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Sphingosine kinase 2 mediates cerebral preconditioning and protects mouse brain against ischemic injury

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Abstract

Background and purpose—Cerebral preconditioning provides insights into endogenous mechanisms that protect the brain from ischemic injury. Hypoxia and the anesthetic isoflurane are powerful preconditioning agents. Recent data show that sphingosine 1-phosphate (S1P) receptor stimulation improves outcome in rodent models of stroke. Endogenous S1P levels are controlled by the expression and activity of sphingosine kinases (SPK). We hypothesize that SPK up-regulation mediates preconditioning induced by isoflurane and hypoxia and reduces ischemic injury.

Methods—Male wild-type C57BL/J, SPK1$^{-/-}$ and SPK2$^{-/-}$ mice were exposed to isoflurane (IsoPC) or hypoxia preconditioning (HPC) before transient middle cerebral artery occlusion. Infarct volume and neurological outcome were measured 24 hours later. SPK inhibitors (SKI-II and ABC294640) were used to test the involvement of SPK2. Expressions of SPK1, SPK2 and HIF1α were determined. Primary cultures of mouse cortical neurons were exposed to isoflurane before glutamate- or hydrogen peroxide-induced cell death.

Results—IsoPC and HPC significantly reduced infarct volume and improved neurological outcome in wild-type and SPK1$^{-/-}$ mice, but not in SPK2$^{-/-}$ mice. Pretreatment with SKI-II or ABC294640 abolished the IsoPC-induced tolerance. Western blot showed a rapid and sustained increase in SPK2 level, whereas SPK1 level was similar between preconditioned mice and controls. HIF1α was up-regulated in wild-type IsoPC mice, but not in SPK2$^{-/-}$. IsoPC protected primary neurons against cell death, which was abolished in ABC294640-treated cells.

Conclusions—Applying genetic and pharmacological approaches, we demonstrate that neuronal SPK2 isoform plays an important role in cerebral preconditioning.

Keywords

Sphingosine kinase 2; preconditioning; isoflurane; hypoxia; cerebral ischemia; neurons; cell death

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Disclosures
C.S. is CEO and President of Apogee Biotechnology Corp.

Introduction

Cerebral preconditioning is a procedure by which a noxious stimulus is applied to a tissue or organ below the threshold of damage. After a recovery period, organs such as brain develop a tolerance to the same or even different 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 isoflurane[3–5], hypoxia[6–8], cortical spreading depression[9, 10], and pro-inflammatory agents (such as lipopolysaccharide)[11, 12]. Isoflurane, an inhalational anesthetic used widely and safely in surgical procedures, induces tolerance to ischemia in many organs, including brain[5, 13, 14], heart[15] and kidney[16, 17].

Stroke is the leading cause of death and disability in developed countries. Despite the accumulating knowledge on the cellular and molecular mechanisms underlying ischemia/reperfusion injury, there is still a lack of effective treatment for stroke[18]. The sphingosine 1-phosphate (S1P) receptor agonist Fingolimod (FTY720) has been shown to be protective in several animal models of cerebral ischemia[19–21]. FTY720 is phosphorylated by sphingosine kinase (SPK) into the active compound phospho-FTY720, which then acts on four of the five known S1P receptor subtypes[22]. In the central nervous system, S1P regulates multiple cellular processes, including proliferation, survival and migration of neurons[23]. Intracellular S1P is tightly regulated by the expression and activity of SPK. Previous reports suggested that SPK plays a role in heart[24, 25], kidney[16, 17] and brain preconditioning[8]. We previously showed that SPK2 is the predominant isoform in brain[26]. The aim of this study was to test the hypothesis that SPK2 is a universal mediator of both isoflurane- and hypoxia-induced preconditioning. In order to test the hypothesis that neuronal SPK2 accounted for preconditioning, we used primary cultures of mouse cortical neurons to examine whether pre-treatment with specific SPK2 inhibitor could block IsoPC-induced protection against cell death *in vitro*.

Materials and methods

Animals

Male C57BL/J mice (23–25 g, Charles River) and age-matched wild-type, SPK1$^{-/-}$[27] and SPK2$^{-/-}$[28] mice were maintained on a 12/12 hours light/dark cycle and fed ad libitum. Experiments were conducted according to protocols approved by the Animal Research Committee of Massachusetts General Hospital and NIH guide for the Care and Use of Laboratory Animals. Mice were randomly allocated; after preconditioning or drug treatments, their identity and genotype were coded with tail marks in order to blind the investigators to the treatment groups; cerebral ischemia, infarct volumes measurement and neurological deficit evaluations were performed in a blinded fashion. Total number of mice included and mortality during surgery are summarized in supplementary tables 1 and 2.

