

Supplemental Material

Sphingosine kinase 2 mediates cerebral preconditioning and protects brains against ischemic injury

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Supplemental Methods

Middle cerebral artery occlusion (MCAo) – Mice were anesthetized with 2% isoflurane, and maintained on 1.5% isoflurane in 30% oxygen and 70% nitrous oxide. Mice were placed on a heating pad to maintain rectal temperature at 37°C and MCAo was performed. The left MCA was occluded for 90 min with a commercially available coated monofilament (Doccol Corporation). At 24 hours after reperfusion, neurological deficit were scored into 4 categories as described¹. Mice were sacrificed with i.p. administration of chloral hydrate (1 g/kg). Brains were harvested and cut into 1 mm-sections. Infarct sizes were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and measured using a computerized image analysis system.

Expression studies for SPK1 and SPK2 - Cortical samples were harvested and frozen immediately. Samples were homogenized and total RNA and protein were extracted. Real-time PCR and western blot analysis were performed to detect SPK1 and SPK2 expressions. Polyclonal antibodies sc-22702 and sc-22704 (1:500, Santa Cruz) were used to detect SPK1 and SPK2 protein levels respectively. Expression levels were normalized to β -actin (1:4000, Sigma) and compared to naïve mice.

Primary culture of mouse cortical neurons – E14-16 embryos of CD1 mice were collected and their brains were harvested in sterile PBS. Cortices were dissected, freed from meninges and choroids plexus, minced and digested in trypsin-EDTA. Trypsin action was stopped with FBS and the tissue were homogenized by trituration with a pipette, passed through a cell strainer and spun down. The pellet were re-suspended in Neurobasal medium (Invitrogen)

with L-glutamine, B27 supplement and penicillin/streptomycin, centrifuged, re-suspended in Neurobasal medium, and plated onto polylysine-coated 24-well plate at a density of 2×10^5 cell/well.

In vitro IsoPC and cell death – After 8 days in culture, neurons were exposed to 2% isoflurane for 30 min in an air-tight chamber and allowed to recover for 24 hours as previously described²⁻⁴. This duration of exposure and concentration of isoflurane did not induce significant neuronal toxicity^{5, 6}. In order to test the involvement of SPK in IsoPC in vitro, cells were treated with SKI-II (0.3 and 1 $\mu\text{mol/L}$)⁷ and ABC294640 (3 and 10 $\mu\text{mol/L}$)⁸ 30 min before IsoPC.

After the 24-hour recovery period, glutamate or H_2O_2 was added to induce cell death. Neurons were treated with 100 $\mu\text{mol/L}$ glutamate for 5 min or 30 $\mu\text{mol/L}$ H_2O_2 (drugs prepared in NBM with no supplement) for 30 min, washed and replaced with fresh pre-warmed NBM⁹. Cell death was quantified with MTT 24 hours later. Neurons were incubated in 200 $\mu\text{g/ml}$ Thiazolyl Blue Tetrazolium Bromide (Sigma, St. Louis) at 37°C for 2 hours. Culture medium was aspirated and cells were lysed in 250 μl DMSO. Color intensity was measured at 570 nm using a Victor³V plate reader (Perkin Elmer, Waltham, MA). Results are expressed as percent absorbance of control wells.

A separate cohort of neurons was fixed with 4% PFA for 10 min and nuclei were stained with Hoechst 33342. Cells undergoing cell death, characterized by condensed nuclei, and the percentages of healthy-looking cells in three random fields were counted in a blinded fashion.

Supplemental Tables

Table 1: Total number of C57BL/J mice subjected to middle cerebral artery occlusion (MCAo).

Group	Included mice	Mortality		Total number of mice
		During MCAo	Within the 24-hour reperfusion period	
Naïve	24	1	3	28
IsoPC	24	2	3	29
PEG	8	1	3	12
PEG + IsoPC	16	2	5	23
SKI-II	8	0	2	10
SKI-II + IsoPC	8	1	3	12
ABC + IsoPC	8	1	1	10
60min-MCAo (Supplementary figure 1)				
Naïve	8	0	0	8
PEG pre-treatment	6	1	1	8
ABC pre-treatment	8	1	2	11
PEG post	6	1	2	9
ABC post	7	1	1	9

Table 2: total number of age-matched wild-type (WT), SPK1^{-/-} and SPK2^{-/-} mice subjected to MCAo.

