Preservation of GABA_\(A\) Receptor Function by PTEN Inhibition Protects Against Neuronal Death in Ischemic Stroke

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**Background and Purpose**—Downregulation of the tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), is thought to be a novel neuroprotective strategy in ischemic stroke, but the underlying mechanisms remain unclear. In this study, we aimed to validate the use of PTEN regulation of \( \gamma \)-aminobutyric acid subtype A receptors (GABA_\(A\)Rs) as a molecular target for the treatment of ischemic stroke. Because suppression of GABA_\(A\)Rs contributes to ischemic neuron death, describing the intracellular signaling that interacts with GABA_\(A\)Rs in ischemic neurons would provide a molecular basis for novel stroke therapies.

**Methods**—We measured surface GABA_\(A\)R expression by immunocytochemical labeling and surface protein biotinylation assay. Knockdown and overexpression approaches were used to test the effects of PTEN on the expression and function of GABA_\(A\)Rs. Neuronal death was detected in both in vitro and in vivo stroke models.

**Results**—The knockdown and overexpression approaches provided the first evidence that PTEN negatively regulated membrane expression and function of GABA_\(A\)Rs in rat hippocampal neurons. Importantly, we demonstrated that a PTEN inhibitor prevented the reduction of surface GABA_\(A\)Rs in injured hippocampal neurons subjected to oxygen-glucose deprivation, an in vitro insult that mimics ischemic injury, whereas a GABA_\(A\)R antagonist significantly reduced this PTEN inhibitor–induced neuroprotection in both in vitro and in vivo ischemic stroke models.

**Conclusions**—Our study provides direct evidence that downregulation of PTEN protects against ischemic neuron death by preserving GABA_\(A\)R function. Targeting this pathway may be an effective strategy for development of selective, potent stroke treatments. (Stroke. 2010;41:1018-1026.)

**Key Words:** GABA_\(A\) receptor ■ PTEN ■ ischemia ■ stroke ■ neuroprotection ■ excitotoxicity

The tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), is a dual-specificity phosphatase. PTEN is involved in the regulation of several basic cellular functions, such as cell cycle progression, cell migration, cell spreading, and cell growth. Recent studies have shown that suppressing PTEN protects against ischemic neuron death through both the enhancement of Akt activation and the inhibition of NR2B subunit–containing N-methyl-D-aspartate receptors. These data suggest that PTEN may be a therapeutic target for stroke treatment. However, the cellular and molecular mechanisms underlying PTEN downregulation–mediated neuroprotection remain largely unknown.

Ischemia-induced loss of excitatory and inhibitory equilibrium contributes to excitotoxicity-mediated neuronal death in ischemic stroke. Suppressed function of \( \gamma \)-aminobutyric acid subtype A receptors (GABA_\(A\)Rs) causes neuronal damage after stroke. Both the GABA uptake inhibitor and the GABA_\(A\)R agonist are neuroprotective in animal models of ischemic stroke. A recent study has shown that cell surface GABA_\(A\)Rs are markedly decreased in neurons treated by oxygen-glucose deprivation (OGD), suggesting that a change in the number of membrane GABA_\(A\)Rs may be a crucial process in enhancing ischemic neuron death. However, use of GABA_\(A\)R agonists as neuroprotective agents has been disappointing in clinical trials owing to uncontrolled, global overactivation of the channels. Thus, identifying the intracellular signaling specifically linked to GABA_\(A\)Rs in ischemic neurons would aid us to understand the cellular and molecular mechanisms underlying PTEN regulation–mediated neuroprotection.
molecular mechanisms underlying ischemia-induced neuronal death, which may ultimately lead to the development of selective, potent therapies for stroke patients.

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Inhibitory synaptic transmission is mediated by postsynaptic GABA$_A$Rs, and the most abundant population of GABA$_A$Rs in the mammalian brain is the $\alpha_1\beta_2\gamma_2$ combination. Recent studies indicate that trafficking of GABA$_A$Rs to and from the membrane surface plays an important role in posttranslational signaling mechanisms that modulate the stability, density, and function of the channels$^{14,15}$ and that the $\gamma_2$ subunit is required for the trafficking of GABA$_A$Rs to the postsynaptic membrane.$^{14,15}$ Interestingly, a recent study has shown that GABA$_A$Rs are upregulated by activation of phosphatidyl inositol 3-kinase/Akt-dependent signaling.$^{16}$ Because Akt activation is negatively regulated by PTEN,$^{17}$ we hypothesized that GABA$_A$Rs may be upregulated by PTEN inhibition.