Cerebral preconditioning

For isoflurane preconditioning (IsoPC), mice were exposed to 1% isoflurane (in 70% nitrogen and 30% oxygen) for 3 hours in an air-tight chamber. Mice were allowed to recover in an incubator (at 28°C) for ~30 minutes and then for 24 hours in their original cages[5]. For hypoxic preconditioning (HPC), mice were kept in an air-tight chamber flushed with 8% O_2 for 4 hours. Mice were allowed to recover for 72 hours[7, 8, 29]. Naïve mice were placed in the air-tight chamber flushed with air for the same duration of time.

Treatment with SPK inhibitors

Fifteen min before IsoPC, mice were administered a specific SPK inhibitor (SKI-II, Chembridge[30]) or an isoform-selective SPK2 inhibitor (ABC294640, Apogee Biotechnology Corporation[31]) at 100 mg/kg via oral gavage (dissolved in 100 μl of polyethylene glycol 400). Dosage, solvent and route of administration were based on the published pharmacokinetics[17, 30, 31].

Transient middle cerebral artery occlusion (MCAo) model

MCA was occluded for 90 min using a commercially available coated monofilament (Doccol Corporation) as reported previously[8, 21, 26] (online supplement).

Expression studies

Mice were exposed to isoflurane (1% in 70% N_2 + 30%O_2, 3 hours) and sacrificed at the following time points: immediately after isoflurane exposure (t = 0), or 1, 6, 24, 48 hours after exposure. Mice were perfused transcardially with cold saline as described before[26] (online supplement).

Primary culture of neurons and IsoPC

Primary cultures of neurons were exposed to IsoPC. The extent of glutamate-and hydrogen peroxide-induced cell death in control and preconditioned neurons was compared (online supplement).

Statistical analysis

Data are expressed as mean±SD. For infarct volumes and cell viability assay, statistical difference between groups was calculated by ANOVA. Neurological deficit score was compared using Mann-Whitney *U* test. Gene and protein expression levels were compared to control by one-way ANOVA. $p<0.05$ was considered statistically significant.

Results

IsoPC reduced infarct volumes and improved neurological outcomes

IsoPC significantly protected brain from transient MCAo, as shown in a representative TTC staining (Fig. 1A). Serial quantitative analysis of infarct volumes revealed that the induced tolerance was observed at all rostro-caudal levels (Fig. 1B), resulting in a smaller total infarct volumes in preconditioned mice (74.5±19.8 vs. 104.5±18.8 mm^3, $p<0.05$, Fig. 1C). IsoPC also improved neurological score ($p<0.05$) in mice at 24 hours after transient MCAo (Fig. 1D). Median values of neurological deficit score of naïve and preconditioned mice were 3 and 2 respectively.

Expression of SPK1 and SPK2

In isoflurane-preconditioned mice, cortical *spk2* mRNA was up-regulated (peak level of approximately 2.4 fold increase at t=0 and 1 hour) in preconditioned mice (Fig. 2A). SPK2 protein was rapidly up-regulated (about 1.7 fold increase at t=0, i.e. immediately after the 3-hour isoflurane exposure) and the peak SPK2 level was found at 1 hour after isoflurane exposure (2.7 fold increase). The up-regulated SPK2 expression was still 2.2 times higher than control at 24 hours (the time at which MCAo was induced) (Fig. 2B). In contrast, cerebral SPK1 mRNA ($p=0.467$) and protein ($p=0.053$) expression remained unchanged at the different time points examined after IsoPC.

Pharmacological approaches

We first established that infarct volumes were unaffected in naïve mice treated with a specific SPK inhibitor (SKI-II at 100 mg/kg, oral gavage) or vehicle (PEG400) 24 hours before cerebral ischemia (Fig. 3A). SKI-II treatment (15 min before isoflurane exposure) abolished the protective effect of preconditioning, resulting in infarct volumes comparable to those seen in naïve mice (111.9±22.6 vs. 107.2±12.8 mm^3 in naïve, Fig. 3A). SKI-II treatment also prevented IsoPC-induced improvement in neurological outcomes (Fig. 3B).

ABC294640 is a novel isoform-selective inhibitor for SPK2[31]. In a preliminary study, we investigated whether this compound was neuroprotective and found similar infarct volumes in mice treated with 100 mg/kg ABC294640 either 24 hours before MCAo, or 30 min after reperfusion (see supplementary figure 1).

Pretreatment with ABC294640 15 min before isoflurane exposure blocked the protective effect of IsoPC; infarct volumes were similar in IsoPC mice treated with ABC294640 and naïve mice (99.0±17.9 vs. 111.5±19.1 mm^3, Fig 3C). ABC294640 also blocked the improvement in neurological score in preconditioned mice (Fig. 3D).

