.; Sigma), which inhibits HIF accumulation (Temes et al. 2005), and dimethylsphingosine (DMS, 0.33 mg/kg, i.v.; Cayman Chemical), which nonselectively inhibits both SphK isoforms, were administered by retro-orbital injection 30 min prior to preconditioning. The S1P₁-specific competitive antagonist W146 (1.0 mg/kg in 0.1 % DMSO, i.p.; Cayman Chemical) was given 1 h prior to preconditioning.

Transient Focal Cerebral Ischemia

Mice were subjected to transient middle cerebral artery occlusion (tMCAO) as described previously (Miller *et al.* 2001, Stowe *et al.* 2011, Wacker *et al.* 2009, Wacker *et al.* 2012, Stowe *et al.* 2012). Briefly, under 2% halothane anesthesia, the left middle cerebral artery (MCA) was exposed by incision of the temporal muscle and baseline blood flow in the cortical MCA territory was measured by laser Doppler flowmetry (LDF; TSI, Inc.). The left common carotid artery was exposed via a ventral midline incision on the neck, and a silicon-coated, 6.0-gauge, nylon suture, 12 mm in length, was advanced 9.0–10.5 mm to transiently block the origin of the MCA. Body temperature was maintained throughout the surgical procedure by a thermoregulated heating pad, and animals were placed in a 34 °C incubator during ischemia. After either 45 min (C57Bl/6, SphK^{-/-}, and CCL2^{-/-} mice) or 1 h (SW-ND4 mice), animals were re-anesthetized, and the suture was withdrawn to reestablish cerebral perfusion. Mice were then recovered from anesthesia for 30 min in a 34 °C incubator until fully ambulatory. Cortical blood flow was again determined at the onset of ischemia and at the end of ischemia to confirm blockage of MCA flow, as well as immediately following and 24 h after suture withdrawal to confirm reperfusion. Mice retaining greater than 15% of baseline perfusion during ischemia, and mice that did not reach at least 50% of baseline flow at 5 min of reperfusion, or 70% of baseline flow at 24 h of reperfusion, were excluded. In addition, animals showing evidence of intracerebral bleeding or subarachnoid hemorrhage upon brain extraction were excluded from the study.

Quantification of Infarction Volume

These endpoints were measured as described in our previous publications (Wacker *et al.* 2012), but only infarct volume data are provided in this study. In brief, at 24 h of reperfusion following tMCAO, neurological deficits were assessed, and then the mice were killed by cervical dislocation, their brains removed, and 1-mm thick coronal sections obtained from a matrix were exposed to a 1% solution (in PBS) of 2,3,5-triphenyl tetrazolium chloride to delineate infarct and noninfarcted brain regions. Edema-corrected infarct volumes were quantified using Sigma Scan Pro 4.

CCL2 Immunoblotting

Animals were sacrificed 6–12 h following preconditioning. Whole cell cortical homogenates lysed in RIPA buffer were immunoblotted using standard protocols. 85 µg of protein/well was loaded and gels were incubated overnight in primary antibody solution (1:1000, CCL2 [Abcam]). IRDye secondary antibody (1:10,000; LI-COR) was imaged and quantified using the LI-COR Odyssey Infrared Imaging System.

Statistical Analyses

Comparisons were done with SigmaStat using 2-way ANOVA for Figure 1 and 1-way ANOVA for Figure 2 and 3. Statistical comparisons between groups were done with the Holm-Sidak method for multiple pair-wise comparisons with $p < 0.05$ accepted as significant.

Results

In experiments from our laboratory that were previously published, we identified both SphK2 (Wacker *et al.* 2012) and CCL2 (Stowe *et al.* 2012) as necessary participants in HPC-induced ischemic tolerance (Figure 1A). As partial confirmation of these findings, and to distinguish between the HIF-dependent and HIF-independent effects of HPC with respect to SphK2 and CCL2 signaling, we assessed whether tolerance induced by the hypoxia-mimetic cobalt chloride also required SphK2 and CCL2. Cobalt induces the accumulation of HIF by blocking the VHL degradation of HIF through either inactivating HIF prolyl hydroxylases

