Preservation of GABA<sub>A</sub> 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 γ-aminobutyric acid subtype A receptors (GABA<sub>A</sub>Rs) as a molecular target for the treatment of ischemic stroke. Because suppression of GABA<sub>A</sub>Rs contributes to ischemic neuron death, describing the intracellular signaling that interacts with GABA<sub>A</sub>Rs in ischemic neurons would provide a molecular basis for novel stroke therapies.

Methods—We measured surface GABA<sub>A</sub>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<sub>A</sub>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<sub>A</sub>Rs in rat hippocampal neurons. Importantly, we demonstrated that a PTEN inhibitor prevented the reduction of surface GABA<sub>A</sub>Rs in injured hippocampal neurons subjected to oxygen-glucose deprivation, an in vitro insult that mimics ischemic injury, whereas a GABA<sub>A</sub>R antagonist significantly reduced this PTEN inhibitor–induced neuroprotection in both the 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<sub>A</sub>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<sub>A</sub> 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 γ-aminobutyric acid subtype A receptors (GABA<sub>A</sub>Rs) causes neuronal damage after stroke. Both the GABA uptake inhibitor and the GABA<sub>A</sub>R agonist are neuroprotective in animal models of ischemic stroke. A recent study has shown that cell surface GABA<sub>A</sub>Rs are markedly decreased in neurons treated by oxygen-glucose deprivation (OGD), suggesting that a change in the number of membrane GABA<sub>A</sub>Rs may be a crucial process in enhancing ischemic neuron death. However, use of GABA<sub>A</sub>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<sub>A</sub>Rs in ischemic neurons would aid us to understand the cellular and molecular mechanisms underlying PTEN downregulation–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 ARs, and the most abundant population of GABA ARs in the mammalian brain is the α1β2γ2 combination. Recent studies indicate that trafficking of GABA ARs to and from the membrane surface plays an important role in posttranslational signaling mechanisms that modulate the stability, density, and function of the channels and that the γ2 subunit is required for the trafficking of GABA ARs to the postsynaptic membrane. Interestingly, a recent study has shown that GABA ARs are upregulated by activation of phosphatidylinositol 3-kinase/Akt–dependent signaling. Because Akt activation is negatively regulated by PTEN, we hypothesized that GABA ARs may be upregulated by PTEN inhibition.

Given the involvement of PTEN and GABA ARs in ischemic neuron damage, the present study tested the effect of PTEN downregulation on GABA AR 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 AR 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. To initiate the OGD challenge, cells were transferred to deoxygenated, glucose-free, extracellular solution (in mmol/L: 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1.0 NaH₂PO₄, 1.8 CaCl₂, and 26 NaHCO₃); introduced into a specialized, humidified chamber; and maintained at 37°C for 40 minutes in 95% O₂/5% CO₂.

To measure the surface expression of GABA ARs, surface protein labeling and Western blot analysis were performed. The neuronal membrane protein preparation was processed and imaged in parallel. Three fields were randomly chosen under bright-field optics, and fluorescence images of each cell in control and OGD groups from the same culture preparation were transferred for analysis. The cells in control and OGD groups from the same culture were used for labeling.

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 fields were randomly selected in each culture. The fluorescence density was analyzed by Image J software (National Institutes of Health, Bethesda, Md). 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 ARs, surface protein biotinylation assays were performed. The neuronal membrane pro-
teins were biotinylated and isolated with a cell surface protein biotinylation and purification kit (Thermo Fisher Scientific Inc, Rockford, Ill). In brief, the same amount of neuronal cells from each experimental group was collected for the assay. The cells were first incubated with sulfo succinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate at 4°C for 30 minutes, and then lysis buffer was added. The lysate was incubated with immobilized NeutrAvidin gel for 60 minutes to isolate the labeled proteins. The GABAAR subunit was quantified by Western blot assay as described previously.

**Colorimetric Assays**

Colorimetric assays were also performed to measure the surface expression of GABAARs in cultured neurons. To label surface GABAAR γ2 subunits, cells were fixed with paraformaldehyde (4% for 10 minutes) under nonpermeabilized conditions and labeled with rabbit anti-GABAAR γ2 antibody (Millipore Corp) raised against the N-terminal extracellular domain of γ2. 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’-AGAGATCGTTAGCAGAAG-3’) and a scrambled sequence (5’-AGAGACAGAAACTCGTTAG-3’) were selected for constructing an siRNA-PTEN green fluorescent protein (GFP) vector and a scrambled PTEN siRNA vector (siRNA-PTEN). Transfection of PTEN siRNAs and wild-type PTEN-GFP (PTEN-GFP) was performed with the use of lipofectamine 2000 (Invitrogen) as described previously. GFP-positive cells were selected for immunostaining and patch-clamp recording.

**Recording of GABA\(_{\gamma2}\)-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\(_2\), 1 mmol/L CaCl\(_2\), 5 mmol/L EGTA, 10 mmol/L HEPES, and 4 mmol/L K\(_2\)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\(_2\), 1 mmol/L MgCl\(_2\), 25 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\(_{\gamma2}\)-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.
Alamar Blue Cell Viability Assay

Neuron viability was measured by the Alamar Blue cell viability assay. 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. 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. 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 arteries were separated, 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 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 mmol/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
experimenter were blinded to group assignment. The animals were returned to their cages for 7 days after ischemia/reperfusion and then used for further experiments.