Genetic tools

The circle of Willis did not show obvious differences in the three mouse strains investigated and naïve wild-type. The change in relative cerebral blood flow during MCAo and reperfusion were similar (supplementary table 3), and $SPK1^{-/-}$ and $SPK2^{-/-}$ mice had similar infarct volumes (104.5±15.3, 98.5±18.2 and 91.9±15.5 mm^3 respectively, Supplementary figure 2). IsoPC reduced infarct volumes in $SPK1^{-/-}$ mice (69.4±10.9 vs. 98.5±18.2 mm^3, $p<0.005$, Fig. 4B) by 30%, comparable to that observed in WT mice. In contrast, infarct volumes in naïve and preconditioned $SPK2^{-/-}$ mice did not differ (91.9±15.5 vs. 84.9±11.9 mm^3, Fig. 4B).

In vitro IsoPC

The extent of cell death was measured by MTT assay. Glutamate (Fig. 5A) and H_2O_2 (Fig. 5B) induced cell death in primary culture of mouse cortical neurons, which were significantly prevented by IsoPC. This protection was not observed when cells were pretreated (30 min before IsoPC) with 1 μmol/L SKI-II or 10 μmol/L ABC294640.

The degree of cell death was also quantified by Hoechst 33342 staining (Supplementary Fig. 3). Neurons with condensed nuclei (i.e. undergoing apoptosis) were counted (Fig. 5C), providing results similar to MTT measurements.

SPK2 – a general mediator for cerebral preconditioning

Hypoxic preconditioning significantly reduced infarct volumes in WT mice (64.8±26.7 vs. 104.5±15.3 mm^3, $p<0.01$, Fig. 6A), whereas infarct volumes were similar in naïve and preconditioned $SPK2^{-/-}$ mice (91.9±15.5 vs. 89.1±14.4 mm^3, Fig. 6A). Neurological outcome was significantly improved in hypoxia-preconditioned WT mice, but not in $SPK2^{-/-}$ mice (Fig. 6B). Cerebral *spk2* mRNA level was up-regulated (Fig. 6C). Western blot revealed an elevated SPK2 protein expression starting at 2 hours, and maintained up to 72 hours after HPC (Fig 6D).

Discussion

Cerebral preconditioning elicited a global neuroprotective effect and reduced infarct volumes. We observed up-regulated cerebral SPK2, but not SPK1 protein expression in preconditioned mice, suggesting that the former isoform may play a role in preconditioning.

Indeed, we also show that the reduced infarct volumes and improved neurological outcomes are absent in mice treated with a selective SPK2 inhibitor or in mice lacking SPK2. As SPK inhibition blocked IsoPC-induced tolerance in primary neurons, we conclude that up-regulation and activation of neuronal SPK2 is essential in cerebral preconditioning and protects the brain against ischemic injury and cell death.

The two SPK isoforms share high sequence homology (80% amino acid homology), yet differ in the central regions and N-termini[32]. SPK1 and SPK2 show different subcellular localizations and enzymatic properties, as well as different expression in various tissues[32]. We have previously observed that the SPK2 isoform predominates in different regions and cell types in the mouse brain[26]. But SPK1 is the more abundant isoform in renal proximal tubules and cardiomyocytes, and it was shown to be up-regulated and activated in preconditioning in the kidney and heart[16, 17, 25]. In contrast, an effect of SPK2 activation in preconditioning has been reported in one study of brain ischemia, which suggests a role of cerebral microvessel SPK2 in hypoxia preconditioning[8]. This study reported that SPK inhibition abolished the induced ischemic tolerance[8]. However the SPK inhibitor used in this study, dimethylsphingosine, is known to inhibit the SPK1 isoform[31, 33], and possibly other enzymes, such as protein kinase C[25]. Following up on this study, we used knockout mice lacking either one of the SPK isoforms and a new selective SPK2 inhibitor, ABC294640, which dose-dependently inhibits SPK2 with an IC_{50} of approximately 60 μmol/L, without affecting the activity of SPK1 at concentrations up to at least 100 μmol/L[31]. Taken together, our study adds further support to the notion that SPK2 is a general mediator in cerebral preconditioning *in vivo* and *in vitro*.

The present data reveal a rapid and sustained up-regulation of SPK2 protein expression in cortical samples in preconditioned mice (~2.2-fold increase at 24 hours after IsoPC and ~2.5-fold increase at 72 hours after HPC). Taking into account that the published therapeutic windows for IsoPC and HPC are 24 hours[5, 13, 14] and 48–72 hours[7, 29] respectively, our data strongly suggest a functional role of SPK2 in mediating preconditioning. Interestingly, Wacker et al., 2009 showed an elevated SPK2 protein expression (1.7-fold increase at 2 hours after HPC) in microvessel-enriched brain extracts, suggesting that the endothelium of cerebral microvessels is the major cellular source for SPK during hypoxia preconditioning[8]. However, this SPK2 up-regulation was transient, as it declined back to baseline in 24 hours in hypoxia-preconditioned mice[8]. We previously observed an elevated spk2 mRNA expression in neurons treated with oxygen-glucose deprivation[26]. We now show that selective SPK2 inhibition (by ABC294640) suppresses the neuroprotective effect of IsoPC in these cells, suggesting an autocrine effect of neuronal SPK2 in response to preconditioning. Cerebral preconditioning requires gene and protein synthesis[34]. IsoPC up-regulates anti-apoptotic protein (Bcl-2)[13] and vascular endothelium growth factor (VEGF)[35] in brain, while hypoxia inducible factor-1alpha (HIF1α) has been shown to mediate HPC[36]. As a master regulator of transcription, HIF binds to hypoxia responsive elements of hypoxia-inducible genes[32]. Hypoxia up-regulates *spk1* in cancer[37] and endothelial cells[38]. However, less is known about the transcription regulation of *spk2*. In attempt to explore the role of HIF1α in cerebral preconditioning, we find an up-regulated HIF1α in cerebral cortex after IsoPC in wild-type mice, but not in $SPK2^{-/-}$ (Supplementary figures 4–5). This supports the previous observations that SPK is activated by hypoxia and SPK stabilizes HIF1α expression.[39, 40] Taken together, our findings pinpoint the crucial role of neuronal SPK as a universal regulator that mediates preconditioning and protects the brain against ischemic injury.