(chelates Fe in catalytic site) (Epstein et al. 2001), or direct binding of HIF (Yuan *et al.* 2001, Yuan *et al.* 2003). As with HPC, the reduction in infarct volume resulting from cobalt preconditioning was prevented in both SphK2- and CCL2-null mice (Figure 1B), implying HIF is upstream of SphK2 and CCL2. Then, given our previous findings that SphK2 protein expression and activity increased 2–4 h after HPC (Wacker et al. 2009), while CCL2 mRNA and protein expression increased 12–24 h after hypoxia (Stowe et al. 2012), we sought to determine whether CCL2 was downstream of SphK2 signaling. To do this, we preconditioned mice using FTY720, a SphK2-specific sphingosine analog that, after becoming phosphorylated by SphK2, acts as an S1P analog by binding to and activating S1P₁, S1P₃, S1P₄, and S1P₅ (but not S1P₂) receptors (Brinkmann *et al.* 2002, Mandala *et al.* 2002). As mentioned earlier, we previously demonstrated that FTY720 was efficacious as a preconditioning drug in our model of transient focal stroke (Wacker et al. 2009). As predicted, in SphK2-null mice, without the enzyme to phosphorylate FTY720, FTY720 preconditioning was completely ineffective in inducing tolerance (Figure 1C). Moreover, FTY720 preconditioning also was impotent as a preconditioning stimulus in CCL2 knockout mice, indicating that CCL2 elaboration is downstream of S1P signaling in the establishment of an ischemia-tolerant phenotype in response to FTY720 preconditioning.

To further elucidate the signaling sequence responsible for the induction of ischemic tolerance following preconditioning with these same three agents, we used pharmacological inhibitors of HIF, SphK, and S1P₁. Neither the PBS nor DMSO vehicles used for solubilizing the inhibitors affected preconditioning with any of the stimuli (data not shown). As shown in Figure 2A, when preconditioning with hypoxia, the typical (~45%) reduction in infarct volume (tolerance) is blocked by the administration of R59949, an inhibitor of both the HIF-1 and HIF-2 isoforms, prior to HPC. R59949 functions by activating the HIF prolyl hydroxylases causing VHL degradation of both HIF isoforms (Temes et al. 2005). The protection induced by HPC is also abrogated if the activity of both SphK isoforms is inhibited by dimethylsphingosine (DMS) given prior to HPC. Finally, HPC-induced tolerance is blocked by the administration of W146, a competitive antagonist of the S1P₁ receptor. Collectively, these findings indicate that HPC acts through HIF and SphK, ultimately leading to an S1P-induced activation of the S1P₁ receptor, in the induction of ischemic tolerance. The ischemic tolerance induced by preconditioning with cobalt, as reflected by a 49% decrease in infarct volume, is also blocked by R59949, DMS, or W146 administered prior to cobalt (Figure 2B). These findings demonstrate that cobalt preconditioning requires HIF1 and/or HIF2, and the SphK-mediated production of S1P, which then, by activating the S1P₁ receptor, induces the changes in gene expression required for establishing the ischemia-tolerant phenotype.

As shown in Figure 2C, preconditioning with the SphK2-specific S1P analog FTY720 resulted in a 33% decrease in infarct volume, consistent with our previous results (Wacker et al. 2009). FTY720-induced tolerance was blocked by DMS, confirming that phosphorylation of FTY720 by SphK2 is necessary for this effect. The efficacy of FTY720 preconditioning was lost in R59949-treated mice, indicating that enhanced HIF-1 and/or HIF-2 expression is a necessary step in preconditioning signaling by FTY720, just as it is with hypoxia and cobalt. Finally, that W146 also blocked FTY720-induced protection indicates that it is the binding of phosphorylated FTY720 to the S1P₁ receptor that mediates the epigenetic response.

While our aforementioned genetic and pharmacologic interventions indicated that CCL2 signaling occurs downstream of HIF activation/stabilization, these experiments did not confirm whether S1P receptor activation is needed for HPC- and FTY720-preconditioning-induced CCL2 expression. Thus, we first measured expression changes in cortical CCL2 protein levels in naïve and HPC-treated mice. As seen previously (Stowe et al. 2012), CCL2

protein expression was significantly upregulated 12 h following HPC. Treating animals with R59949 to inhibit HIF accumulation during HPC significantly blocked the HPC-induced increase in CCL2 protein (Figure 3A). In addition, administering W146 prior to HPC to prevent S1P signaling through the S1P₁ receptor also abrogated the elevation in CCL2 protein expression induced by HPC (Figure 3A). These results, in parallel with those shown in Figure 1A and 2A, indicate that the HPC-induced increase in CCL2 expression is HIF-dependent. Moreover, they demonstrate that the activation of the S1P₁ receptor is required for the elevated CCL2 expression occurring in response to HPC.