Group	Included mice	Mortality		Total number of mice
		During MCAo	Within the 24-hour reperfusion period	
SPK1^{-/-}				
Naïve	7	1	1	9
IsoPC	7	1	1	9
SPK2^{-/-}				
Naïve	8	1	1	10
IsoPC	7	0	2	9
WT				
Naïve	8	0	1	9
HPC	8	1	2	11
SPK2^{-/-}				
Naïve	8	0	1	9
HPC	8	0	2	10

Supplemental Figures

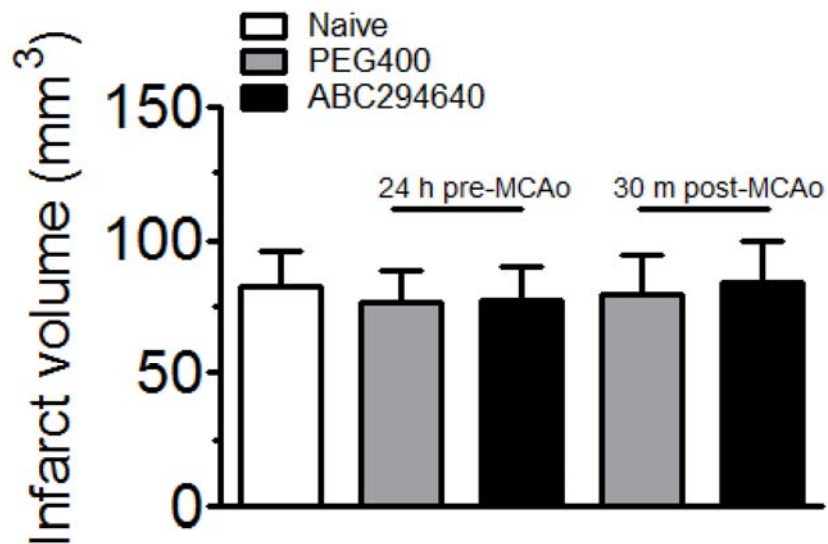


Figure S1. Effect of ABC294640 on infarct volumes. ABC294640 was given (100 mg/kg, oral gavage) either 24 hours before or 30 min after a 60 min-MCAo. There was no difference in infarct volume in naïve mice receiving ABC294640 treatment. PEG400 is the vehicle control. Data are mean \pm SD (n=6-8).

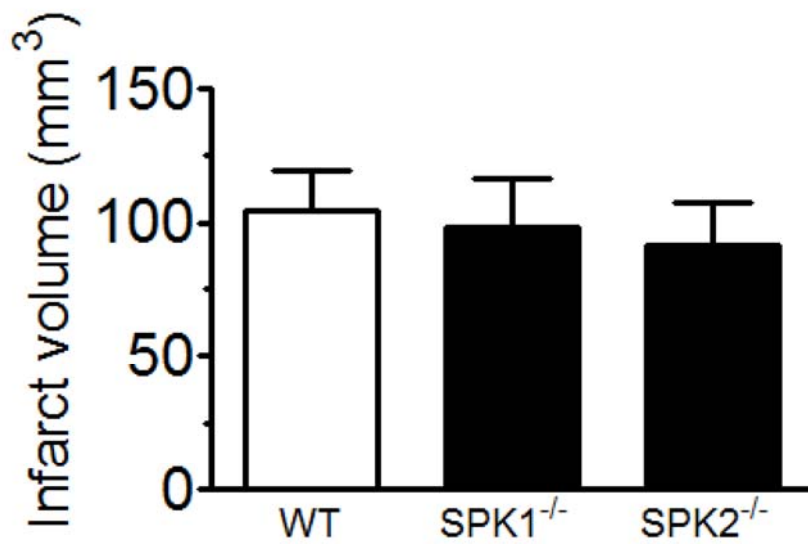


Figure S2. Infarct volumes of WT, SPK1^{-/-} and SPK2^{-/-} mice. Mice underwent a 90 min-MCAo and a 24 hr-reperfusion. The researcher performing MCAo and infarct volume measurements was blinded from the mouse genotypes. Data are mean±SD (n=7-8).

