Supporting Information

Numajiri et al. 10.1073/pnas.1103503108

SI Materials and Methods

Biotin-Switch Assay for SNO-P. Cell lysates and brain tissue extracts were prepared in HEN buffer (250 mM Hepes, pH 7.5, 1 mM EDTA, 0.1 mM neocuproine, and 0.1% SDS), containing 1% Triton X-100. Protein concentration ranges were tested by the assay; typically, 0.8 mg of cell lysate and up to 1.6 mg of tissue extract was used. Blocking buffer [2.5% SDS, 20 mM methylmethane thiosulphonate (MMTS) in HEN buffer] was mixed with the samples and incubated for 30 min at 50 °C to block free thiol groups. After removing excess MMTS by acetone precipitation, nitrosothiols were then reduced to thiols with 1 mM ascorbate. The newly formed thiols were then linked to the sulfhydryl-specific biotinylating reagent *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP). We then pulled down the biotinylated proteins using streptavidin-agarose beads and performed Western blot analysis to detect the SNO-P remaining in the samples. Alternatively, eluted biotinylated samples were subjected to 4–12% SDS/PAGE, and the gels were stained with silver.

Screen to Detect SNO-Ps. SH-SY5Y and 293T cells expressing NOS1 were incubated with or without 5 μM A23187 for 30 min, and then lysates were prepared in HEN buffer containing Triton X-100. Mixed lysates (0.8 mg) were used for screening. Blocking buffer was added, and samples were incubated for 30 min at 50 °C to block free thiol groups. After removing excess MMTS by acetone precipitation, nitrosothiols were reduced to thiols by using 1 mM ascorbate. The newly formed thiols were then linked to the sulfhydryl-specific biotinylating reagent biotin-HPDP. Each sample was applied to antibody arrays (LAB VISION and Sigma) and incubated for 30 min at room temperature according to the manufacturer's instructions. Array slides were incubated with streptavidine-Cy3 for 1 h and then washed with washing buffer five times. The dried antibody array slides were scanned by using a microarray scanner (ScanArray 5000, PerkinElmer), and fluorescent intensities were analyzed with QuantArray v.2.01 software (GSI Lumonics).

Fluorometric Detection of *S*-Nitrosothiols. Recombinant proteins were incubated with 100 μM SNOC in the dark for 30 min at room temperature. Residual nitrite and SNOC degradation products were then removed by a desalting column (Sigma) pre-equilibrated in PBS. After addition of 100 μM 2,3-diaminonaphthalene (DAN) and 100 μM $HgCl_2$, samples were incubated for 30 min in the dark. Fluorescent 2,3-naphthotriazole (NAT) generated from this reaction was measured at an excitation wavelength of 375 nm and an emission wavelength of 450 nm by using a spectrofluorometer. NAT formation stoichiometrically reflects NO released from SNO-Ps, providing a quantitative measure of *S*-nitrosothiol formation on PTEN. The fluorescence intensity curve of serial NAT dilutions was used to construct a standard curve (1, 2).

In Vitro PTEN Activity. In vitro GST-PTEN protein (300 ng) was assayed for lipid phosphatase activity against PI(3,4,5)P3 (Eschelon Biosciences). In brief, WT PTEN or SNO-PTEN was incubated in an enzyme reaction buffer (10 mM Hepes, pH 7.2, 150 mM NaCl) with 50 μM diC8-PI(3,4,5)P3 for 10 min at 37 °C. The reaction was stopped by adding 50 mM *N*-ethylmaleimide (NEM), and the samples were centrifuged at 15,000 g for 20 min at 4 °C. Phosphate released into the supernatant was detected colorimetrically with Biomol Green Reagent (Biomol Research Laboratories).

Assay for eNOS Activity. F2 cells were pretreated with 10 μM Akt inhibitor IV or control for 30 min and then exposed to 10 μM SNOC for 30 min. Cell lysates were used for Western blot analysis with anti phospho-specific eNOS and eNOS antibodies, and for eNOS activity. Enzyme activity was determined with a NOS Assay Kit (Calbiochem) according to the manufacturer's instructions.

