α7 Nicotinic Acetylcholine Receptor Agonism Confers Neuroprotection Through GSK-3β Inhibition in a Mouse Model of Intracerebral Hemorrhage

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Background and Purpose—Perihematomatol edema formation and consequent cell death contribute to the delayed brain injury evoked by intracerebral hemorrhage (ICH). We aimed to evaluate the effect of α7 nicotinic acetylcholine receptor (α7nAChR) stimulation on behavior, brain edema, and neuronal apoptosis. Furthermore, we aimed to determine the role of the proapoptotic glycogen synthase kinase-3β (GSK-3β) after experimental ICH.

Methods—Male CD-1 mice (n = 109) were subjected to intracerebral infusion of autologous blood (n = 88) or sham surgery (n = 21). ICH animals received vehicle administration, 4 or 12 mg/kg of α7nAChR agonist PHA-543613, 12 mg/kg of α7nAChR agonist PNU-282987, 6 mg/kg of α7nAChR antagonist methyllycaconitine (MLA), 15 μg/kg of phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, or PHA-543613 combined with MLA or wortmannin. Behavioral deficits and brain water content were evaluated at 24 and 72 hours after surgery. Western blotting and immunofluorescence staining were used for the quantification and localization of activated Akt (p-Akt), GSK-3β, and cleaved caspase-3 (CC3). Neuronal cell death was quantified through terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL).

Results—α7nAChR stimulation improved neurological outcome and reduced brain edema at 24 and 72 hours after surgery (P < 0.05 compared with vehicle). Furthermore, PHA-543613 treatment increased p-Akt and decreased p-GSK-3β and CC3 expressions in the ipsilateral hemisphere (P < 0.05, respectively), which was reversed by MLA and wortmannin. P-Akt, p-GSK-3β, and CC3 were generally localized in neurons. PHA-543613 reduced neuronal cell death in the perihematomatol area (P < 0.05).

Conclusions—α7nAChR stimulation improved functional and morphological outcomes after experimental ICH in mice. PHA-543613 reduced the expression of proapoptotic GSK-3β through the PI3K-Akt signaling pathway. (Stroke. 2012; 43:00-00.)

Key Words: α7 nicotinic acetylcholine receptor ■ glycogen synthase kinase-3 ■ intracerebral hemorrhage ■ apoptosis ■ caspase-3 ■ PHA-543613 ■ PHA-282987

The prognosis of spontaneous intracerebral hemorrhage (ICH) is often less favorable than that of similar-sized ischemic strokes.1 Approximately 15% of all cerebrovascular accidents are hemorrhagic in nature, not including the considerable proportion of ischemic brain lesions (30%) that will eventually undergo hemorrhagic transformation.2,3 The expanding blood clot as well as the subsequent formation of brain edema induce cellular and molecular processes, which provoke neuronal apoptosis, thus aggravating the injury after ICH.3–5 Consequently, prospective treatments reducing brain edema or apoptotic cell death may provide a neuroprotective strategy for ICH patients.

α7 nicotinic acetylcholine receptors (α7nAChR) are expressed in neurons, neuroglia, and endothelial cells of the mammalian brain.6–8 In addition to their effects as ligand-gated ion channels, α7nAChR stimulation attenuates neuronal apoptosis in various disease models, particularly through activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway.7–9 Activated Akt inhibits the glycogen synthase kinase-3β (GSK-3β) in neurons and nonneuronal cells by serine-9 phosphorylation.10 Several proapoptotic stimuli, including oxygen-glucose deprivation and growth factor withdrawal, have been reported to increase the activity—as well as the tyrosine-216 phosphorylation—of GSK-3β.10–12 Phosphorylated GSK-3β (p-GSK-3β, Tyr216) induces caspase-3 activation, an early step in the execution phase of cellular apoptosis.13 Furthermore, stimulated GSK-3β has been found...
to aggravate the brain injury after experimental ischemic stroke and to study the role of GSK-3β in ICH.

In the present study, we aim to investigate 2 hypotheses: (A) Selective α7nAChR agonists, PHA-543613 and PNU-282987, ameliorate behavioral and morphological outcomes (brain edema) after experimental ICH in mice and (B) α7nAChR stimulation reduces activated GSK-3β through the PI3K-Akt signaling pathway, eventually decreasing neuronal apoptosis.