We then repeated these CCL2 protein expression measurements following preconditioning with FTY720 (Figure 3B). CCL2 protein expression increased significantly in response to FTY720, with the highest expression observed at 6 h following FTY720 injection. Administration of R59949 prior to FTY720 preconditioning prevented this increase, as did W146 dosing prior to FTY720 preconditioning. These results demonstrate that, even when initiating the preconditioning induction pathway at the stage of S1P signaling, the activation of HIF by FTY720 is required for CCL2 elaboration, as is the FTY720-induced activation of the S1P₁ receptor; in turn, such findings reveal a significant degree of cross-talk or positive feedback between S1P and one or both HIF isoforms that defines this signaling cascade.

Discussion

The mechanisms by which preconditioning signals the gene expression required for ischemic tolerance remain to be fully elucidated. Using genetic and pharmacologic approaches, results from the present study indicate that SphK2 signaling and CCL2 production are both required for inducing the genomic reprogramming responsible for endogenous ischemic tolerance in response to HPC, as well as in response to cobalt preconditioning and preconditioning with the sphingosine analog FTY720. The involvement of CCL2 protein was also confirmed by measuring elevated levels of this cytokine following HPC and FTY720 preconditioning. Furthermore, we demonstrated that HIF is a required part of the signaling cascade induced by these preconditioning stimuli, as is activation of the S1P₁ receptor. Cross-talk or feed-forward interactions between S1P and HIF aid in the upregulation of CCL2 and is necessary for successful preconditioning. Together, our findings implicate HIF, SphK, S1P₁, and CCL2 in the induction of ischemic tolerance by hypoxia, as well as by FTY720.

Previously published findings on the interaction of HIF and SphK (or S1P) are varied and seemingly conflicting, but likely due, in large part, to the cells/tissues examined, as well as which SphK and HIF isoforms were under investigation (Schnitzer et al. 2009, Ralph et al. 2004, Ader *et al.* 2008, Ader *et al.* 2009, Anelli et al. 2008, Cho *et al.* 2011, Cu villier & Ader 2011, Pchejetski *et al.* 2011, Schwalm et al. 2008). With respect to SphK being under the control of the transcriptional and metabolic regulator HIF, it was reported that SphK1 mRNA was upregulated (identified as part of a larger microarray study) in hypoxia-stressed neurons overexpressing HIF-2 α (Ralph et al. 2004). A HIF-2 α -mediated upregulation of SphK1 was later corroborated in U87MG glioma cells in response to hypoxia or chemical stabilization of HIF (Anelli et al. 2008). Moreover, in the human endothelial cell line EA.hy926, upregulation of SphK1, but not SphK2, gene transcription by both HIF-1 α and HIF-2 α was demonstrated following hypoxia (Schwalm et al. 2008). Finally, a role for HIF in regulating the hypoxia-induced expression of SphK2 was advanced in studies of A549 lung cancer cells (Schnitzer et al. 2009). HIF regulation of SphK1 and SphK2 is not unexpected, as both isoforms contain HREs in their respective promoter regions. Collectively, these studies suggest that, while HIF-mediated upregulation of SphK is common in many cell types, the isoform dependence of both appears to be cell- and tissue-specific. Our present results indicate that, in the mouse cortex, the expression and/or activity

of SphK2 is upregulated secondary to the stabilization of HIF levels by hypoxia or cobalt. Because the HIF inhibitor that we used, R59949, is nonspecific (Temes et al. 2005), our findings do not allow for the identification of the specific HIF isoform that regulates SphK2. However, given the well-established rapid increase in HIF-1 α that occurs in response to hypoxia (within minutes) (Jewell *et al.* 2001), and our previous finding that SphK2 is upregulated within 2–4 h of HPC (Wacker et al. 2009), the HIF-1 α isoform is implicated in this signaling cascade.