PTEN Oxidation. HEK cells were treated with indicated concentrations of SNOC or H_2O_2 for 30 min. The cells were then washed with ice-cold PBS and incubated with lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% deoxycholic acid, 0.1% SDS, 1 mM EDTA) containing 40 mM NEM. Lysates were sonicated and then centrifuged at 12,000 × g for 10 min at 4 C. The supernatant (20 μg) was subjected to SDS/PAGE under nonreducing conditions, and the separated proteins were transferred to a nitrocellulose membrane. Then, the membrane was subjected to Western blot analysis with anti-PTEN antibody.

Transient Focal Cerebral Ischemia. Mice were anesthetized with 1.0–1.5% isoflurane (Merck Hoei Co. Ltd.) in air (21% O_2) via a face mask. Focal cerebral ischemia was induced by introducing an 8-0 monofilament (Ethicon) coated with silicone hardener mixture (Xantopren; Bayer Dental) into the left common carotid artery and advancing it along the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery (MCA). Two hours after ischemia, animals were briefly reanesthetized with isoflurane, and the filament was withdrawn to allow reperfusion through the common carotid artery. During surgery, body temperature was maintained between 37.0 and 37.5 °C with the aid of a heating lamp and heating pad. Mice with an intracranial hemorrhage, pulmonary insufficiency, and/or asphyxia and/or without an ischemic brain infarct were excluded from analysis.

Colorimetric Detection of NO_2^- Accumulation in Brain Tissue. Mice were deeply anesthetized and decapitated at 3 h after ischemia-reperfusion (I/R). Brains were quickly removed, cut into 4-mm coronal sections (between 4 and 8 mm from the frontal forebrain), and separated into core and penumbra regions. Brain samples were homogenized in 4 volumes of sample buffer [100 mM PBS, pH 7.4, 10% glycerol, 1% Triton X-100, 4 mM EGTA, protease inhibitors (Roche)], and the protein concentration was measured by protein assay kit according to manufacturer's instruction (Pierce Biotechnology). Samples were then mixed with the neutral Griess reagent [57 mM sulfanilamide and 1.2 mM *N*-(1-Naphthyl)ethylenediamine] with the addition of $HgCl_2$ to a final concentration of 100 μM. The mixture incubated for 20 min at room temperature. Fluorescence was measured by using a SkanIt RE for Varioskan Flash 2.4 (a microplate reader; Thermo Fisher Scientific) at 540 nm and converted to NO_2^- content by using a nitrate standard curve.

Double Immunostaining. At 2 h after MCA occlusion (MCAO), 8 h after I/R mice were injected with sodium pentobarbital (50 mg/kg, i.p.), then perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed after 15-min perfusion fixation at 4 °C, then immersed in the same fixative solution overnight at 4 °C. They were then immersed in 25% sucrose in 0.1 M PB for 24 h and finally frozen in liquid nitrogen. Coronal brain sections (14 μm) were cut on a cryostat. For immunofluorescent double staining, sec-

tions were incubated overnight at 4 °C with the primary antibodies: rabbit anti-PTEN antibody (1:100; Cell Signaling Technology) and mouse anti-phospho-Akt antibody (1:1,000; Millipore). Then, they were incubated for 3 h with Alexa Fluor 488 F (ab')$_2$ fragment of goat anti-rabbit IgG (H+L) antibody, and Alexa Fluor 546 F (ab')$_2$ fragment of goat anti-mouse IgG (H+L) antibody. At the end of immunostaining, Hoechst 33342 (1:1,000) was added to the samples for 10 min to visualize the nuclei. The images were taken using a confocal microscope (FV10i, Olympus).

TUNEL Staining. The TUNEL assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals). Ischemic areas of cortical brain sections 0.4–1.0 mm anterior to bregma (through the anterior commissure) were excised and used. The brains were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 1 d in 25% sucrose with PBS. The brains were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek). Cerebral sections 10-μm thick were cut on a cryostat at −25 °C, and stored at −80 °C until staining. After twice washing with PBS, sections were incubated with terminal deoxyribonucleotidyl transferase enzyme at 37 °C for 1 h. The sections were washed three times in PBS for 1 min at room temperature. Sections were subsequently incubated with an anti-fluorescein antibody-peroxidase conjugate at room temperature in a humidified chamber for 30 min and then developed by using DAB tetrahydrochloride peroxidase substrate.

Cell Counting. To quantify the number of DNA-fragmented cells and phospho-Akt positive cells present after MCAO, the numbers of these cells in the caudate-putamen (as the ischemic core) and cortex (as the ischemic penumbra; two areas) were counted in a high-power field (×200) on a section through the anterior commissure by a masked investigator. Each count was expressed as number per mm^2 (n = 4–5).