Given the involvement of PTEN and GABA$_A$Rs in ischemic neuron damage, the present study tested the effect of PTEN downregulation on GABA$_A$R expression and function in the in vitro OGD and in vivo ischemic stroke models. We provide direct evidence that PTEN downregulation protects against ischemic neuron death by preventing GABA$_A$R suppression in ischemic neurons.

Materials and Methods

Hippocampal Neuron Culture and OGD Treatment

To create an in vitro ischemia-like injury model, hippocampal neuron cultures were treated by OGD. The cultures were prepared from Sprague-Dawley rats at gestation day 18, and detailed methods are described in our previous study.$^{18}$ To initiate the OGD challenge, cells were transferred to deoxygenated, glucose-free, extracellular solution (in mmol/L: 116 NaCl, 5.4 KCl, 0.8 MgSO$_4$, 1.0 NaH$_2$PO$_4$, 1.8 CaCl$_2$, and 26 NaHCO$_3$); introduced into a specialized, humidified chamber (Plas-Labs, Lansing, Mich); and maintained at 37°C in 85% N$_2$/10% H$_2$/5% CO$_2$ for 40 minutes.$^{18}$ For “sham” treatment, neuron cultures were treated by OGD. The cultures were prepared using a maximum projection. For all experiments, we analyzed the fluorescence signal in regions of interest by measuring the average fluorescence intensity per unit area. All images in all experiments were acquired from a single plane and transferred for analysis. Three independent experiments were performed, and a minimum of 25 randomly selected cells per group per experiment were analyzed by an investigator blinded to treatment group assignment. Scale bar=10 μm. B, Top, Western blot from protein biotinylation assay showing that surface expression of membrane GABA$_A$R $\gamma_2$ subunits is decreased after OGD treatment. Bottom, Bar graph showing the significant reduction of $\gamma_2$ expression in the OGD group (n=6 for each group, $^t$<0.05, Student t test). C, Top, Representative images of Western blot showing the total expression of whole-cell GABA$_A$R $\gamma_2$ subunits in control and OGD groups. β-Actin was used as a loading control. Bottom, Bar graph of data in the upper panel (n=5 for each group; $^t$<0.05, Student t test).

Immunofluorescence Labeling, Image Acquisition, and Analysis

To measure the surface expression of GABA$_A$R $\gamma_2$ subunits in control and OGD-treated neurons,$^{18,19}$ unpermeabilized cells were labeled with rabbit anti-$\gamma_2$ primary antibody against the extracellular domain of the $\gamma_2$ subunit (Millipore Corp, Billerica, Mass), and Alexa Fluor 594 (red fluorescence) secondary antibody (Invitrogen Canada Inc, Burlington, Canada). To examine PTEN expression, neurons were permeabilized by treatment with 4% paraformaldehyde in phosphate-buffered saline for 20 minutes and then with 0.3% Triton X-100 for 10 minutes. Rabbit anti-PTEN primary antibody (Cell Signaling Technology Inc, Danvers, Mass) and Alexa Fluor 594 (red fluorescence) secondary antibody (Invitrogen Canada Inc) were used for labeling.

Fluorescence-labeled neurons were imaged with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany) and analyzed as described previously.$^{18,19}$ Images were acquired with a Zeiss Axio-Cam digital camera in the linear range with constant settings. Each image was a z-series of 6 to 13 images, taken at 0.75-μm-depth intervals. The resultant stack was “flattened” into a single image by using a maximum projection. For all experiments, we analyzed the fluorescence signal in regions of interest by measuring the average fluorescence intensity per unit area. All images in all experiments were analyzed according to identical acquisition parameters. During data acquisition and analysis, the investigator was blinded to treatment group assignment. In each experiment, neurons were selected randomly under bright-field optics, and fluorescence images of each neuron acquired from a single plane were transferred for analysis. The cells in control and OGD groups from the same culture preparation were processed and imaged in parallel. Three images were randomly selected in each culture. The fluorescence density was analyzed by Image J software (National Institutes of Health, Bethesda, Md).$^{18}$ The expression of surface receptors and whole-cell proteins represented by labeled fluorescence densities in treated groups was normalized versus that in control groups. The n value refers to the number of cells analyzed.

