Preconditioning Stimuli Induce Autophagy via Sphingosine Kinase 2 in Mouse Cortical Neurons*

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Background: Preconditioning provides insights into endogenous mechanisms that could be used to protect brain from injury.

Results: Preconditioning stimuli up-regulate sphingosine kinase 2, leading to autophagy.

Conclusion: Sphingosine kinase 2 mediates autophagy and preconditioning, possibly by disrupting Beclin 1/Bcl-2 interaction.

Significance: The discovery of new signaling independent of SPK2 catalytic activity provides medicinal chemists with novel “druggable” targets important for neuroprotection.

Sphingosine kinase 2 (SPK2) and autophagy are both involved in brain preconditioning, but whether preconditioning-induced SPK2 up-regulation and autophagy activation are linked mechanistically remains to be elucidated. In this study, we used in vitro and in vivo models to explore the role of SPK2-mediated autophagy in isoflurane and hypoxic preconditioning. In primary mouse cortical neurons, both isoflurane and hypoxic preconditioning induced autophagy. Isoflurane and hypoxic preconditioning protected against subsequent oxygen glucose deprivation or glutamate injury, whereas pretreatment with autophagy inhibitors (3-methyladenine or KU55933) abolished preconditioning-induced tolerance. Pretreatment with SPK2 inhibitors (ABC294640 and SKI-II) or SPK2 knockdown prevented preconditioning-induced autophagy. Isoflurane also induced autophagy in mouse in vivo as shown by Western blots for LC3 and p62, LC3 immunostaining, and electron microcopy. Isoflurane-induced autophagy in mice lacking the SPK1 isoform (SPK1−/−), but not in SPK2−/− mice. Sphingosine 1-phosphate and the sphingosine 1-phosphate receptor agonist FTY720 did not protect against oxygen glucose deprivation in cultured neurons and did not alter the expression of LC3 and p62, suggesting that SPK2-mediated autophagy and protections are not S1P-dependent. Beclin 1 knockdown abolished preconditioning-induced autophagy, and SPK2 inhibitors abolished isoflurane-induced disruption of the Beclin 1/Bcl-2 association. These results strongly indicate that autophagy is involved in isoflurane preconditioning both in vivo and in vitro and that SPK2 contributes to preconditioning-induced autophagy, possibly by disrupting the Beclin 1/Bcl-2 interaction.

Preconditioning is a procedure by which a noxious stimulus is applied to a tissue or organ below the threshold of damage and induces tolerance to the same or different subsequent noxious stimuli given above the threshold of damage (1, 2). Studying cerebral preconditioning may provide insight into endogenous protective mechanisms that could be exploited therapeutically. Known preconditioning stimuli include inhalational anesthetics, hypoxia, brief ischemia, cortical spreading depression, and proinflammatory agents. Isoflurane, used widely and safely in surgical procedures, induces tolerance to ischemia in many organs, including brain (1).

In the central nervous system, sphingosine 1-phosphate (S1P) regulates multiple cellular processes, including proliferation, survival, and migration of neurons (3). Intracellular S1P levels are regulated by the expression and activity of sphingosine kinases (SPKs), which have been shown to play a role in preconditioning of the heart (4–7), kidney (8), and brain (9). We previously found that SPK2, but not SPK1, mediates hypoxia- and isoflurane-induced brain preconditioning, possibly via hypoxia-inducible factor-1α (9), but the mechanisms involved were not elucidated.

Autophagy is a regulated process for the removal of cellular proteins and damaged organelles (10, 11). Autophagy is induced during preconditioning in heart (12, 13) and is involved in ischemic preconditioning of neurons and rat brain (14, 15). We thus hypothesized that isoflurane and hypoxic preconditioning stimuli...
tioning might also induce autophagy in an SPK2-dependent manner to protect neurons.