Cerebral ischemia/reperfusion triggers acute cellular injury (for example neuronal cell death took place within hours) and late phase tissue damage (such as inflammatory responses progress and peak in days after ischemic insult)[18]. Although numerous reports support the

notion that preconditioning could protect brain cells (including neurons, endothelial cells and astrocyte) from cell death, there is limited information regarding the role of SPK in mediating the acute neuroprotection by preconditioning. To this end, we evaluate the effects of cerebral preconditioning on stroke outcomes (infarct volumes and neurological deficit score) at 24 hrs after transient MCAo. It will be interesting to follow-up on the potential roles of SPK in mediating long-term neuroprotection (such as angiogenesis and neurogenesis) that may lead to improved motor function and recovery.

A review of preconditioning literature finds that a large number of pathways seem to mediate this phenomenon[1, 2]. This suggests that these pathways might act via common mediators to induce tolerance. Interestingly, SPK is known to be activated by a wide array of stimuli, including cell depolarization, G protein receptor agonists (muscarinic receptor agonists, formyl peptide, nucleotides, bradykinin, cannabinoids, lysophosphatidic acid and S1P), agonists at receptor tyrosine kinases (PDGF, EGF, NGF, VEGF), cross-linking of immunoglobulin receptors, TNF-α, TGF-β, interleukins, Ca^{2+} increasing agents and phorbol ester[41]. Furthermore, sphingolipids stimulate many signaling pathways (including HIF signaling[39, 40], see above) and modulate most cellular functions[42]. It is therefore tempting to speculate that sphingolipid signaling plays a central role in the many pathways involved in preconditioning.

In summary, the present data demonstrates that SPK2 is a universal mediator in isoflurane- and hypoxia-induced preconditioning. Further investigation of the cross-talk between the SPK/S1P axis and HIF is likely to provide insights into the endogenous signaling that could protect the brain against ischemia/reperfusion injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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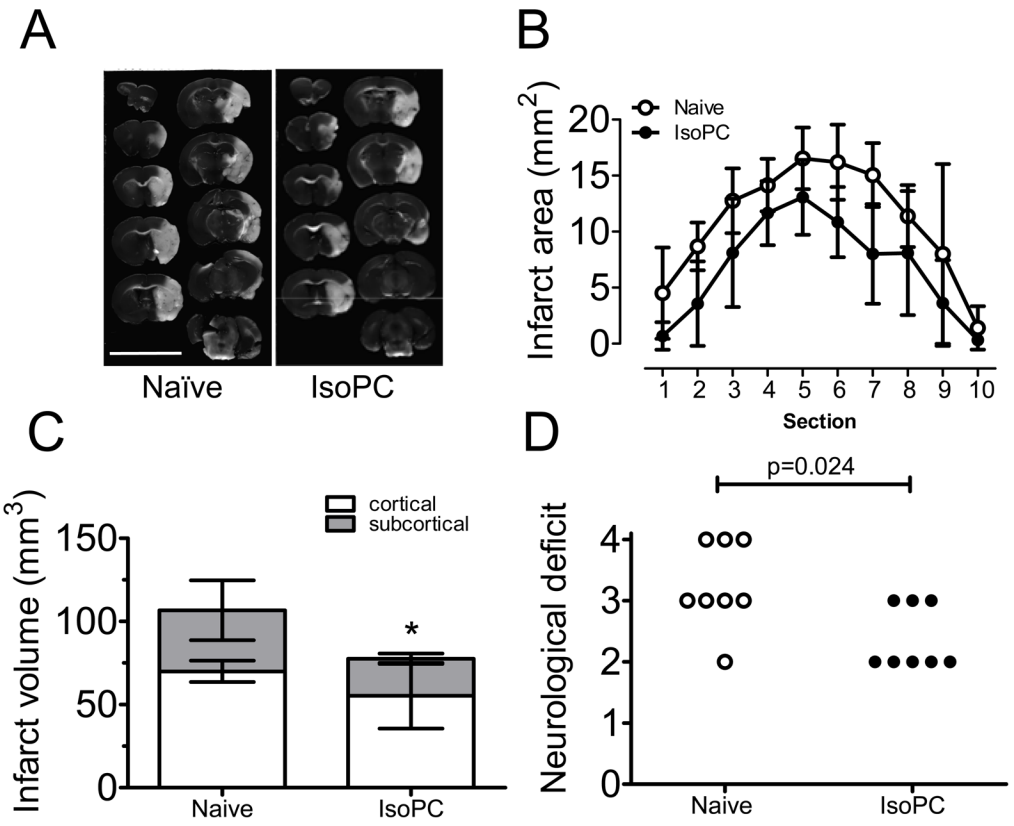