Surface Protein Biotinylation and Western Blot Assays

To quantify the surface expression of GABA$_A$Rs, surface protein biotinylation assays were performed. The neuronal membrane pro-
**Colorimetric Assays**

Colorimetric assays were also performed to measure the surface expression of GABA<sub>A</sub>Rs in cultured neurons. To label surface GABA<sub>A</sub>_R y2 subunits, cells were fixed with paraformaldehyde (4% for 10 minutes) under nonpermeabilized conditions and labeled with rabbit anti-GABA<sub>A</sub>_R y2 antibody (Millipore Corp) raised against the N-terminal extracellular domain of y2. Cells were then incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Baie D’urfe, Canada), washed 5 times with phosphate-buffered saline to minimize nonspecific reactivity, and incubated in 1 mL of the horseradish peroxidase substrate o-phenylenediamine (Sigma, St. Louis, Mo) for 2 to 5 minutes. Reactions were stopped with the addition of 0.2 mL of 3N HCl, and the optical density of the supernatant was read on a spectrophotometer at 492 nm. Analysis was performed on 6 plates per group.

**PTEN siRNA Expression Vectors**

PTEN small interfering RNA (siRNA) was used to knockdown PTEN protein expression in cultured hippocampal neurons. An expression vector that directs the synthesis of siRNAs was constructed as described previously. A PTEN siRNA target sequence (5′-AGAGATCGTTGAGCAGAAC-3′) and a scrambled sequence (5′-AGAGACAGAACTCGTTAG-3′) were selected for constructing siRNA-PTEN-GFP or SsiRNA-PTEN-GFP with or without potassium bisperoxo(pyridine-2-carboxylato)oxovanadate(V) [bpV(pic)] treatment. The recording-electrode resistance was 3 to 5 MΩ when filled with solution containing 140 mmol/L CsCl, 2 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, 5 mmol/L EGTA, 10 mmol/L HEPES, and 4 mmol/L K<sub>2</sub>ATP, titrated to pH 7.3 with CsOH, and the osmolality was 280 to 290 mOsm. For bpV(pic) treatment, 100 nmol/L bpV(pic) was added into the pipette filling solution. Bath solutions contained 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 5 mmol/L HEPES, and 33 mmol/L glucose, titrated to pH 7.4, with an osmolality of 300 to 320 mOsm. Tetrodotoxin (0.5 μmol/L) was added to the bath solution to block voltage-gated sodium channel currents. Neurons were held at −60 mV under voltage clamp. GABA<sub>A</sub>_R-mediated whole-cell currents were recorded by pressure application of 100 μmol/L GABA (20 kPa, 20 ms) from a micropipette with its tip located 20 μm from the recorded cell. Drugs were delivered at intervals of 30 seconds. Data were acquired with an Axopatch 200B amplifier and pClamp 10 software interfaced to a Digidata 1322A acquisition board (Molecular Devices), and signals were filtered at 2 kHz and digitized at 10 kHz.

**Recording of GABA<sub>A</sub>_R-Mediated Whole-Cell Currents**

Recordings were performed in cultured neurons transfected with siRNA-PTEN-GFP or SsiRNA-PTEN-GFP with or without potassium bisperoxo(pyridine-2-carboxylato)oxovanadate(V) [bpV(pic)] treatment. The recording-electrode resistance was 3 to 5 MΩ when filled with solution containing 140 mmol/L CsCl, 2 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, 5 mmol/L EGTA, 10 mmol/L HEPES, and 4 mmol/L K<sub>2</sub>ATP, titrated to pH 7.3 with CsOH, and the osmolality was 280 to 290 mOsm. For bpV(pic) treatment, 100 nmol/L bpV(pic) was added into the pipette filling solution. Bath solutions contained 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 5 mmol/L HEPES, and 33 mmol/L glucose, titrated to pH 7.4, with an osmolality of 300 to 320 mOsm. Tetrodotoxin (0.5 μmol/L) was added to the bath solution to block voltage-gated sodium channel currents. Neurons were held at −60 mV under voltage clamp. GABA<sub>A</sub>_R-mediated whole-cell currents were recorded by pressure application of 100 μmol/L GABA (20 kPa, 20 ms) from a micropipette with its tip located 20 μm from the recorded cell. Drugs were delivered at intervals of 30 seconds. Data were acquired with an Axopatch 200B amplifier and pClamp 10 software interfaced to a Digidata 1322A acquisition board (Molecular Devices), and signals were filtered at 2 kHz and digitized at 10 kHz.