Surprisingly, inhibition of HIF blocked FTY720-induced tolerance, suggesting HIF is downstream of S1P-related signaling. Several recent studies on the interaction of HIF and SphK in a variety of cell/tissues suggest bi-directional cross talk between these molecules, with SphK signaling also facilitating HIF stabilization and thus HIF-induced changes in gene expression. In particular, evidence indicates that SphK1 regulates HIF-1 α expression and activity in several tumor cell lines (PC-3 prostate cancer cells, A549 lung cancer cells, SUM-159PT breast adenocarcinoma cells, and U87 glioblastoma cells) secondary to stabilizing the HIF-1 α isoform under hypoxic conditions (Ader et al. 2008). Consistent with such a finding, melatonin suppresses HIF-1 α accumulation by inhibiting SphK1 activity in PC3 cells (Cho et al. 2011), and LPS induces a Toll-like receptor 4-mediated accumulation of HIF-1 α secondary to SphK1 upregulation in macrophages (Pchejetski et al. 2011). However, given that S1P induces HIF-1 α in vascular endothelial and smooth muscle cells (Michaud et al. 2009), and that S1P is produced by both SphK isoforms, promotion of HIF stabilization may not be unique to the SphK1 isoform. Indeed, recent reports show elevations in cerebral HIF-1 α protein in response to isoflurane preconditioning were not observed in SphK2-null mice (Yung et al. 2011), demonstrating a SphK2-specific upregulation of HIF. Likewise, our results support a SphK2-specific signaling pathway in mouse brain. Thus, taken together, the prevailing evidence for bi-directional signaling between HIF and SphK-derived S1P is consistent with our current genetic and pharmacologic results indicating that SphK2 activity appears to be both downstream of HIF (with cobalt preconditioning being ineffective in SphK2-null or DMS-treated mice) and upstream of HIF (with preconditioning initiated at the stage of S1P signaling with FTY720 being ineffective when HIF is inhibited). This perplexing finding may be explained by the existence of a positive feedback loop from S1P to HIF that functions either to further increase the expression of both molecules, or to extend the time frame over which the increased expression of both molecules persists; such an interaction may represent one of many positive feedback loops regulating HIF activity (Prabhakar & Semenza 2012). Alternatively, this finding could represent paracrine signaling wherein HIF-induced S1P released from one cell-type acts on neighboring cells to increase their expression of HIF. From our findings, we contend that cross-talk between HIF and S1P is integral to the gene expression changes induced by HPC.

In previous work from our laboratory, we utilized CCL2-null mice to demonstrate that HPC-induced tolerance to transient focal stroke was CCL2-dependent (Stowe et al. 2012). This result was surprising, given that previous studies had indicated pharmacologic inhibition or genetic knockout of CCL2 protected against stroke, particularly in a permanent ischemia model. In the present study, we confirmed that CCL2-null mice were not protected against transient ischemia when preconditioned with hypoxia, cobalt, or FTY720, and extended these findings to document that HIF activation secondary to preconditioning with either hypoxia or the hypoxia-mimetic cobalt is required for the resultant CCL2 production. Such a dependency is not unexpected, given that CCL2 is also a hypoxia-responsive, HIF-dependent gene (Chao *et al.* 2010, Marisa *et al.* 2005, Mojsilovic-Petrovic et al. 2007, Xu *et al.* 2007). Because FTY720 is not phosphorylated/activated by the SphK1 isoform (Paugh et al. 2003), our additional findings herein that FTY720 also elevated CCL2 expression in a HIF-dependent manner, and that the FTY720 preconditioning-induced reduction in infarct

volume was HIF-dependent, underscore the aforementioned bi-directional regulatory link between HIF and SphK2-derived S1P.

Finally, based on our results with the S1P₁-specific receptor antagonist W146 (Zhu *et al.* 2010, Sanna *et al.* 2006), we also advance the contention that the upregulation in CCL2 expression by S1P, as well as by phosphorylated FTY720, occurs through this bioactive lipid receptor. Studies in peripheral tissues already support an S1P-mediated upregulation of CCL2 (Lin *et al.* 2006, Oskeritzian *et al.* 2008, Yopp *et al.* 2004). In macrophages, this upregulation was reported to be dependent on the S1P₃ receptor (Keul *et al.* 2011), in mast cells, the S1P₂ receptor (Oskeritzian *et al.* 2010), and in peripheral endothelial cells, both the S1P₁ and S1P₃ receptors (Lin *et al.* 2007). Although we did not assess the effects of other S1P receptor-specific antagonists, our documentation that W146 completely abrogated ischemic tolerance induced by hypoxia, cobalt, and FTY720, as well as robustly inhibited the upregulation of CCL2 protein by hypoxic and FTY720 preconditioning, would suggest a prominent role for the S1P₁ receptor in brain, and may serve to highlight again the unique tissue/cell-dependent regulatory features of S1P signaling. Which cells in brain express this receptor is also not addressed by the present study, or the specific cell types that respond to hypoxia, cobalt, and FTY720 with a HIF-dependent production of S1P. But given our previous immunohistochemical finding that neurons and endothelial cells upregulate CCL2 expression in a time-dependent fashion in response to HPC (Stowe *et al.* 2012), we would contend that the S1P so formed in response to these preconditioning stimuli acts in an autocrine and/or paracrine manner to generate CCL2. In turn, CCL2 produced in neurons and endothelial cells could signal gene expression changes in these respective cells in an autocrine manner and/or CCL2 released by these cells could, in a paracrine fashion, induce adaptive changes in gene expression in neighboring glial and vascular smooth muscle cells. Given CCL2's prototypical role as a monocyte chemoattractant, perhaps monocytes also participate in this inter-cellular signaling as well. Clearly, further studies are warranted to elucidate the nature of such CCL2-regulated epigenetic responses.