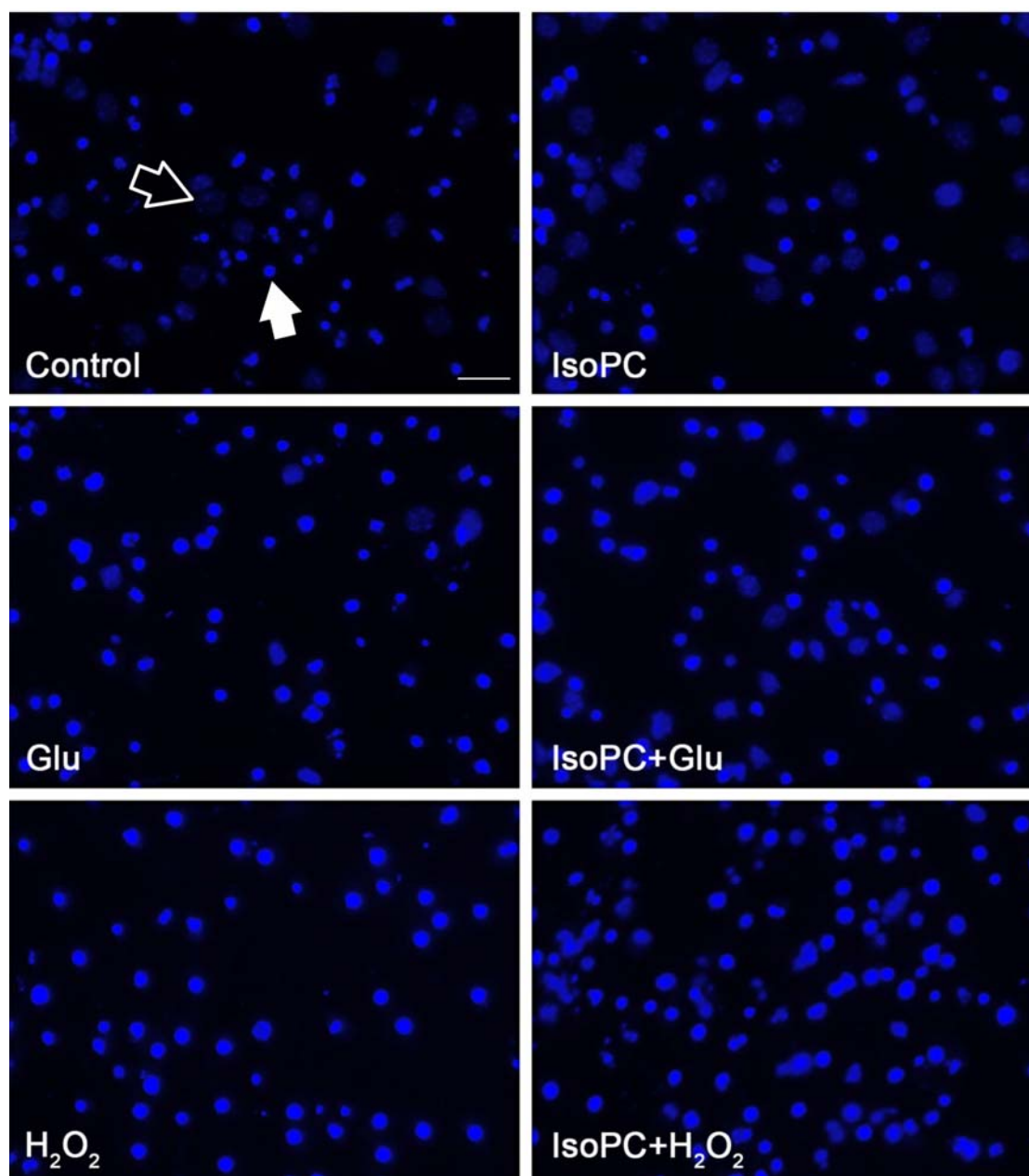


Figure S3. Extent of cell death as shown by nuclear staining. After exposure to glutamate (100 $\mu\text{mol/L}$ for 5 min) or H_2O_2 (30 $\mu\text{mol/L}$ for 30 min), neurons were fixed with 4% PFA and stained with Hoechst 33342. Filled and open arrows indicate dead and healthy neurons respectively. Scale bar (15 μm) applies to all panels.