**Figure 2.** Downregulation of PTEN increases the surface expression of GABA<sub>A</sub>_R y2 subunits. A, Left, Representative images showing the downregulation of PTEN expression in neurons transfected with siRNA-PTEN-GFP. Arrowheads indicate successfully transfected neurons. Right, Bar graph showing that PTEN protein expression is significantly decreased in neurons transfected with siRNA-PTEN-GFP compared with those transfected with SsiRNA-PTEN-GFP (n = 35 cells for siRNA-PTEN group and n = 24 cells for SsiRNA-PTEN group; *P < 0.05, ANOVA). Three independent experiments were performed, and a minimum of 8 randomly selected cells per group per experiment were analyzed. Scale bar = 15 μm. B, Left, Representative images showing that surface expression of GABA<sub>A</sub>_R y2 subunits is increased in neurons transfected with siRNA-PTEN-GFP. Right, Bar graph showing that surface expression of GABA<sub>A</sub>_R y2 subunits was significantly increased in neurons transfected with siRNA-PTEN-GFP compared with those transfected with SsiRNA-PTEN-GFP (n = 39 cells for siRNA-PTEN group and n = 28 cells for SsiRNA-PTEN group; *P < 0.05, ANOVA). Three independent experiments were performed, and a minimum of 8 randomly selected cells per group per experiment were analyzed by an investigator blinded to the transfected constructs. Scale bar = 15 μm.
medium containing 100 nmol/L bpV(pic) and 50
period, and the medium was replaced with fresh maintenance
cultures were returned to the CO2 incubator. Viability was
cultures and replaced with maintenance medium containing 10%
the recovery period in a CO2 incubator.

The cultures were treated with bpV(pic) (100 nmol/L in maintenance

bpV(pic) group, cells were given 100 nmol/L bpV(pic) in glucose-free extracellular solution during OGD
For the OGD challenge

bpV(pic) medium was replaced with normal maintenance medium.

Alamar Blue Cell Viability Assay
Neuron viability was measured by the Alamar Blue cell viability assay.22 The maintenance medium was removed from the neuronal cultures and replaced with maintenance medium containing 10% (vol/vol) Alamar Blue (BioSource International, Camarillo, Calif), and cultures were returned to the CO2 incubator. Viability was assayed when the solution in the control wells turned from blue to pink, which typically required ~3 hours. Alamar Blue fluorescence was measured with a Perkin-Elmer Victor 3 MultiLabel plate reader (Perkin-Elmer Life and Analytic Sciences, Inc) with excitation at 530 nm and emission at 590 nm. For each experiment, the data were normalized as a percentage of the averaged value in control plates.

Drug Preparation and Treatment
The PTEN inhibitor bpV(pic) was purchased from Calbiochem.23 The cultures were treated with bpV(pic) (100 nmol/L in maintenance medium) for 2 hours at 37°C in a 5% CO2 incubator, and then the bpV(pic) medium was replaced with normal maintenance medium. For the OGD challenge+bpV(pic) group, cells were given 100 nmol/L bpV(pic) in glucose-free extracellular solution during OGD treatment, and the medium was replaced with fresh maintenance medium containing 100 nmol/L bpV(pic) during the recovery period in a CO2 incubator. In the OGD challenge+bpV(pic)+bicuculline (Bic) group, cells were given 100 nmol/L bpV(pic) and 50 μmol/L Bic (Sigma) in glucose-free extracellular solution during the OGD period, and the medium was replaced with fresh maintenance medium containing 100 nmol/L bpV(pic) and 50 μmol/L Bic during the recovery period in a CO2 incubator.