In conclusion, based on the genetic and pharmacologic studies presented herein, we propose a novel signaling cascade for preconditioning-induced cerebral ischemic tolerance (Figure 4). Both hypoxic and cobalt preconditioning stabilize HIF; most likely, it is the HIF-1 α , and not the HIF-2 α , isoform that is involved initially, given the isoform-specific kinetics for HIF revealed in other studies (Jewell *et al.* 2001, Stroka *et al.* 2001, Holmquist-Mengelbier *et al.* 2006) and the rapid timecourse we reported previously for SphK2 upregulation/activity in response to HPC (Wacker *et al.* 2009). Then, by a mechanism yet to be defined, HIF-1 α facilitates SphK2 protein expression and/or activity leading to an increase in S1P formation. This S1P then acts in an autocrine and/or paracrine fashion through the S1P₁ receptor to upregulate CCL2 mRNA and protein expression. While HIF-mediated, survival-promoting changes in gene expression are well-described, at this juncture it remains unclear how CCL2 regulates the genomic reprogramming required for ischemic tolerance. S1P signaling also serves in another yet-to-be-detailed manner to stabilize HIF-1 α and/or HIF-2 α through autocrine- or paracrine-based cross-talk, further enhancing this signaling cascade. Preconditioning by the sphingosine analog FTY720 transduces this epigenetic response network similarly: After being phosphorylated by SphK2, its activation of the S1P₁ receptor also drives the production of CCL2. Additionally, activated FTY720 signaling feeds back to promote HIF-dependent signaling, another step vital to inducing ischemic tolerance. The extent to which this overall signaling cascade is activated by other well-established preconditioning stimuli is presently unclear. Although intentional hypoxic gas breathing is used by some athletes for training purposes, and although FTY720 is clinically approved as a treatment for patients with multiple sclerosis, the continued elucidation of preconditioning signaling pathways as we have done here provides an expanded number of downstream

genomic reprogramming effector molecules that one might therapeutically target (Simon *et al.* 2012) to induce tolerance in humans.

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Abbreviations

HIF	hypoxia inducible factor
CCL2	chemokine (C-C motif) ligand 2
S1P	sphingosine 1-phosphate
FTY720	2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride
HPC	hypoxic preconditioning
HRE	hypoxia response element
pVHL	von Hippel–Lindau protein
VEGF	vascular endothelial growth factor
SphK	sphingosine kinase
CCR2	chemokine (C-C motif) receptor 2
CNS	central nervous system
LDF	laser Doppler flowmetry
CoCl₂	cobalt chloride
DMSO	dimethyl sulfoxide
PBS	phosphate buffered saline
D M S	N, N-dimethylsphingosine
W146	3-amino-4-(3-hexylphenylamino)-4-oxobutyl phosphonic acid
R59949	3-[2-[4-(bis(4-Fluorophenyl)methylene)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone
RIPA	radioimmunoprecipitation assay
tMCAO	transient middle cerebral artery occlusion
mRNA	messenger ribonucleic acid

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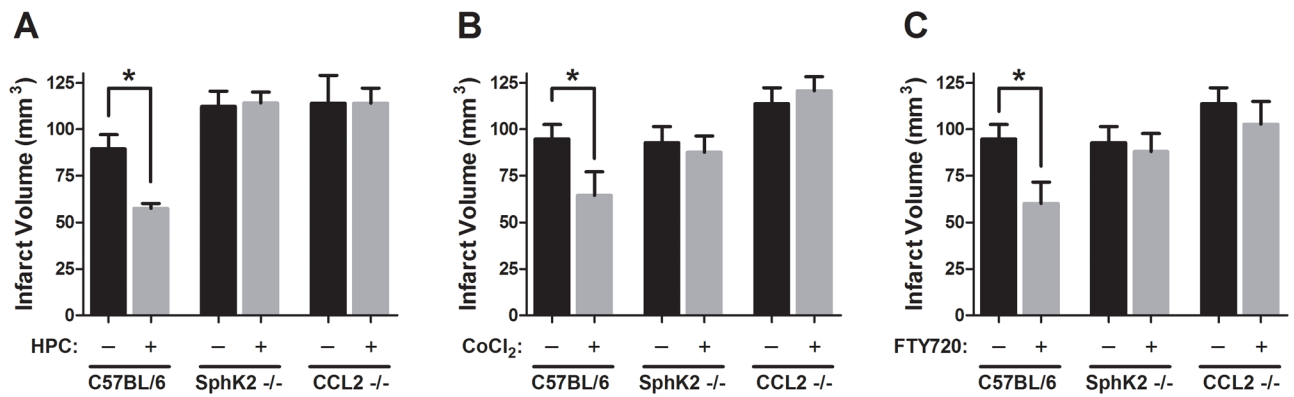