**EXPERIMENTAL PROCEDURES**

The experiments were conducted according to protocols approved by the Animal Research Committee of Massachusetts General Hospital and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

*Primary Culture of Mouse Cortical Neurons*—Embryonic day 15 and 16 embryos of CD1 mice were collected, and their brains were harvested in sterile PBS. Cortices were dissected, freed from meninges and choroid plexus, minced, and digested in trypsin. The action of trypsin was stopped with DMEM containing 10% fetal bovine serum, and tissues were homogenized by trituration with a pipette, passed through a cell strainer, and spun down. Pellets were resuspended in Neurobasal medium (Invitrogen; 21103) with L-glutamine, B27 supplement (Invitrogen; 17504-044), and penicillin/streptomycin; centrifuged; resuspended in Neurobasal medium; and plated onto polyethyleneimine-coated 6-well (6×10^5 cells/well) or 24-well (1.0×10^5 cells/well) dishes (16).

*In Vitro Isoflurane Preconditioning (ISO) and Hypoxic Preconditioning (HP) Model*—After 7 days in culture, neurons were exposed to 2% isoflurane (Abbott Laboratories; 26675-46-2) for 30 min in an airtight chamber and harvested 6, 12, 24, and 48 h later. For HP, neurons were exposed to 4% oxygen for 8 h in an airtight chamber and harvested 12, 24, 48, and 72 h later. These conditions were based on previous reports and did not induce significant neuronal toxicity (17–19).

*Cell Viability Analysis*—Cell death was induced by OGD or exposure to glutamate 24 h after exposure to ISO, or 48 h after exposure to hypoxia. To induce Glu toxicity, neurons were treated with 10 μM L-glutamic acid (Sigma; 49449) for 5 min (drugs prepared in medium), washed and placed in fresh prewarmed Neurobasal medium. For OGD, cultures were washed three times with N2-bubbled Hank’s balanced salt solution and placed in an airtight chamber aerated with 95% N2/5% CO2 for 4 h. Cells were then removed from the anaerobic chamber, washed and then placed in Neurobasal medium. Cell viability was quantified by MTT assay 24 h after OGD or Glu exposure. Neurons were incubated in 200 μg/ml thiazolyl blue tetrazolium bromide (MTT; Sigma; M2128) at 37 °C for 2 h. Culture medium was aspirated, and cells were lysed in 200 μl of DMSO. Color intensity was measured at 570 nm using a Victor3 plate reader (PerkinElmer Life Sciences). The results are expressed as a percentage of absorbance of control wells. Separate cultures of neurons were fixed with 4% paraformaldehyde for 10 min, and the nuclei were stained with Hoechst 33342; cells undergoing cell death were characterized by condensed nuclei, and the percentages of healthy-looking cells were counted in a blinded fashion in four random fields.

*Isoflurane Preconditioning in Mice*—Male C57BL/J mice (23–28 g, 6–8 weeks of age; Charles River, Wilmington, MA) and age-matched wild-type, SPK1−/−, and SPK2−/− mice were maintained on a 12-h light/12-h dark cycle and fed ad libitum. The mice were randomly allocated to treatment groups: they were exposed to 1% isoflurane (in 70% N2 and 30% O2) for 3 h in an airtight chamber, recovered in an incubator (at 28 °C) for ~30 min, and then returned to their cage (9, 20), whereas control mice were placed in the airtight chamber flushed with air for the same duration of time. For Western blot analysis, 6, 24, or 48 h after isoflurane exposure, mice were euthanized and perfused transcardially with cold PBS. The cortex, striatum, and hippocampus were harvested and frozen immediately.

*Western Blot Analysis*—Samples from *in vivo* and *in vitro* experiments were homogenized, and the total proteins were extracted. Protein concentrations were determined by Bradford assay. A 30-μg (*in vivo*) or 20–30-μg (*in vitro*) aliquot of proteins from each sample was loaded. Western blot analysis was performed to detect LC3 (1:1000; Abcam; ab62721), p62 (1:500; Enzo; BML-PW9860), and SPK2 (1:100; Abcam; ab37977) expression. Expression levels were normalized to β-actin (1:10,000; Sigma; A2228).