Figure 1.

Effect of isoflurane preconditioning on infarct volumes and neurological deficit scores in mice that underwent a 90-min middle cerebral artery occlusion (MCAo). A, Representative pictures of 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal brain slides (1 mm-thick each) from naïve and preconditioned (IsoPC) mice. B, Infarct areas in consecutive coronal slices. C, Cortical and subcortical infarct volumes in naïve and preconditioned mice were measured and compared. Data are mean±SD (n=8). P value for cortical, subcortical and total infarct volumes were 0.063, 0.041 and 0.026 respectively. D, Neurological deficit was evaluated and scored based on four categories: *grade 0*: no observable neurological deficit (normal); *grade 1*: failure to extend forepaw fully on lifting the whole body by the tail (mild); *grade 2*: circling to the contralateral side (moderate); *grade 3*: falling to one side (severe); *grade 4*: no spontaneous walking, depressed level of consciousness (very severe).

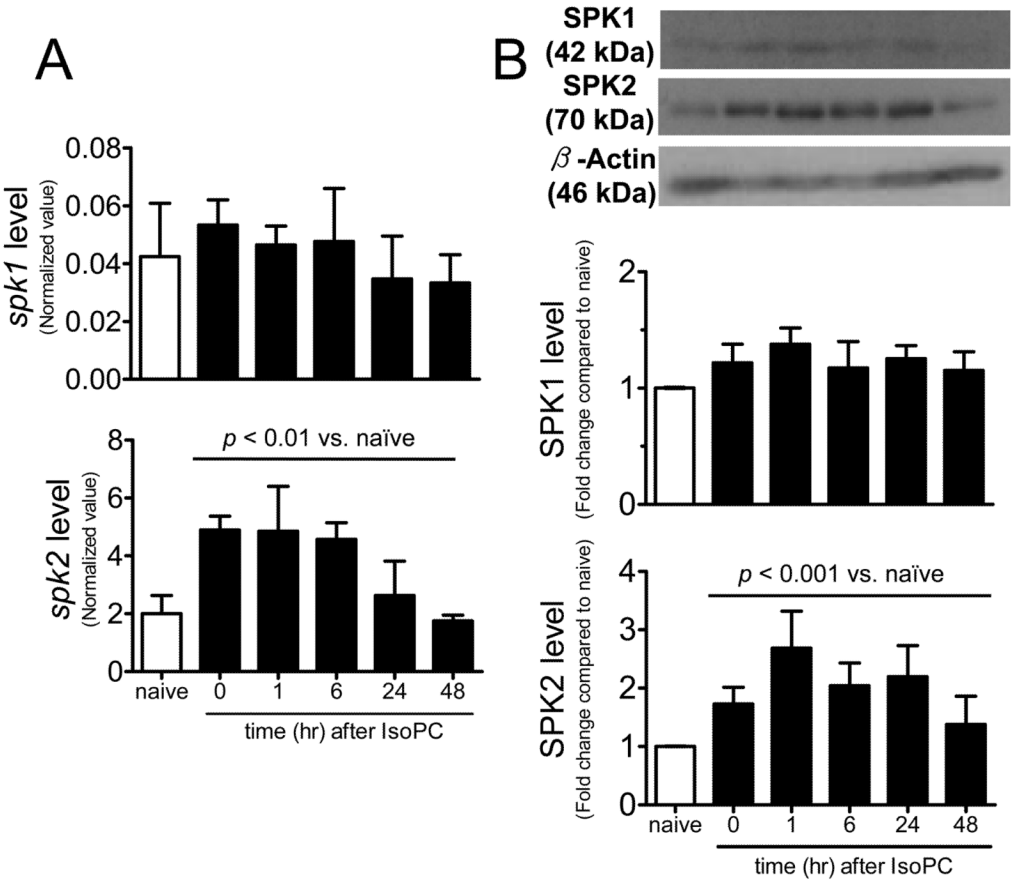

Figure 2.