Figure 1. The requirement for SphK2 and CCL2 for preconditioning for ischemic tolerance by hypoxia, cobalt, and the sphingosine analog FTY720

(A) Previously published data showed that hypoxic preconditioning (HPC) reduces infarct volume 24 h after transient middle cerebral artery occlusion (tMCAO) in wildtype (C57BL/6) mice, and the loss of such protection in SphK2 knockout (data from (Wacker et al. 2012)) and CCL2 knockout mice (data from (Stowe et al. 2012)). (B) Cobalt (CoCl₂) preconditioning reduces infarct volume 24 h after tMCAO in wildtype (C57BL/6) mice, but this protection is not achieved in SphK2 knockout or CCL2 knockout mice. (C) FTY720 preconditioning also reduces infarct volume 24 h after tMCAO in wildtype (C57BL/6) mice, but as with hypoxia and cobalt, tolerance is not realized in SphK2 knockout or CCL2 knockout mice. Data represent mean±SEM. N=5–13 per group. (*) P<0.05 between groups.

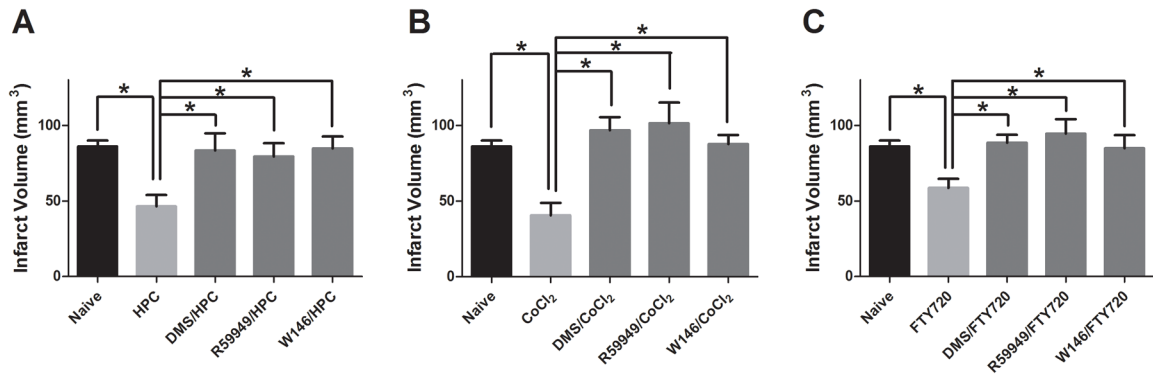


Figure 2. The requirement for SphK-, HIF-, and S1P₁-based signaling in preconditioning by hypoxia, cobalt, and FTY720

(A) Hypoxic preconditioning (HPC) reduces infarct volume 24 h after transient middle cerebral artery occlusion (tMCAO) in naive mice, but this protection is blocked by the inhibition of HIF (R59949), SphK (DMS), or S1P₁ (W146) just prior to HPC. Similarly, both cobalt (CoCl₂) preconditioning (B) and FTY720 preconditioning (C) reduce infarct volume 24 h after tMCAO in naive mice, but this protection is also blocked when HIF (R59949), SphK (DMS), or S1P₁ (W146) are inhibited prior to preconditioning. Data represent mean±SEM. N=4–18 per group. (*) P<0.05 between groups.