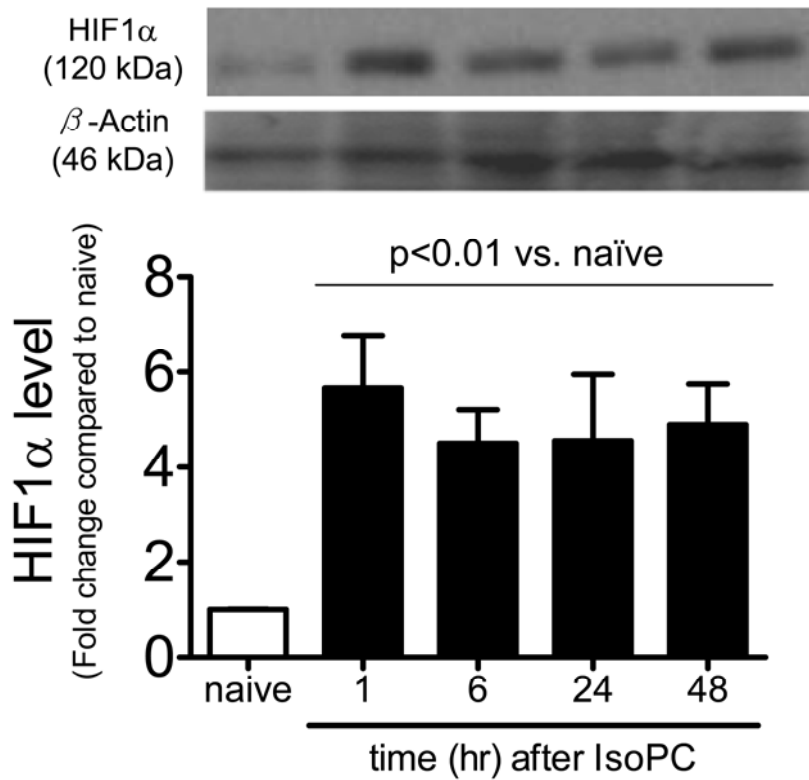


Figure S4. Expression of hypoxia inducible factor 1alpha (HIF1α) in cerebral cortex harvested at different time points after isoflurane preconditioning (IsoPC). The primary antibody against HIF1α (Sigma, H6536) was used at 1:1000. HIF1α protein expression was normalized to loading control (β-actin) and expressed as fold compared to naïve control. Bars represent mean±SD (n=3). ** p<0.01 compared to naïve control by one-way ANOVA.

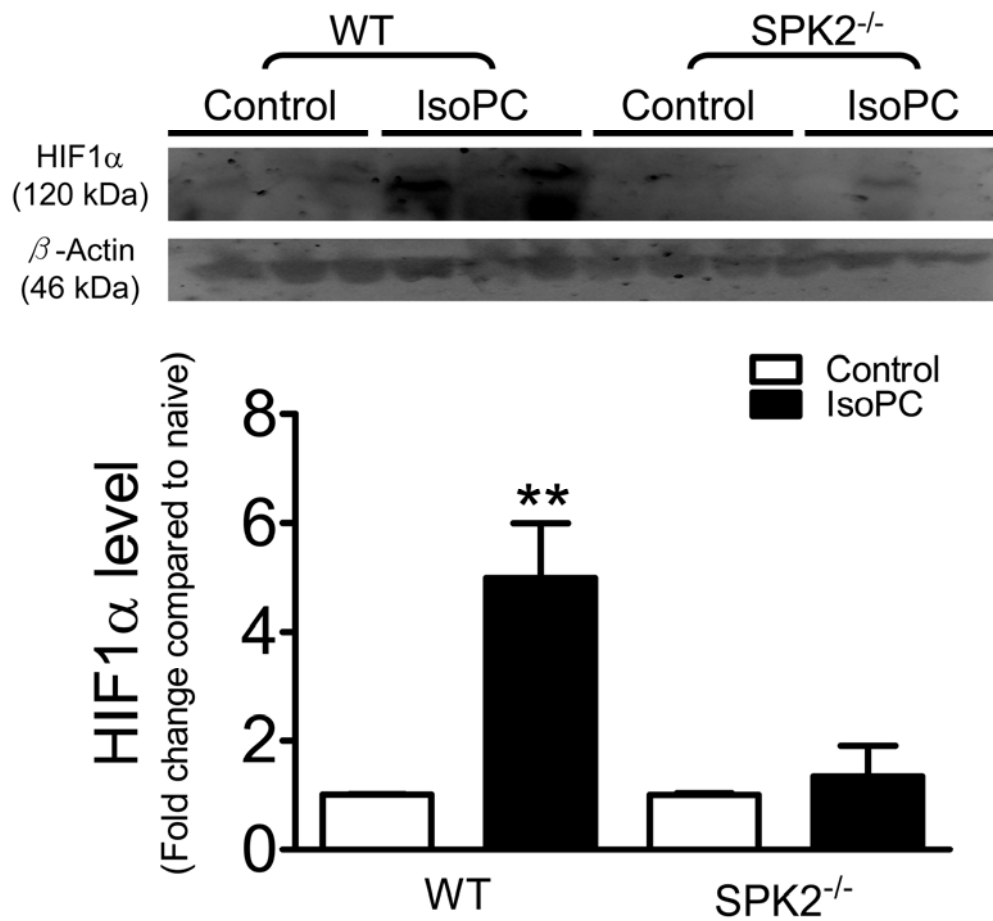


Figure S5. Protein expression of HIF1 α in control and preconditioned wild-type and SPK2^{-/-} mice. Cortical samples were collected 1 hr after isoflurane preconditioning. HIF1 α level was determined and normalized to loading control (β -actin) and expressed as fold compared to naïve control. Bars represent mean \pm SD (n=3).

Supplemental References

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