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’s t test was used. Significance was placed at P<0.05.

**Results**

**OGD Insult Reduces Surface Expression of GABA\(_{\text{A}}\)Rs on the Neuron Membrane Surface**

Because reduced expression of surface GABA\(_{\text{A}}\)Rs may represent a novel mechanism underlying the effect of suppressed GABA\(_{\text{A}}\)R function on ischemic neuron death,11 we first tested whether an ischemic insult could cause reduced expression of membrane GABA\(_{\text{A}}\)Rs. The membrane expression of GABA\(_{\text{A}}\)R \(\gamma\) 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 \(\gamma\) subunits was labeled by a specific antibody recognizing the extracellular domain of \(\gamma\) subunits under nonpermeable conditions.18 As expected, the surface expression of \(\gamma\) 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 \(\gamma\) 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\(_{\text{A}}\)R \(\gamma\) subunits was not significantly altered by OGD insult (Figure 1C), indicating that the specific reduction of membrane \(\gamma\) subunit expression is not due to inhibited protein synthesis or increased degradation of \(\gamma\) subunits.

**PTEN Regulates Membrane Expression of GABA\(_{\text{A}}\)Rs**

We next determined whether PTEN regulates the surface expression of GABA\(_{\text{A}}\)Rs. We first examined the effect of PTEN downregulation on surface GABA\(_{\text{A}}\)R 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 \(\gamma\) 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\(_{\text{A}}\)Rs in hippocampal neurons.

To validate the modulation of GABA\(_{\text{A}}\)R 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 \(\gamma\) expression (Figure 3B). Taken together, these results provide the first evidence that the surface expression of GABA\(_{\text{A}}\)Rs is negatively regulated by PTEN in hippocampal neurons.

**PTEN Regulates GABA\(_{\text{A}}\)R Function**

On the basis of the observed modulation of GABA\(_{\text{A}}\)R expression by PTEN (Figures 2 and 3), we hypothesized that PTEN might regulate the function of GABA\(_{\text{A}}\)Rs in hippocampal neurons. We therefore recorded GABA\(_{\text{A}}\)R-
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\(_\text{A}\) currents than did those expressing SsiRNA-PTEN (Figure 4A and 4B). Recent evidence indicates that at nanomolar concentrations, bpV(pic) selectively inhibits PTEN activity.\(^{23}\) 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\(_\text{A}\) 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\(_\text{A}\)-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\(_\text{A}\)Rs is negatively regulated by the phosphatase PTEN in hippocampal neurons.

**PTEN Inhibition Prevents OGD-Induced Decrease of Membrane GABA\(_\text{A}\)Rs**

The negative modulation of GABA\(_\text{A}\)R expression by PTEN supports the possibility that the OGD-induced decrease of membrane GABA\(_\text{A}\)Rs 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 \(\gamma\) subunits in cultured hippocampal neurons subjected to OGD insult for 40 minutes. Our data showed that the OGD-induced decrease in surface expression of \(\gamma\) 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 \(\gamma\) subunits in neurons subjected to OGD.\(^{27}\) As expected, the cell ELISA assay yielded the same results as those in the biotinylation assay (Figure 5B). As a control experiment, we showed that the total expression of whole-cell GABA\(_\text{A}\)R \(\gamma\) subunits was not altered in the presence of OGD and/or bpV(pic) in rat hippocampal neurons. Therefore, suppressing PTEN prevents OGD-induced reduction of surface GABA\(_\text{A}\)Rs in hippocampal neurons.

**PTEN Inhibition–Induced Neuroprotection Occurs by Preserving GABA\(_\text{A}\)R Function in Ischemic Neurons**

Given our findings that GABA\(_\text{A}\)R function is negatively regulated by PTEN and that OGD-induced decrease of membrane GABA\(_\text{A}\)Rs is reduced by PTEN inhibition, we
reasoned that upregulation of GABA$_A$R 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$_A$R antagonist Bic (Figure 6A). These results suggest that the upregulated GABA$_A$Rs 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$_A$R 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/
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<sub>A</sub>R 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<sub>A</sub>R function in ischemic neurons. These results provide direct evidence suggesting that PTEN/GABA<sub>A</sub>R-dependent signaling may be a potential therapeutic target for stroke treatment.

**Discussion**

Coordinated balance of inhibitory and excitatory neurotransmission, mediated respectively by GABA<sub>A</sub>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<sub>A</sub>R function is suppressed in OGD-treated neurons and that the reduced GABA<sub>A</sub>R expression may contribute to OGD insult–induced suppression of GABA<sub>A</sub>R function. Thus, preserving or enhancing GABA<sub>A</sub>R function and expression may be a potential strategy to protect against ischemic neuron death. Although enhancing GABA<sub>A</sub>R function by GABA<sub>A</sub>R agonists has been shown to protect against delayed neuronal death in experimental models of cerebral ischemia, use of GABA<sub>A</sub>R 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<sub>A</sub>Rs, which would not only lead to an antiexcitotoxic effect but also result in unwanted activities mediated by GABA<sub>A</sub>R overactivation. The present results suggest that investigating intracellular signaling such as PTEN linked to GABA<sub>A</sub>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<sub>A</sub>R 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<sub>A</sub>R 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 neuroprotective 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.

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**Disclosures**

None.

**References**


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