*LC3 and SPK2 Immunofluorescence in Neurons*—Cortical neurons were grown onto coverglasses in 24-well plates. After treatment, they were fixed for 15 min using 4% paraformaldehyde and incubated with PBS containing 0.1% Triton X-100 for 30 min. After blocking with 2% BSA for 1 h at room temperature, the cells were then incubated with antibodies against LC3 (1:800; Novus; NB600-1384) or SPK2 (1:500; Abgent; AP 7238a) at 4 °C for 24 h, and with Cy3-conjugated anti-rabbit IgG (1:400; Jackson ImmunoResearch; 711-165-152) for 2 h. Afterward, the cells were incubated with 0.5 μg/ml 4,6-diamidino-2-phenylindole (Sigma; D9564) for 10 min and mounted on slides, and images of fluorescence were acquired.

*LC3 Immunohistochemistry in Mice*—Twenty-four hours after ISO, mice were perfused with PBS followed by PBS containing 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight. Forty-micron-thick coronal sections were cut with a vibratome. Sections were blocked in 5% normal goat serum, 1% BSA, 0.2% Triton X-100, 0.03% H2O2 in PBS for 1 h at room temperature and incubated with anti-LC3-antibody (1:800; Novus; NB600-1384) for 48 h at 4 °C; after washing with PBS, they were incubated with biotinylated anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch; 711-065-152) for 2 h at room temperature, washed, and incubated with ABC reagent (Vector ABC kit; PK-6100) for 90 min at room temperature. After washing, sections were stained with dianisobenzidin (Vector Laboratories; SK-4100) for 1–2 min, washed, and sealed with a coverslip (21).

*Transmission Electron Microscopic Examination*—Twenty-four hours after ISO, mice were perfused with PBS followed by PBS containing 2% paraformaldehyde/2% glutaraldehyde. The brains were postfixed overnight in PBS containing 2% paraformaldehyde/2% glutaraldehyde. Fifty-micron-thick coronal sections were cut with a vibratome. The sections were incubated in 1% osmium tetroxide for 1 h, dehydrated in graded ethanol, incubated in 1% uranyl acetate for 1 h, dehydrated in graded ethanol, and embedded in epon. Polymerization was performed at 60 °C for 24 h. Based on our immunohistochemistry results, layer V (internal pyramidal layer) of the parietal cortex was selected for analysis. Blocks were cut on an ultramicrotome (50 nm) and examined using a JEOL 1011 electron microscope. To
RESULTS

Autophagy Contributes to the Neuroprotection Elicited by ISO and HP in Cortical Neurons—Activation of autophagy was first examined in primary cultured mouse cortical neurons by immunoblotting LC3 and p62 (28, 29). The LC3II/LC3I ratio was increased after ISO (Fig. 1A), whereas p62 was down-regulated (Fig. 1B), with maximal effects observed at 24 h. SPK2 was also up-regulated after ISO, and the peak SPK2 levels were seen 12–24 h after ISO (Fig. 1C). LC3 and SPK2 up-regulation was confirmed by immunofluorescence (Fig. 2). Hypoxia, the other preconditioning stimulus, also increased LC3II/LC3I ratio and down-regulated p62 in neurons (Fig. 1, D and E), but maximal effects were seen at 48 h after HP, with a corresponding peak in SPK2 expression at 24–48 h (Fig. 1F).

Either 4-h oxygen/glucose deprivation (OGD) or 5-min exposure to Glu decreased cell viability (Fig. 3, A and B). ISO greatly attenuated OGD- or Glu-induced cell death. Pretreatment with 3-MA or KU55933, at concentrations known to effectively block autophagy (10 μM and 2 μM) (22, 23), abolished ISO-induced protection both in the OGD and the Glu models. Hypoxia also induced tolerance to OGD or Glu (Fig. 3, C and D), in a 3-MA- and KU55933-sensitive manner. The degree of cell death was also quantified by Hoechst 33342 staining, providing results similar to MTT (thiazolyl blue tetrazolium bromide) measurements (data not shown). Although 3-MA and KU55933 both abolished HP-mediated neuroprotection against OGD, only KU55933 significantly inhibited HP-mediated tolerance against glutamate, whereas the inhibition seen in the presence of 3-MA did not reach statistical significance. In contrast, both 3-MA and KU55933 abolished preconditioning by isoflurane, against the effects of OGD and glutamate toxicity. This could be due to the fact HP induces higher levels of SPK2 (Fig. 1) and induces a more robust neuroprotection (9), which might therefore be more difficult to inhibit using autophagy inhibitors. In control experiments (not shown), we established that cortical neurons were unaffected by either 10 mM 3-MA or 2 μM KU55933, added alone; we also ruled out possible neuroprotective effects of these agents (in the absence of preconditioning), finding similar cell viability when neurons were treated with 3-MA, KU55933, or their vehicle 24 h before exposure to OGD or Glu.