Expression of sphingosine kinase isoforms in mouse cortex extracts after isoflurane exposure. A, *spk1* and *spk2* mRNA levels were normalized to 18S RNA (n=3). B, Protein levels were normalized to loading control (β-actin) and fold changes compared to control were calculated (n=4). Data are mean±SD. Expressions were compared to naïve control by one-way ANOVA and p values as indicated.

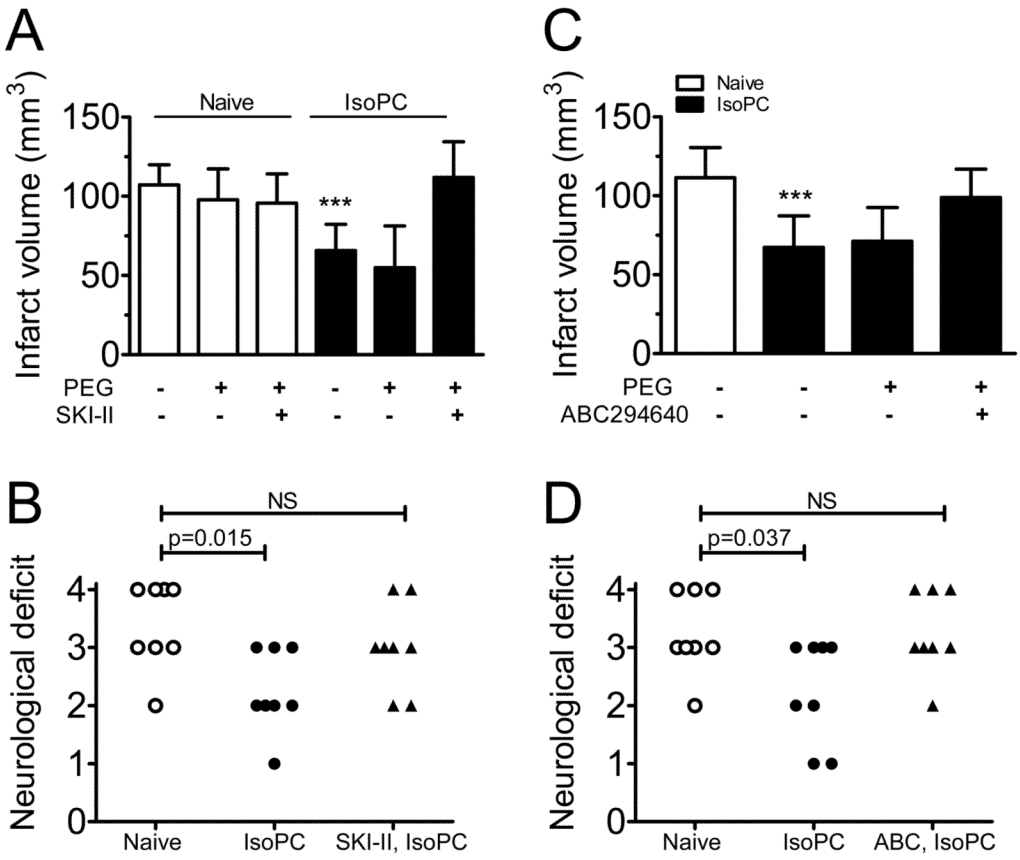

Figure 3.

Treatment with two SPK inhibitors abolished the protective effects of isoflurane preconditioning. A specific SPK inhibitor (SKI-II, A and B) and an SPK2 isoform-selective inhibitor (ABC294640, C and D) were used to verify the role of SPK2 in cerebral preconditioning. Mice were treated with either inhibitor at 100 mg/kg or vehicle (PEG400) by oral gavage at 15 min before preconditioning (IsoPC) and allowed to recover for 24 hours before a 90 min-MCAo. Neurological scores were evaluated at 24 hours after reperfusion (B and D). Data are mean±SD (n=8). *** indicates $p<0.001$ when compared to naïve mice. NS indicates not statistically significant.