Animal Model of Transient Global Ischemia and Histology Analysis
A four-vessel occlusion model of cerebral ischemia was performed in adult male Sprague-Dawley rats weighing 200 to 230 g, as described in our previous study.24 This work was approved by the animal care and use committee of the Medical College of Georgia. In brief, while the rats were under chloral hydrate (350 mg/kg IP) anesthesia, the vertebral arteries were electrocauterized. Both common carotid artery separations were performed, a silicone elastomer ligature was placed loosely around each artery without interrupting blood flow, and the incision was then closed. Twenty-four hours later, the animals were reanesthetized in a jar containing 3% isoflurane. The rats were removed from the jar, and the common carotid arteries were re-exposed and clipped with aneurysm clips for 10 minutes. Rats whose pupils were dilated and unresponsive to light during ischemia were selected for the experiments. An electroencephalogram was monitored to ensure isoelectricity within 30 seconds after carotid artery occlusion. Carotid artery blood flow was restored by releasing the clips. With use of an overhead infrared lamp and a heating pad throughout the experiment, the pericranial temperature recorded at the left auricular tube was maintained at 37°C, and the rectal temperature was maintained at 36.5 to 37.5°C during and for 2 hours after ischemia. Sham control animals received the same surgical procedures except that the common carotid arteries were not occluded. The bpV(pic) (6 μL, 10 μmol/L) and/or Bic (3 μL, 10 nmol/L) were injected intracerebroventricularly into both cerebral ventricles (from the bregma: 0.8 mm posterior, ±1.5 mm lateral, and 3.5 mm deep) with a Hamilton microsyringe at a rate of 1 μL/min 1 hour after ischemia while the rats were under anesthesia. The animals were randomly assigned to experimental groups, and the

Figure 3. Overexpression of PTEN enhances surface expression of GABA<sub>A</sub> R y2 subunits. A, Left, Representative images showing the expression of PTEN in neurons transfected with PTEN-GFP. Arrowheads indicate successfully transfected neurons. Right, Bar graph showing that PTEN protein expression was significantly increased in neurons transfected with PTEN-GFP compared with those transfected with GFP (n=21 cells for PTEN-GFP group and n=31 cells for GFP group; *P<0.05, ANOVA). Three independent experiments were performed, and a minimum of 7 randomly selected cells per group per experiment were analyzed by an investigator blinded to the transfected constructs. Scale bar=15 μm. B, Left, Representative images showing that surface expression of GABA<sub>A</sub> R y2 subunits is decreased in neurons transfected with PTEN-GFP. Right, Bar graph showing that surface expression of GABA<sub>A</sub> R y2 subunits is significantly decreased in neurons transfected with PTEN-GFP compared with those transfected with GFP (n=24 cells for PTEN-GFP group and n=27 cells for GFP group; *P<0.05, ANOVA). Three independent experiments were performed, and a minimum of 8 randomly selected cells per group per experiment were analyzed by an investigator blinded to the transfected constructs. Scale bar=15 μm.
OGD Insult Reduces Surface Expression of GABA<sub>A</sub>Rs on the Neuron Membrane Surface

Because reduced expression of surface GABA<sub>A</sub>Rs may represent a novel mechanism underlying the effect of suppressed GABA<sub>A</sub>R function on ischemic neuron death, we first tested whether an ischemic insult could cause reduced expression of membrane GABA<sub>A</sub>Rs. The membrane expression of GABA<sub>A</sub>γ2 subunits was measured in cultured rat hippocampal neurons subjected to OGD, a well-established in vitro insult that mimics ischemic injury. The surface expression of γ2 subunits was labeled by a specific antibody recognizing the extracellular domain of γ2 under nonpermeable conditions. As expected, the surface expression of γ2 subunits was significantly reduced 24 hours after 40 minutes of OGD challenge (Figure 1A). By performing quantitative membrane protein biotinylation assays, we also confirmed that the protein level of membrane γ2 subunits was significantly decreased in injured neurons (Figure 1B). However, results from the same neuron samples tested by immunoblotting assay indicated that the protein level of whole-cell GABA<sub>A</sub>γ2 subunits was not significantly altered by OGD insult (Figure 1C), indicating that the specific reduction of membrane γ2 subunit expression is not due to inhibited protein synthesis or increased degradation of γ2 subunits.