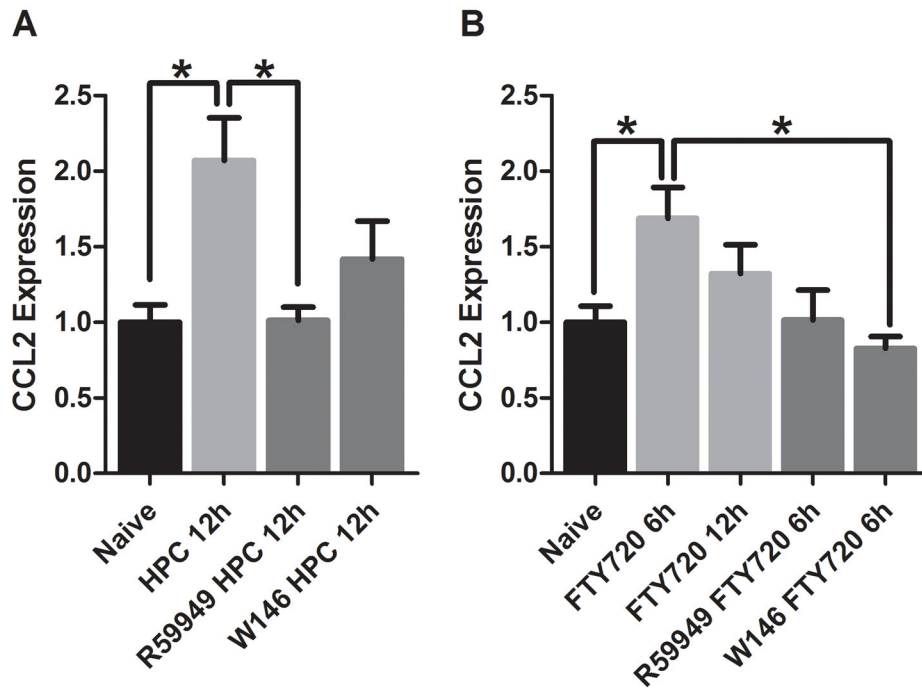


Figure 3. HIF- and S1P₁-based regulation of CCL2 expression in response to preconditioning by hypoxia and FTY720

(A) HPC increases CCL2 protein expression when measured 12 h after the cessation of HPC, but this increase is attenuated if both HIF isoforms are inhibited during HPC by R59949 or if the S1P₁ receptor is antagonized during HPC by W146. (B) FTY720 increases CCL2 protein expression 6 h after FTY720 administration, but this increase is abrogated by inhibition of HIF prior to FTY720 administration with R59949, or antagonism of S1P₁ prior to FTY720 administration with W146. Data represent mean±SEM. N=4–8 per group. (*) P<0.05 between groups.