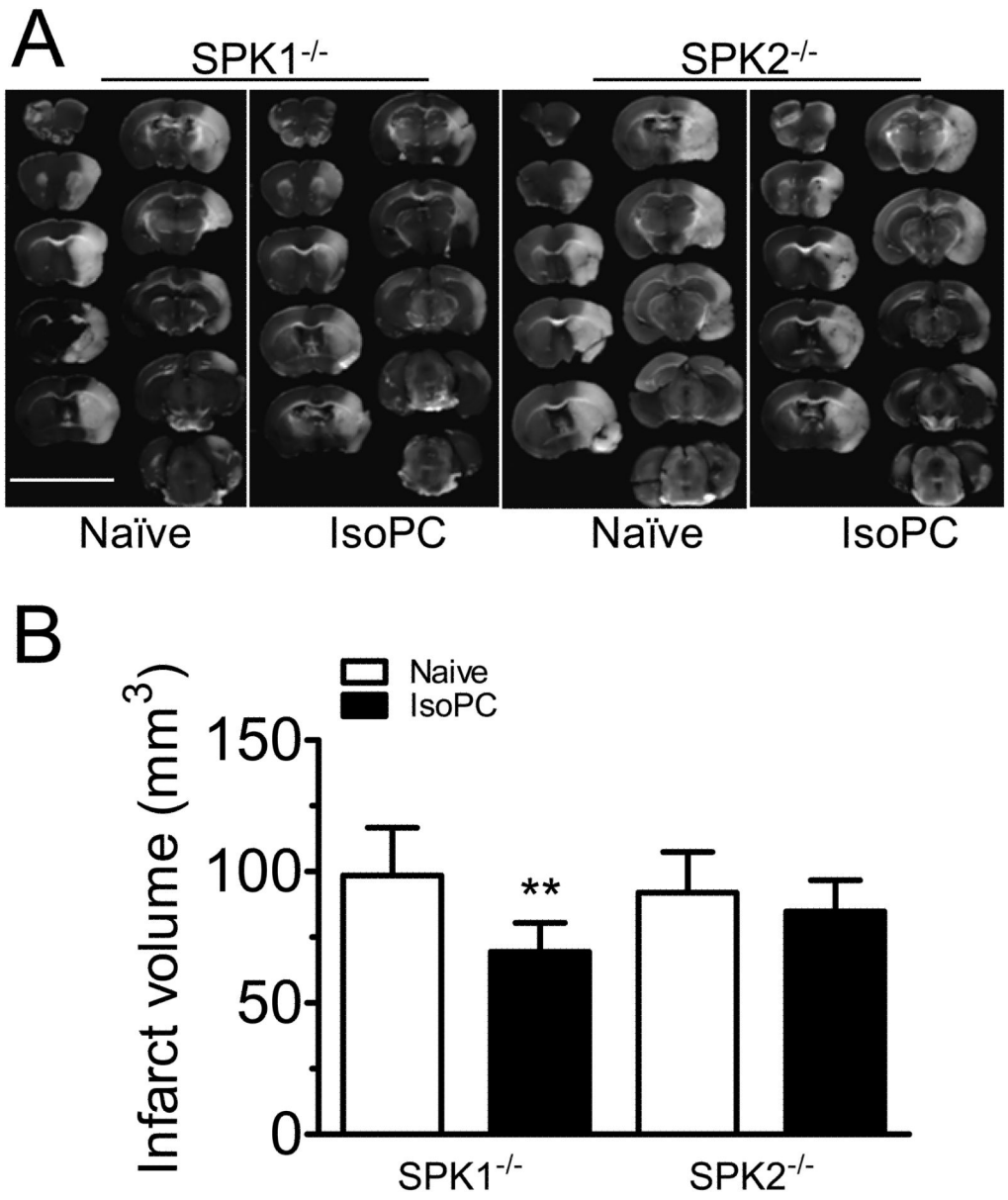

Figure 4.

Effect of IsoPC on infarct volumes in SPK knockout mice. A, Representative pictures of TTC-stained brain sections. B, Summarized data for infarct volumes from naïve and preconditioned mice lacking either SPK isoforms. Data are mean±SD (n=7–8). ** indicates $p<0.01$ compared to naïve $SPK1^{-/-}$ mice.

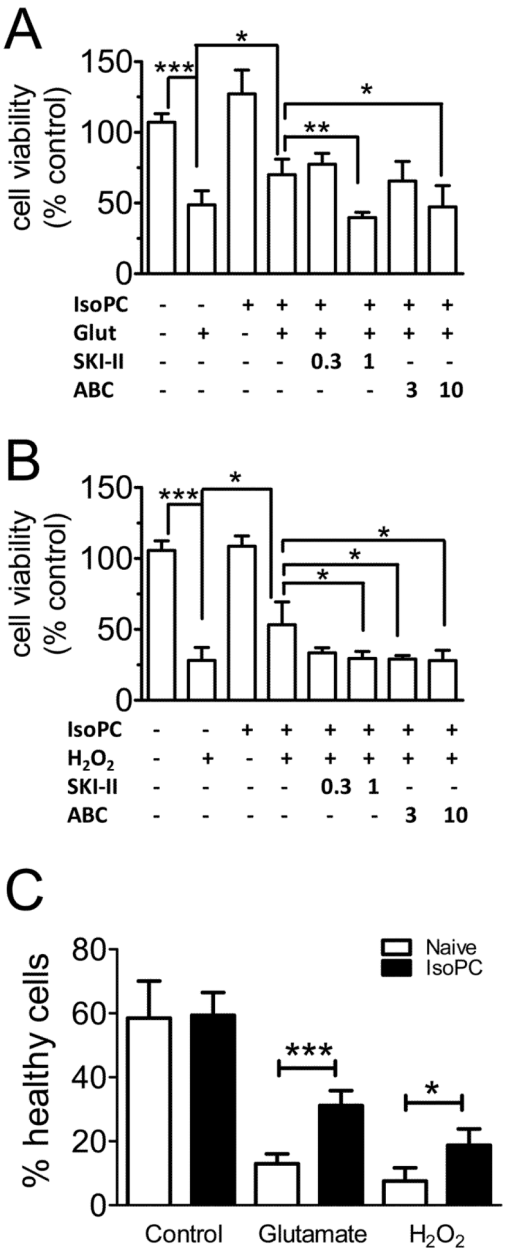

Figure 5.