SPK2 Inhibition Prevents Preconditioning-induced Autophagy in Cortical Neurons—To explore whether SPK2 is involved in preconditioning-induced autophagy, we used two SPK2 inhibitors, SKI-II (4-[4-(4-chlorophenyl)-thiazol-2-yl-amino]-phenol) and ABC294640 (3-(4-chlorophenyl)-adaman-tane-1-carboxylic acid (pyridin-4-yl-methyl) amide), on cortical neurons. SKI-II is a specific SPK inhibitor but does not discriminate between isoforms, whereas ABC294640 is an SPK2-selective inhibitor (24, 25). We have previously shown that these inhibitors abolish ISO-induced tolerance both in vivo and in vitro (9). In the present study, isoflurane significantly increased the LC3II/LC3I ratio and decreased p62 levels (Fig. 4, A and B), whereas pretreatment with 1 μM SKI-II or 10 μM ABC294640 reduced LC3II/LC3I ratio and restored p62 levels. Because high concentrations of ABC294640 (50 μM) or the SPK inhibitor SKI-I have been reported to activate autophagy in

quantify the number of double-membrane vacuolar structures, four mice in each group and 25 neurons from each block were examined in a blinded manner. The number of large double-membrane vacuolar structures (typical of autophagosomes) was counted in lower magnification images of randomly selected neurons, and the autophagosomal nature of the structures was confirmed using higher magnification images. Cortical neurons were identified by their large, round, and light nucleus with obvious nucleolus; they often contained randomly scattered rosettes of RNA particles and dispersed profiles of endoplasmic reticulum and could be recognized by the presence of neural filaments.

Co-immunoprecipitation—Twenty-four hours after ISO, neurons were harvested and lysed in radioimmune precipitation assay buffer. The lysates were precleared with protein A/G-agarose (Santa Cruz; sc-2003) for 1 h, incubated with anti-Bcl-2 antibody (Santa Cruz; sc-7382) overnight, and then subjected to immunoprecipitation with protein A/G-agarose for 3 h. The immunoprecipitates were analyzed by immunoblot with anti-Beclin antibody (Santa Cruz; sc-11427).

Drug Treatment—Cortical neurons were pretreated with autophagy inhibitors 3-methyladenine (3-MA; Sigma; M9281) (22) or KU55933 (Tocris; 3544) (23) or SPK inhibitors SKI-II (Cayman, 10009222) (24) or ABC294640 (Apogee Biotechnology Corporation, Hummelstown, PA) (25) 30 min before preconditioning. Neurons were treated with autophagy inducer rapamycin (Sigma; R0395) (15), S1P (Avanti Polar Lipids; 860942P), or the S1P receptor agonist FTY720 (Novartis Pharma AG, Basel, Switzerland) (26) for 24 h.

siRNA—Neurons were transfected on day 6 in vitro. Four μl of Lipofectamine 2000 (Invitrogen) was diluted in 200 μl of Opti-MEM (Invitrogen) at room temperature and 5 min later was combined with mouse SPK2 siRNA1 (sense: 5’-GAGCAUGGAAACCACUUCATT-3’, antisense: 5’-UGAAGUGGUAUUAAGCUCTT-3’), SPK2 siRNA2 (sense: 5’-GCUCUGCUAUUAUGGUCAATT-3’, antisense: 5’-UGAACCUAUAGGCAGCCTT-3’) (80 nM; Genepharma, Shanghai, China), Beclin 1 siRNA1 (sense: 5’-GGAGUGGAUUAGAAUAUACTT-3’, antisense: 5’-AUUAAUUCUCAUCCACCTT-3’), or Beclin 1 siRNA2 (sense: 5’-GAAUCCUGACGGGGUACCTT-3’, antisense: 5’-GGUGACCCGGUACAGACTT-3’) (40 nM). In all experiments, the neurons were also transfected with a control scrambled RNA targeting a sequence not sharing homology with the mouse genome (negative control (NC); sense: 5’-UUCUCGGAACGUGUCAGUTT-3’, antisense: 5’-ACGUGACAGUUCGAGAATT-3’) in 200 μl of Opti-MEM. Incubation was continued for 20 min at room temperature, and the mixture was applied to culture wells (27).