Cell Death Assay. Human neuroblastoma SH-SY5Y cells were transduced with a WT or mutated PTEN genes plus enhanced green fluorescent protein (EGFP) with expression constructs and then incubated for 24 h. Cells were exposed to SNOC and then 60 min later treated with 0.5 μM staurosporine for an additional 7 h. To analyze morphological nuclear changes indicative of apoptosis, the cells were fixed with 1% glutaraldehyde for 30 min, rinsed three times with PBS, and stained with DAPI for 30 min. Stained cells were observed under a fluorescent microscope. The numbers of EGFP-positive apoptotic cells in cultures were counted by two investigators who were blinded to the experimental treatments. Results were expressed as the percentage of EGFP-positive apoptotic cells/total EGFP-positive cells.

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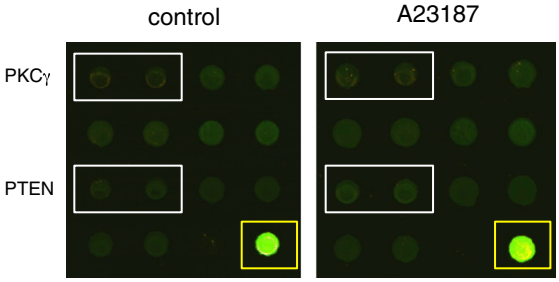

Fig. S1. Detection of SNO-Ps by antibody array. Cells were incubated with or without 5 μM A23187 for 30 min, and cell lysates were subjected to the biotin-switch method. The samples were applied to antibody arrays, and slides were incubated with streptavidin-Cy3 for 1 h. Then, the dried antibody array slides were scanned by using a microarray scanner (ScanArray 5000, PerkinElmer). A representative block of antibodies spotted in duplicate is illustrated. SNO-Ps were identified by increased fluorescence intensity after A23187 treatment. Positions of positive Cy3 controls are indicated by yellow squares.

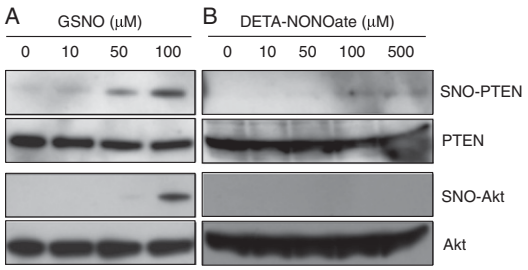

Fig. S2. *S*-nitrosylation of PTEN by GSNO and DETA–NONOate. HEK293T cells were exposed for 30 min to various concentrations of GSNO (*A*) and DETA–NONOate (*B*). SNO-PTEN and SNO-Akt were detected by the biotin-switch assay.

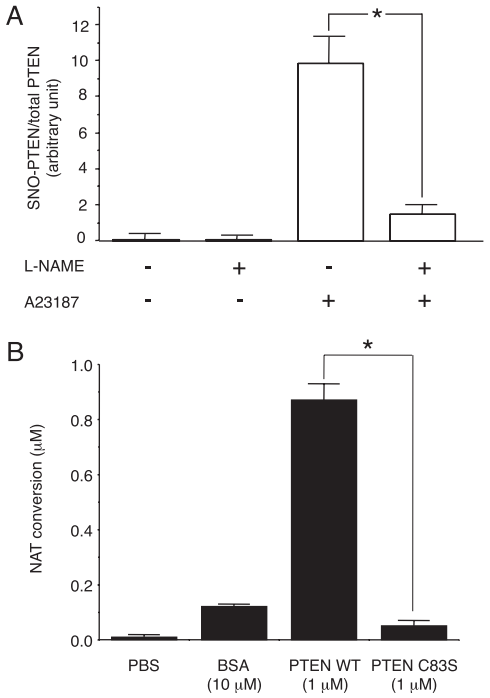

Fig. S3. SNO-PTEN formation. (*A*) HEK cells expressing nNOS were assayed for endogenous SNO-PTEN. nNOS was activated by Ca^{2+} ionophore A23187 (5 μM) in the presence or absence of NOS inhibitor (L-NAME, 1 mM). The results were quantified by densitometry. The relative ratio of SNO-PTEN to total PTEN was calculated for each sample. Values are means ± SEM, $n = 3$; *$P < 0.01$ by ANOVA. (*B*) In vitro SNO-PTEN formation. Recombinant PTEN protein (1 μM) was incubated with SNOC (100 μM) for 30 min at room temperature. SNO-PTEN thus generated was assessed by NO release, causing the conversion of DAN to the fluorescent compound NAT. SNOC itself quickly decayed, resulting in insignificant *S*-nitrosothiol readings in this assay. BSA (10 μM) was used as the positive control. Values are mean ± SEM, $n = 4$; *$P < 0.01$ by ANOVA.