PTEN Regulates Membrane Expression of GABA<sub>A</sub>Rs

We next determined whether PTEN regulates the surface expression of GABA<sub>A</sub>Rs. We first examined the effect of PTEN downregulation on surface GABA<sub>A</sub>γ2 expression in cultured hippocampal neurons transfected with PTEN siRNA. As illustrated in Figure 2A, the protein expression of PTEN was remarkably decreased in neurons transfected with siRNA-PTEN, but not with the SsiRNA-PTEN, indicating a specific knockdown of PTEN expression by PTEN siRNA introduction.4 Our data further showed that the surface expression of γ2 subunits was remarkably increased in neurons transfected with siRNA-PTEN, but not in neurons transfected with SsiRNA-PTEN (Figure 2B). These results suggest that endogenous PTEN may regulate the surface expression of GABA<sub>A</sub>Rs in hippocampal neurons.

To validate the modulation of GABA<sub>A</sub>γ2 expression by PTEN, we transfected the cDNAs of wild-type PTEN in cultured hippocampal neurons (Figure 3A). We showed that overexpression of PTEN in cultured neurons resulted in a significant reduction of surface γ2 expression (Figure 3B). Taken together, these results provide the first evidence that the surface expression of GABA<sub>A</sub>Rs is negatively regulated by PTEN in hippocampal neurons.

**Results**

OGD Insult Reduces Surface Expression of GABA<sub>A</sub>Rs on the Neuron Membrane Surface

PTEN Regulates Membrane Expression of GABA<sub>A</sub>Rs

**Conclusion**

The histological examination method has been described in detail in our previous study.25 To label the surviving cells, the mouse anti–neuron-specific nuclear protein (NeuN) monoclonal antibody (1:500, Millipore Corp) and the Alexa Fluor 594 donkey anti-mouse antibody (1:500, Invitrogen Canada Inc) were used. The fluorescent dye FluoroJadeB (Millipore Corp) was used to stain the neurons for signs of neurodegeneration. Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss) as described previously.26 The number of NeuN- or FluoroJadeB-positive CA1 neurons per 1-mm length of the medial CA1 pyramidal cell layer was counted bilaterally in 5 sections per animal. Cell counts from the right and left hippocampus on each of the 5 sections were averaged to provide the mean value.

**Statistics**

All data are expressed as mean±SEM. Statistical analysis was performed with a 2-way ANOVA followed by the Student-Newman-Keuls test. When only 2 groups were compared, a Student t test was used. Significance was placed at P<0.05.

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**Notes**

1. PTEN Regulates GABA<sub>A</sub>R Function

On the basis of the observed modulation of GABA<sub>A</sub>γ2 expression by PTEN, we hypothesized that PTEN might regulate the function of GABA<sub>A</sub>Rs in hippocampal neurons. We therefore recorded GABA<sub>A</sub>γ2 currents, compared with those in SsiRNA-PTEN (n=7) and control (n=6; *P<0.05, ANOVA) groups. Three independent transfection experiments were performed, and a minimum of 2 cells per group per experiment were recorded; (2) Inhibition of PTEN by bpV(pic) application to control neurons or neurons transfected with SsiRNA-PTEN marked enhanced GABA<sub>A</sub>R-mediated currents compared with those before bpV(pic) application. However, bpV(pic) application to neurons transfected with siRNA-PTEN caused no significant increase in GABA<sub>A</sub>R currents compared with those before bpV(pic) application, indicating that bpV(pic) specifically inhibits PTEN activity in rat hippocampal neurons.