Statistical Analysis—All assessments were performed in a blinded fashion. For in vivo experiments, mice were randomly allocated. The number of mice in each group was based on power analysis assuming a treatment effect of 30% and an S.D. of 25%. The data are expressed as means ± S.D. Statistical analysis was carried out by one-way analysis of variance, followed by the Newman-Keuls multiple-comparison tests. p < 0.05 was considered to be significant.
tumor cells or mouse embryonic fibroblasts, resulting in autophagic or apoptotic cell death (30, 31), we also treated neurons with SPK inhibitors alone. LC3II/LC3I ratio and p62 levels were not altered by 10 \( \mu \)M ABC294640 or 1 \( \mu \)M SKI-II, suggesting that they have no direct effect on autophagy under our experimental conditions. As with ISO, pretreatment with ABC294640 or SKI-II abolished the changes in LC3II/LC3I ratio and p62 induced by HP (Fig. 4, C and D). To confirm these data obtained with drug inhibitors, we also transfected neurons with SPK2 siRNA and found that SPK2 siRNA prevented ISO-mediated increases in the LC3II/LC3I ratio (Fig. 5). Taken together, these results suggest that SPK2 mediates preconditioning-induced autophagy.

**Isoflurane Preconditioning (ISO) Induces Autophagy in Vivo**—To ascertain the in vivo significance of our findings, we examined LC3 and p62 expression in the cortex, striatum,
and hippocampus of C57 mice 6, 24, and 48 h after exposure to isoflurane. The LC3II/LC3I ratio was significantly increased in cortex at 24 h, whereas p62 was down-regulated, with peak effects observed at 24 h in cortex and striatum (Fig. 6, A and B). Other changes in immunoblots did not reach statistical significance (Fig. 6), but the fact that these time-related increases in LC3II/LC3I ratios were consistently observed in the three brain regions examined and were mirrored by time-related decreases in LC3 levels (also consistent between brain regions) strongly suggests that isoflurane induces autophagy in vivo. We also evaluated autophagy by visualizing LC3 immunoreactivity with immunofluorescence and diaminobenzidin staining in cortex 24 h after ISO. In control mice, LC3 immunoreactivity in cortex was low. Strong LC3 staining in cortical neurons was observed in mice exposed to ISO (Fig. 6C). Many LC3-positive neurons showed a punctate pattern of immunofluorescence (data not shown), suggesting induction of autophagy. We then used electron microscopy to evaluate ultrastructural changes and autophagosome formation in cortical neurons. Neurons in control cortex appeared normal with relatively healthy-looking organelles and nuclei (Fig. 6D). Twenty-four hours after ISO, neuron organelles and nuclei also seemed normal without appreciable injury, but some engulfment of cytoplasmic materials by double-membrane vacuolar structures was found, suggesting possible autophagy induction after ISO. Quantitative analysis showed that 32.5 ± 6.8% of cortical neurons had double-membrane vacuolar structures in the control group, whereas 62.0 ± 4.8% of neurons showed these structures in the ISO group (p = 0.011; Fig. 6E), confirming that ISO induces autophagy not only in primary neurons but also in vivo.

Preconditioning-induced Autophagy Activation Is Absent in SPK2 Knock-out Mice—To expand on our in vivo data and confirm that SPK2 is involved in preconditioning-induced autophagy in vivo, we used SPK1−/− (32) and SPK2−/− (33) mice. Because of the limited number of available mice, in some cases we only observed trends without reaching statistical significance, but we did observe that in WT mice, ISO significantly increased LC3II/LC3I ratio and decreased p62 in cortex or striatum at 24h (Fig. 7, C and D), whereas these changes were not seen in SPK2 knock-out mice. In contrast, LC3II/LC3I ratio and p62 expression in WT and

![Image](image-url)
SPK1−/− mice did not differ at 24 h after ISO (Fig. 7, A and B). These results suggest that the SPK2, but not the SPK1 isoform is involved in ISO-induced autophagy.