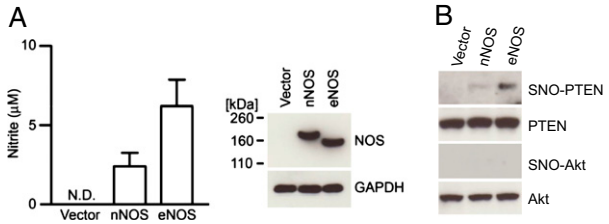

Fig. S4. SNO-PTEN formation in eNOS/nNOS expressing cells. (*A*) HEK293T cells were transfected with a C-terminal myc tagged-eNOS or -nNOS expression vector and incubated for 24 h. The cells were then stimulated with 5 μM A23187 for 1 h. Nitrite accumulation in culture medium was monitored by the Griess assay, and expression of nNOS and eNOS were estimated by immunoblotting with anti-myc antibody. (*B*) Biotin-switch assay for SNO-PTEN and SNO-Akt after exposure to A23187.

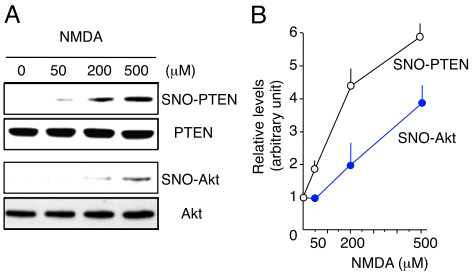

Fig. S5. SNO-P formation in primary cortical cultures. (*A*) SNO-PTEN and SNO-Akt were detected in cultured rat cortical neurons after exposure to various concentrations of NMDA by the biotin-switch assay. (*B*) SNO-Ps were quantified by densitometry. The relative ratio of SNO-P to total protein was calculated for each sample. Values are means ± SEM, $n = 3$.

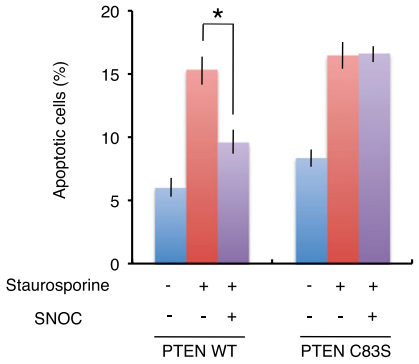

Fig. S6. Low concentrations of SNOC afford in vitro neuroprotection against staurosporine insult. Human SH-SY5Y cells were transduced for 24 h with expression vectors for WT-PTEN or C83S-PTEN plus EGFP. Cultures were exposed to 10 μM SNOC and then stimulated with 0.5 μM staurosporine for 7 h. After fixation, the cells were stained with DAPI to assess neuronal nuclei. Values are means ± SEM for n = 3,500 cells counted in five experiments, $*P < 0.01$ by ANOVA.

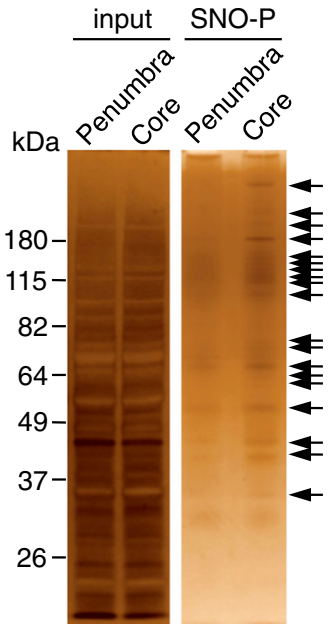

Fig. S7. Comparison of SNO-P levels in the core and penumbral regions of ischemic brains. Samples derived from the ischemic core and penumbra were biotinylated by using the biotin-switch method, purified on streptavidin-agarose, and then eluted by using 2-mercaptethanol. The samples were then subjected to SDS/PAGE, and the gels were stained with silver. A significant increase in the number of SNO-Ps (arrows) was evident in the ischemic core compared with the penumbra.