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**Figure 4.** Suppression of PTEN by PTEN siRNA or PTEN inhibitor enhances GABA<sub>A</sub>R-mediated whole-cell currents. A, Representative tracings of GABA<sub>A</sub>R-mediated whole-cell currents in cultured rat hippocampal neurons. B, Bar graph for the study in A, showing that: (1) Downregulation of PTEN expression by siRNA-PTEN (n=7) significantly increased GABA<sub>A</sub>R-mediated currents, compared with those in SsiRNA-PTEN (n=7) and control (n=6; *P<0.05, ANOVA) groups. Three independent transfection experiments were performed, and a minimum of 2 cells per group per experiment were recorded; (2) Inhibition of PTEN by bpV(pic) application to control neurons or neurons transfected with SsiRNA-PTEN markedly enhanced GABA<sub>A</sub>R-mediated currents compared with those before bpV(pic) application. However, bpV(pic) application to neurons transfected with siRNA-PTEN caused no significant increase in GABA<sub>A</sub>R currents compared with those before bpV(pic) application, indicating that bpV(pic) specifically inhibits PTEN activity in rat hippocampal neurons.
mediated whole-cell currents in cultured hippocampal neurons in which PTEN expression was suppressed by PTEN siRNAs. Our data showed that neurons expressing siRNA-PTEN exhibited significantly larger peak amplitudes of GABA<sub>AR</sub> currents than did those expressing SsiRNA-PTEN (Figure 4A and 4B). Recent evidence indicates that at nanomolar concentrations, bpV(pic) selectively inhibits PTEN activity. We also showed that inhibition of PTEN by the PTEN inhibitor bpV(pic) (100 nmol/L) resulted in a significant increase in the peak amplitudes of GABA<sub>AR</sub> currents (Figure 4A and 4B). By treating neurons expressing siRNA-PTEN with bpV(pic), we provide evidence that introduction of PTEN siRNAs occluded the bpV(pic)-induced potentiation of GABA<sub>AR</sub>-mediated whole-cell currents, indicating the specificity of bpV(pic) in inhibiting PTEN activity in our experimental conditions. These results suggest that the functional activity of GABA<sub>AR</sub>s is negatively regulated by the phosphatase PTEN in hippocampal neurons.

**PTEN Inhibition Prevents OGD-Induced Decrease of Membrane GABA<sub>AR</sub>s**

The negative modulation of GABA<sub>AR</sub> expression by PTEN supports the possibility that the OGD-induced decrease of membrane GABA<sub>AR</sub>s might be blocked by PTEN inhibition. To address this possibility, a protein biotinylation assay was performed to determine the effect of bpV(pic) on the surface expression of γ2 subunits in cultured hippocampal neurons subjected to OGD insult for 40 minutes. Our data showed that the OGD-induced decrease in surface expression of γ2 subunits in ischemic neurons was significantly reduced by application of bpV(pic) (Figure 5A). We also performed cell ELISA assays (colorimetric assays) to examine the effect of bpV(pic) on the surface expression of γ2 subunits in neurons subjected to OGD. As expected, the cell ELISA assay yielded the same results as those in the biotinylation assay (Figure 5B). A control experiment, we showed that the total expression of whole-cell GABA<sub>AR</sub> γ2 subunits was not altered in the presence of OGD and/or bpV(pic) in rat hippocampal neurons. β-Actin was used as a loading control.  

**PTEN Inhibition–Induced Neuroprotection Occurs by Preserving GABA<sub>AR</sub> Function in Ischemic Neurons**

Given our findings that GABA<sub>AR</sub> function is negatively regulated by PTEN and that OGD-induced decrease of membrane GABA<sub>AR</sub>s is reduced by PTEN inhibition, we
reasoned that upregulation of GABA_3R function by PTEN inhibition might protect against ischemic neuron death. By performing an in vitro neuron viability assay, we demonstrated that treatment with the PTEN inhibitor bpV(pic) remarkably reduced OGD-induced neuronal death and that this neuroprotective effect was significantly blocked by the selective GABA_3R antagonist Bic (n=6 for each group; *P<0.05, ANOVA). These results suggest that the upregulated GABA_3Rs in injured neurons contribute to PTEN inhibition-induced neuroprotection.

To provide in vivo evidence for the role of PTEN suppression in protecting against ischemic neuron death through preservation of GABA_3R function, we tested the effect of bpV(pic) in an animal model of global cerebral ischemia. NeuN was used to label neurons, and FluoroJadeB (a marker of neuronal degeneration) was used to label the neurodegenerative cells. Our data showed that NeuN-positive neurons were significantly decreased, whereas FluoroJadeB-positive neurons were markedly increased 7 days after ischemia/