**SPK2 Inhibition Abolish ISO-induced Disruption of Beclin 1/Bcl-2**—To determine whether the preconditioning effect of SPK2 depends on its catalytic activity, we examined whether S1P or the S1P receptor agonist FTY720 protects neurons against OGD-induced cell death. OGD induced significant cell injury (Fig. 8A), which neither S1P (1 or 3 μM) nor FTY720 (30 or 100 nM) were able to prevent, indicating a lack of direct neuroprotective effect by these agents. S1P or FTY720 did not alter LC3II/LC3I ratio and p62 levels, suggesting that neither S1P nor FTY720 has direct effects on autophagy (Fig. 8, B and C). Pretreatment with SKI-II and ABC294640 had no effect on basal SPK2 levels but significantly reduced preconditioning-induced SPK2 up-regulation in cortical neurons (Fig. 8D). Taken together, these results suggest that autophagy activation mediated by SPK2 during preconditioning may be independent of its catalytic activity.

To determine the role of Beclin 1, we knocked it down in neurons using two siRNA sequences (Fig. 9A). Both siRNAs prevented ISO-mediated increases in the LC3II/LC3I ratio (Fig. 9B), suggesting that ISO preconditioning induces autophagy via Beclin 1. Considering that SPK2 is a BH3-only protein that induces cell death when overexpressed in different cell types (34), we hypothesized that SPK2 might disrupt the interaction between Bcl-2 and Beclin 1 by a mechanism previously described for the atypical BH3-only proteins BNIP3/BNIP3L (35). We therefore quantified Bcl-2/Beclin 1 association by co-immunoprecipitation in lysates of cortical neurons. ISO decreased the amount of co-immunoprecipitated Bcl-2/Beclin 1, whereas ABC294640 and SKI-II increased co-immunoprecipitation of Bcl-2 and Beclin 1 (Fig. 9C), indicating that ISO might disrupt the interaction between Bcl-2 and Beclin 1, and initiate autophagy, whereas SPK2 inhibitors abolish preconditioning-induced disruption of Bcl-2/Beclin 1.

**DISCUSSION**

We used two preconditioning stimuli to explore the role of SPK2 in preconditioning-induced autophagy. In primary neurons, both ISO and HP induced autophagy and tolerance to subsequent OGD- or Glu-induced injury, whereas pretreatment with autophagy inhibitors abolished this tolerance, suggesting that autophagy is involved in the preconditioning process. Pretreatment with SPK2 inhibitors abolished preconditioning-induced autophagy. ISO also increased autophagy in the cortex of wild-type C57 mice but only induced autophagy in SPK1−/− mice, not in SPK2−/− mice.

Our data show increased SPK2 levels, LC3II/LC3I ratio, and down-regulation of p62 in primary neurons after preconditioning. In agreement with our in vitro data, in mice exposed to isoflurane, LC3II/LC3I ratio is increased in cortex, whereas p62 is down-regulated in both cortex and striatum. The occurrence of autophagy was further confirmed in vivo using both LC3

*FIGURE 3. Autophagy inhibitors abolished preconditioning-induced neuroprotection in primary cultured cortical neurons.* Neurons were preincubated with 3-MA (10 mM) or KU55933 (2 μM) 30 min before the onset of ISO or HP. Twenty-four hours after ISO or 48 h after HP, neurons were exposed to OGD for 4 h or 100 μM Glu for 5 min and cultured again under normal conditions for 24 h. Cell injury was evaluated by MTT assay. 3-MA and KU55933 abolished neuroprotection by ISO in the OGD (A and Glu models (B). Similarly, 3-MA and KU55933 abolished neuroprotection by HP in the OGD (C) and Glu models (D). The data are shown as means ± S.D. (n = 3 independent experiments). **, p < 0.01 versus control group; #, p < 0.05; ##, p < 0.01 compared with the OGD or Glu group; $, p < 0.05; $$, p < 0.01 versus preconditioning + OGD or preconditioning + Glu group. CON, control.
immunostaining and electron microscopy. We have previously observed up-regulated SPK2 protein expression after ISO in vivo (9); these results and the current data strongly implicate both autophagy and SPK2 in the mechanism of preconditioning. Indeed, we have found that ISO and HP protect against OGD- or Glu-induced injury, whereas pretreatment with
autophagy inhibitors 3-MA or KU55933 blocks preconditioning-induced tolerance in primary neurons. We thus conclude that activation of autophagy is essential in preconditioning and protects against cell death.