SPK2 mediates isoflurane preconditioning in primary culture of mouse cortical neurons. Glutamate- (A) and hydrogen peroxide-induced (B) cell death in naïve and preconditioned (IsoPC) neurons were measured by MTT assay. Neurons were pre-treated with SPK inhibitors (0.3 and 1 μmol/L SKI-II or 3 and 10 μmol/L ABC294640) 30 min before IsoPC. C, percentage of cell death was summarized. Bars are mean±SD (n=4). Statistical significance among the groups was measured and calculated as shown. *p<0.05, **p<0.01, ***p<0.001 compared to corresponding naïve control. Glut, glutamate; H_2O_2, hydrogen peroxide; ABC, ABC294640

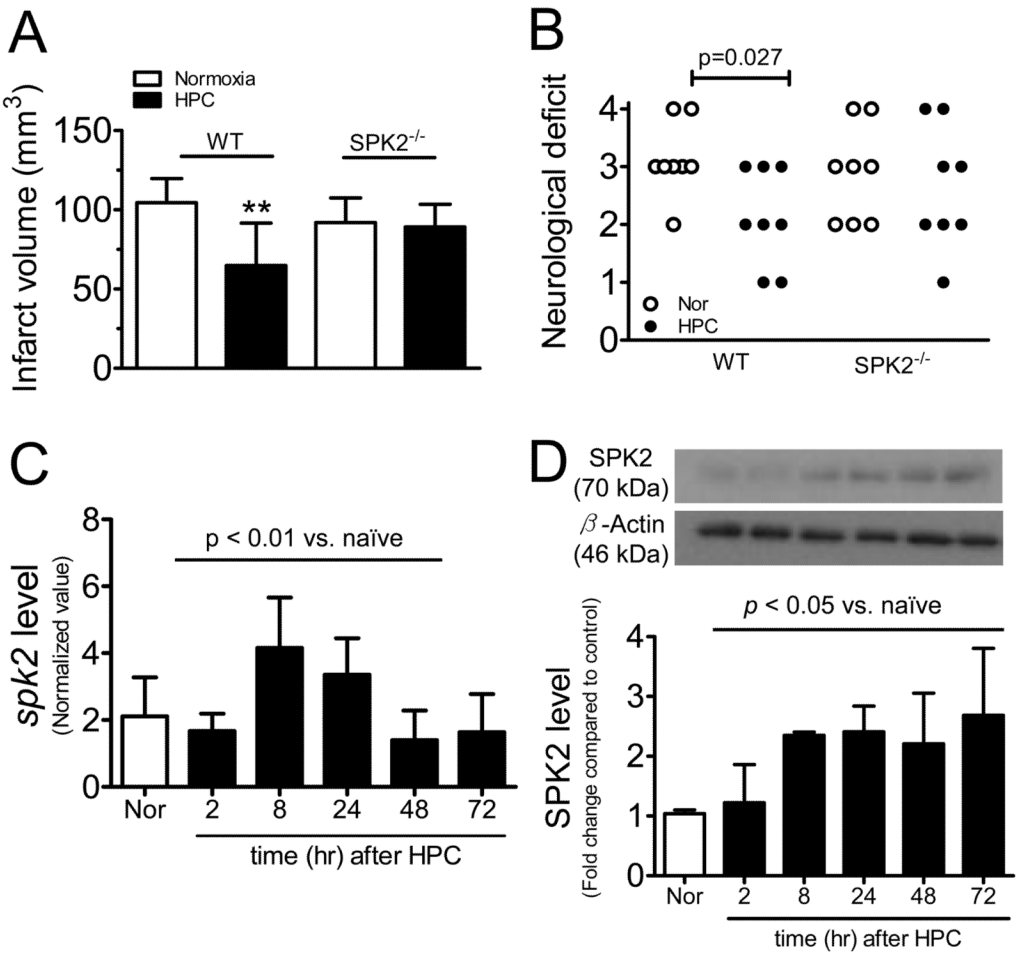

Figure 6.

SPK2 also mediates hypoxic preconditioning (HPC). A, Infarct volumes in naïve and preconditioned wild-type (WT) and SPK2$^{-/-}$ mice (n=8, **p<0.01). B, Neurological score was evaluated at 24 hours after MCAo. C, *spk2* mRNA level were normalized to 18s (n=4). D, SPK2 expression was quantified (n=3). Data are mean±SD. mRNA and protein levels were compared to control by one-way ANOVA.