Figure 6. PTEN inhibition protects neurons from OGD-induced neuronal death by enhancing GABA_3R function. A, Bar graph of data from the neuron viability assay. Data from each group were normalized to that of controls (=100%). The significant decrease of neuronal viability in the OGD group was reduced by bpV(pic) treatment. However, the neuroprotective effect of bpV(pic) was attenuated by the GABA_3R antagonist Bic (n=6 for each group; *P<0.05, ANOVA). B1, Representative images showing NeuN antibody–labeled normal neurons and degenerated cells (labeled with FluoroJadeB) in the CA1 region of rat hippocampus under control conditions (sham group), reperfusion after global ischemia (I/R7 day group), bpV(pic) treatment after global ischemia (bpV group), and bpV(pic)+Bic treatment after global ischemia (bpV+Bic group). Magnification in the left column of each labeling is ×5, scale bar=200 μm, and in the right columns is ×40, scale bar=50 μm. B2, Bar graphs of study in B1, showing that PTEN inhibition reduced ischemia/reperfusion-induced neuronal death and that this neuroprotective effect was attenuated by inhibition of GABA_3Rs in rat hippocampus (sham group, n=5; I/R7 day group, n=5; bpV group, n=5; bpV+Bic group, n=7; *P<0.05, ANOVA).
reperfusion compared with the sham group (Figure 6B), indicating significant ischemic neuron death in the present experimental model. Our data further showed that PTEN inhibition by bpV(pic) treatment remarkably reduced ischemia-induced neuronal death (Figure 6B). However, treatment with the GABA_A receptor antagonist Bic significantly attenuated the bpV(pic)-mediated neuroprotective effect (Figure 6B). Taken together, this study demonstrates that downregulating PTEN protects against neuronal death by preventing the suppression of GABA_A function in ischemic neurons. These results provide direct evidence suggesting that PTEN/GABA_A-dependent signaling may be a potential therapeutic target for stroke treatment.

Discussion

Coordinated balance of inhibitory and excitatory neurotransmission, mediated respectively by GABA_A Rs and glutamate receptors, controls neuronal communication in normal brain function. However, an ischemic insult profoundly shifts the balance in the direction of overexcitation due to the excess release of excitatory amino acids and/or suppression of the GABA-ergic system. The present study provides evidence supporting the notion that GABA_A function is suppressed in OGD-treated neurons and that the reduced GABA_A expression may contribute to OGD insult–induced suppression of GABA_A function. Thus, preserving or enhancing GABA_A function and expression may be a potential strategy to protect against ischemic neuron death.

Although enhancing GABA_A function by GABA_A agonists has been shown to protect against delayed neuronal death in experimental models of cerebral ischemia, use of GABA_A agonists as neuroprotective agents has been disappointing in clinical trials. One simple explanation is that the adverse effects may be caused by agonist-induced global overactivation of GABA_A Rs, which would not only lead to an antiepileptoid effect but also result in unwanted activities mediated by GABA_A overactivation. The present results suggest that investigating intracellular signaling such as PTEN linked to GABA_A Rs in ischemic neurons is a critical approach in future research.

An increasing number of studies have provided evidence suggesting that the tumor suppressor PTEN is critically involved in the cellular process of neuronal death and that suppressing PTEN enhances the potential for neuronal survival. However, how PTEN inhibition exerts its neuroprotective effects in injured neurons remains largely unknown. In the present study, we provide the first evidence that PTEN inhibition, through enhancing GABA_A expression and function, protects against neuronal death in the in vitro OGD and in vivo ischemic stroke models. As our previous finding indicated that inhibition of NR2B subunit containing N-methyl-D-aspartate receptors contributes to PTEN suppression–induced neuroprotection, PTEN suppression may restore the balance between excitatory and inhibitory signaling in ischemic neurons by both preserving GABA_A function and inhibiting overactivated N-methyl-D-aspartate receptors. Our previous evidence also indicates that PTEN suppression protects against ischemic neuron death, at least in part, through activation of an Akt-dependent cell survival–promoting pathway. Thus, a differential therapeutic strategy should be considered for stroke patients on the basis of the multiple neuroprotection mechanisms mediated by PTEN suppression. In addition to hippocampal neurons, neurons in other areas could be affected by ischemic stroke. Our future studies will investigate the effects of PTEN suppression on neurons in other areas, such as the cortex, after ischemic stroke.

Acknowledgment

We thank K.M. Yamada for the PTEN plasmid.

Source of Funding

This work was supported by grants from the Heart and Stroke Foundation of Canada and the Canadian Institutes of Health Research to Q.W.

Disclosures

None.

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*Stroke*. 2010;41:1018-1026; originally published online April 1, 2010; doi: 10.1161/STROKEAHA.110.579011

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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