Primary Responding Cell

Secondary Responding Cell

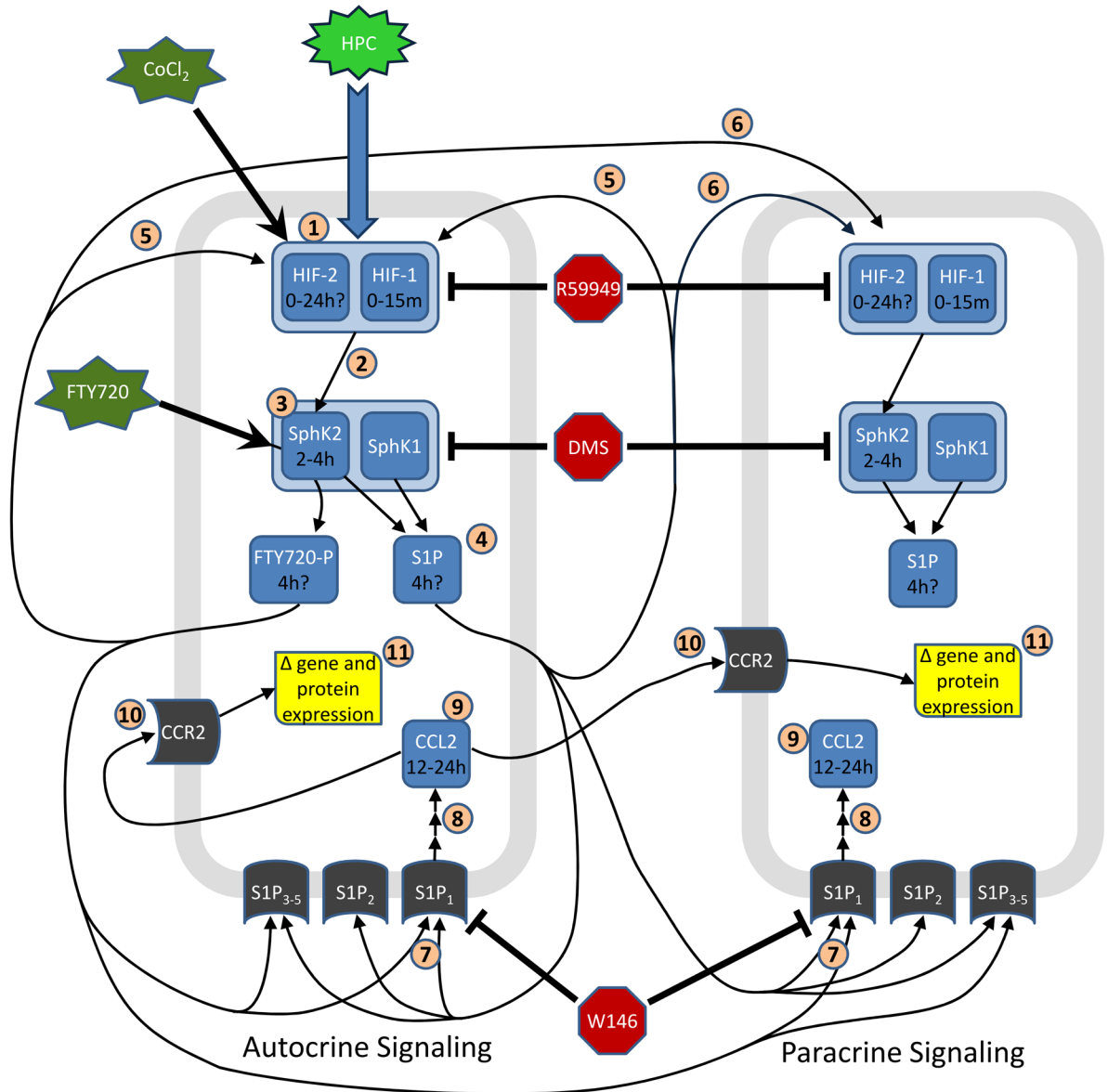


Figure 4. Proposed signaling cascade for preconditioning by hypoxia, cobalt, and FTY720
 “Working hypothesis” schematic showing the proposed signaling pathways and molecules involved in the ischemic tolerance achieved by the different preconditioning stimuli tested in this study (green), as well as the pharmacologic manipulations used (red) to elucidate this cascade. Both primary and secondary responding cells are shown to highlight the potential autocrine and paracrine actions of S1P, FTY720-P, and CCL2. Estimated time periods over which the expression of some of the primary mediators in the signaling cascade changes are also shown. Evidence for the inclusion of each numbered molecule or numbered interaction in this signaling pathway is derived from the present study, or others, as follows: [1] Extensive literature supports an increase in HIF expression in response to hypoxia or CoCl_2

treatment (Bergeron *et al.* 2000, Yuan *et al.* 2003, Yuan *et al.* 2001, Semenza 2009, Jewell *et al.* 2001, Semenza 2001, Lahiri *et al.* 2006). [2] Wacker *et al.* (2009) showed that HPC leads to a microvascular SphK2 increase 2–4 h later, and that inhibition of this increased activity blocks HPC-induced protection. [3] HPC-, CoCl₂-, and FTY720-induced protection is blocked in SphK2-null mice (Figure 1). [4] Inhibition of SphK2 activity blocks preconditioning (Figures 1 and 2), so presumably S1P or FTY720-P formation is required for this process. The timing of increased microvascular SphK2 activity shown in Wacker *et al.* (2009) suggests S1P increases around 4 h after HPC. [5] FTY720 preconditioning is blocked by HIF inhibition (Figure 2C), indicating it acts on HIF either via an autocrine feedback loop, or [6] via paracrine signaling on another cell. [7] Competitive antagonism of S1P₁ with W146 blocked preconditioning-based protection by HPC, CoCl₂, and FTY720 (Figure 2). [8] Increased CCL2 expression following HPC or FTY720 treatment did not occur when the S1P₁ receptor was antagonized with W146 (Figure 3). [9] CCL2-null mice exhibited larger baseline infarcts than naïve mice following tMCAO (Figure 1). Stowe *et al.* (2012) showed increases in CCL2 expression 12–24 h following HPC, primarily in neurons. [10] CCR2 is the predominant receptor for CCL2, so the involvement of this receptor in CCL2-mediated preconditioning is presumed. However, paradoxically, CCR2-null mice have reduced baseline infarcts relative to naïve mice (Dimitrijevic *et al.* 2007); this latter effect may be due to the activity of a different CCR2 ligand, or maybe CCL2-mediated protection functions via a receptor other than CCR2. [11] Synthesis of new proteins secondary to changes in gene expression is known to be required for establishing the preconditioning-induced, ischemia-tolerant phenotype (Gidday 2006, Sharp *et al.* 2004).