These results add to previous reports indicating that autophagy is induced by hypoxia and ischemic preconditioning in heart (12, 13), neurons or brain (14, 15, 24) and now point to SPK2 as a potential key mediator of these effects. To explore whether SPK2 is involved in preconditioning-induced autophagy, we used SPK2 inhibitors in cultured neurons. Although SKI-II is not thought to be isoform-specific, ABC294640 inhibits preferentially SPK2 (24, 25). In our study, both preconditioning paradigms increased LC3II/LC3I ratio and decreased p62, and pretreatment with SKI-II and ABC294640 reduced LC3II/LC3I ratio and restored p62 level.

We then applied genetic approaches in vivo, by using SPK1−/− and SPK2−/− mice (32, 33). We have previously observed that SPK2 predominates in different regions and cell types in the mouse brain (36). Both neuronal (9) and microvascular SPK2 (37–39) might play a role in brain preconditioning. In the present study, we showed that knocking out SPK2, but not SPK1, abolished preconditioning-induced autophagy. These data, combined with our observations in primary neurons, suggest that neuronal SPK2 plays a key role in preconditioning-induced autophagy; the role of similar pathways in other brain cell types, in particular the vasculature, remains to be investigated.

We cannot rule out that increased SPK2 activity might reduce sphingosine levels and indirectly decrease ceramide levels (because sphingosine can be converted to ceramide in ER). To the best of our knowledge, however, ceramide induces autophagy (40, 41), it is therefore unlikely that autophagy activation via SPK2 would be related to a decreased levels of ceramide. Conflicting findings have been published on the effects of S1P on autophagy in different tumor cell lines (42–44). S1P has anti-apoptotic properties in many cell types (45), whereas the agonist FTY720, which acts on four of the five known S1P

**FIGURE 6. ISO induced autophagy activation in vivo.** C57 mice were exposed to 1% isoflurane for 3 h to induce ISO. Cortex, striatum, and hippocampus were dissected 6, 24, and 48 h after ISO. A and B, the protein levels of LC3 (A) and p62 (B) were detected with immunoblotting. β-Actin levels were used as loading control. The data are shown as means ± S.D. (n = 6 mice). *, p < 0.05 versus control group. In a separate series of experiments, mice were exposed to 1% isoflurane for 3 h and decapitated 24 h later. Layer V (internal pyramidal layer) of the parietal cortex was selected for observation and analysis. C, brain sections were labeled with the anti-LC3 antibody and processed with diaminobenzindin (DAB) staining. Scale bars, 100 μm. Note that LC3 immunoreactivity was increased in ISO group. D and E, electron microscopy images show increased number of double-membrane vacuolar structures in cortical neurons of ISO mice. Scale bars, 500 nm. Arrows indicate nascent autophagosomes. N, nucleus. The data are shown as percentages of neurons displaying typical features of autophagosomes (double-membrane vacuolar structures; n = 4 mice). *, p < 0.05 versus control group. Con or CON, control.
Sphingosine Kinase 2 Mediates Autophagy

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20853

receptor subtypes, is protective in several animal models of cerebral ischemia (46). SPK2 and S1P1 receptors have been shown to participate in the signaling associated with hypoxic and FTY720 preconditioning (39), but this study did not investigate which cell type(s) express the relevant SPK2 and S1P1 receptors. To investigate potential mechanisms by which SPK2 contributes to autophagy activation, we examined the effects of S1P on autophagy and preconditioning. We found that neither tolerance nor autophagy induction by ISO were affected by FTY720 or by S1P. These negative results are in agreement with previous findings suggesting that anti-inflammatory mechanisms and vasculo-protection, rather than direct effects on neurons, underlie the beneficial effects of FTY720 in mouse stroke models (26). However, it is worth mentioning that when tested on mixed cortical cell cultures, FTY720, P-FTY720, and S1P were recently reported to be neuroprotective when applied prior to NMDA-induced cell death (47); it is unclear whether differences in cell type, noxious stimulus, and/or pre- versus post-treatment paradigms account for the difference between these and our findings.