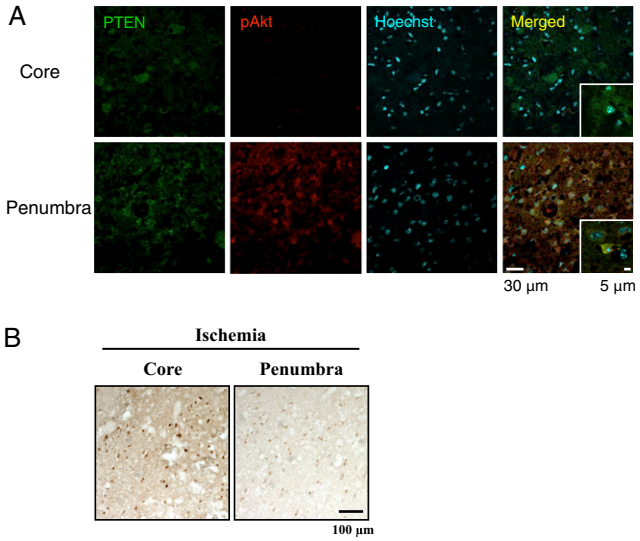

Fig. S8. Detection of pAkt protein and TUNEL-positive cells in ischemic brains. (*A*) Immunohistochemical analysis of PTEN and pAkt protein in ischemic brains. Immunofluorescent labeling of PTEN (green), phospho-Akt (red), and Hoechst 33342 (blue) in the core and penumbral regions. [Scale bars: 30 μm (low magnification) and 5 μm (high magnification)]. (*B*) TUNEL staining performed 8 h after I/R of mouse brain. Cryostat sections (10 μm in thickness) were cut at −25 °C and stored at −80 °C until staining. After washing twice with PBS, sections were incubated with terminal deoxyribonucleotidyl transferase enzyme at 37 °C for 1 h. Sections were then washed three times in PBS, incubated with anti-fluorescein antibody-peroxidase conjugate at room temperature in a humidified chamber for 30 min, and developed by using DAB tetrahydrochloride peroxidase substrate. (Scale bar: 100 μm.)

Table S1. Detection of known and predicted *S*-nitrosylated proteins by antibody array screening

		Fluorescent intensity, arbitrary units			
Protein	Refs.	A: Vehicle (control)	B: A23187	Δ(B – A)	Δ(B/A)
Actin	(1–3)	3,976	4,521	545	1.14
Actinin	(4, 5)	2,679	2,938	259	1.10
Annexin VII	(4)	1,441	1,632	191	1.13
Caspase-3	(6)	2,250	2,634	384	1.17
Caspase-6	—	1,683	1,964	281	1.17
Caspase-7	—	1,094	1,248	154	1.14
Caspase-8	(7)	580	750	170	1.29
Caspase-9	—	332	438	106	1.32
Caspase-10	—	607	773	166	1.27
Caspase-11	—	601	827	226	1.38
Cofilin 1	(4, 8–10)	3,219	3,391	172	1.05
eNOS	(11)	1,225	1,433	208	1.17
ERK1/2	(3, 4)	571	920	349	1.61
Filamin	(4, 5)	614	773	159	1.26
HDAC2	—	730	1,039	309	1.42
JNK	(12)	496	762	266	1.54
Myosin	(4)	1,198	1,377	179	1.15
Peroxiredoxin	(4, 8, 9, 13)	992	1,183	191	1.19
PKC β	(9, 14)	621	958	337	1.54
PP1A	(9, 15)	1,380	1,573	193	1.14
PP2A	(4, 9, 15)	1,040	1,225	185	1.18
PTEN	(16–22)	535	1,275	740	2.38
Tubulin α	(1, 3, 4, 9, 10, 13)	2,193	2,462	269	1.12
UCH-L1	(3, 4, 9)	2,377	2,656	279	1.11
14-3-3	(3, 9, 23, 24)	782	1,011	229	1.29

Mean fluorescence values (arbitrary units) obtained from analysis of arrays treated with vehicle (A) and A23187 (B) are shown. Refs., references.

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