The lack of effect of S1P on autophagy and neuroprotection suggests that the effect of SPK2 may not depend on its catalytic activity, suggesting an alternative, possibly BH3 domain-dependent, mechanism by which SPK2-mediated preconditioning might be linked to autophagy. Indeed, ISO decreased the interaction between Bcl-2 and Beclin-1, suggesting that autophagy is involved in isoflurane preconditioning both in vivo and in vitro and that preconditioning-associated SPK2 up-regulation may promote Beclin 1-dependent autophagy by disrupting association between Bcl-2 and Beclin 1. The fact that SPK2 inhibitors prevented the preconditioning-induced disruption of Beclin 1/Bcl-2 interaction would seem to invalidate this hypothesis. However, SPK inhibitors, at least for the SPK1 isoform, can also lead to proteasomal degradation of the enzyme, in addition to blocking its catalytic activity (48, 49). Indeed, in the current study, SKI-II or ABC294640 had no effect on basal SPK2 levels, but they significantly reduced preconditioning-induced SPK2 up-regulation, suggesting that these inhibitors not only block SPK2 catalytic activity but also act at the level

FIGURE 7. Isoflurane-induced autophagy activation was seen in SPK1, but not SPK2 knock-out mice. The mice were exposed to 1% isoflurane for 3 h. Cortex, striatum, and hippocampus were dissected 24 h later. Levels of LC3 and p62 were measured by immunoblotting. LC3 (A) and p62 (B) expression in SPK1−/− mice after ISO (n = 4). LC3 (C) and p62 (D) expression in SPK2−/− mice after ISO (n = 5). *, p < 0.05 versus control group. #, p < 0.05 versus ISO group. CON, control.
of SPK2 expression in neurons. Taken together, all these results indicate that SPK2-mediated autophagy activation in preconditioning may not depend on its catalytic activity. S1P-independent actions of SPK2 are not unprecedented: SPK2 regulates IL-2 pathways in T cells independently of S1P (50), and previous studies have shown that SPK2 is a BH3-only protein that induces apoptosis when overexpressed in different cell types (34, 35). BNIP3 is another BH3 domain protein that is up-regulated by hypoxia via hypoxia-inducible factor-1α; up-regulated BNIP3 displaces Beclin 1 from Bcl-2/Beclin 1 or Bcl-XL/Beclin 1 complexes, releasing Beclin 1, thereby initiating mitochondrial autophagy and decreasing reactive oxygen species production (51, 52).

The literature suggests that although hypoxia-induced up-regulation of SPK2 is protective (53), SPK2 overexpression induces apoptosis (34). Interestingly, such dual effects have similarly been reported for BNIP3/BNIP3L (35, 54). It is therefore tempting to speculate that the effect of SPK2 on cell fate might 1) be critically dependent on its levels, on the levels of interacting molecules or on the cellular environment and 2) involve a mechanism similar to that described for BNIP3/BNIP3L. Our co-immunoprecipitation experiments indeed support the notion that SPK2 is another BH3-only protein up-regulated by preconditioning that can displace Beclin 1 from Bcl-2/Beclin 1 complexes, release Beclin 1, and initiate autophagy. In addition, we found that cortical neurons transfected with Beclin 1 siRNA did not show preconditioning-mediated autophagy activation, suggesting that ISO is associated with Beclin 1-dependent autophagy.

Taken together, our results suggest that autophagy is involved in preconditioning in cortical neurons both in vivo and in vitro and that SPK2 contributes to preconditioning-induced autophagy by disrupting Bcl-2/Beclin 1 complexes. Although most current drugs act either on receptors or on enzymes, usually interacting with their ligand binding or catalytic sites (55), the discovery of new signaling properties independent of SPK2...
catalytic activity hints at novel “druggable” targets involving protein-protein interactions.

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