**Methods**

**Animals and Intracerebral Blood Infusion**

All procedures were conducted following an institutionally approved protocol in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male CD-1 mice (n = 109, weighing 35–45 g; Charles River, Wilmington, MA) were housed in a light- and temperature-controlled environment with unlimited access to food and water.

Hemorrhagic stroke in mice was induced by using a modified double infusion model of autologous whole blood (30 µL) as previously reported. Briefly, mice were anesthetized through the use of intraperitoneal coinjection of ketamine (100 mg/kg) and xylazine (10 mg/kg) in a 2:1 vol/vol ratio. With the mouse positioned prone onto a stereotactic head frame (Kopf Instruments, Tujunga, CA), a craniotomy was performed and a 27-gauge needle was inserted into the right hemisphere (stereotactic coordinates from bregma: 0.2 mm anterior, 2.0 mm lateral and 3.0 mm in depth). Autologous whole blood (5 µL), collected from the central tail vein, was infused at a rate of 2 µL/min. The needle was then lowered to the target position of 3.7 mm in depth. After a waiting period of 5 minutes, 25 µL of autologous whole blood was infused into the right basal ganglia. The needle was left in place for an additional 10 minutes after the completed infusion, before being withdrawn at a rate of 1 mm/min. The craniotomy was sealed with bone wax and the scalp was sutured. All mice were allowed to fully recover under observation. Sham-operated animals were subjected to needle insertion only.

**Experimental Groups and Pharmacological Interventions**

Experimental groups consisted of mice subjected to ICH (n = 88) or sham surgery (n = 21). After ICH induction, animals were randomly selected and treated with either 4 or 12 mg/kg of α7nAChR agonist PHA-543613 (PHA-4 mg, PHA-12 mg) or with 12 mg/kg of α7nAChR agonist PNU-282987 (PNU-12 mg). All treatments were administered as an intraperitoneal injection at 1 hour after surgery. Alternatively, ICH mice received 6 mg/kg of the α7nAChR antagonist methyllycaconitine (MLA) intraperitoneally at 15 minutes, or the PI3K inhibitor wortmannin (15 µg/kg) intravenously, at 90 minutes before PHA-543613 (12 mg/kg) injection (PHA + MLA, PHA + Wort). MLA and wortmannin were also administered without consequent α7nAChR agonist treatment (MLA-6 mg, Wort-15 µg). Sham and vehicle animals received an equivalent volume of normal saline, as given to animals in the intervention groups (0.2 mL). All drugs were purchased from Sigma-Aldrich (St Louis, MO) and processed according to previously published protocols.

**Assessment of Behavioral Outcome**

Behavioral outcomes were assessed in a blinded fashion at 24 and 72 hours after surgery. The sensorimotor Garcia test was modified for use in mice after experimental hemorrhagic stroke. This composite assessment consists of 7 individual tests evaluating spontaneous activity (I), axial sensation (II), vibrissae proprioception (III), symmetry of limb movement (IV), lateral turning (V), forelimb outstretching (VI), and climbing (VII). Each test received a score between 0 (worst performance) and 3 (best performance), and a total García score was calculated as the sum of all subtests (maximum = 21 points). For the corner turn test, mice were allowed to advance into a 30° angled corner. To exit the corner, mice could turn either to the right or to the left. The choice of turning side was recorded for each animal. A coronal brain section of 4-mm thickness was separated 2 mm anterior and posterior of the needle tract and then further divided into ipsilateral and contralateral cortex and basal ganglia. The cerebellum was additionally collected as an internal control. All brain specimens were weighed using an analytic microbalance (APX-60, Denver Instruments, Bohemia, NY) to obtain the wet weight. Samples were then dried at 100°C for 24 hours before determining the dry weight. The brain water content (%) was calculated as (wet weight – dry weight)/wet weight × 100.

**Western Blotting**

Mice were euthanized at 24 hours after surgery, and ipsilateral hemispheres were isolated and processed as previously described.

Equal amounts of protein (50 µg) were separated by SD&A PAGE, then transferred onto nitrocellulose membranes and incubated with the respective primary and secondary antibodies (1:1000). The following primary antibodies were obtained from Cell Signaling Technology: anti-p-Akt (Ser473), anti-Akt, anti–GSK-3β, and anti–cleaved caspase-3 (CC3). Anti–p–GSK-3β (Tyr216), anti–β-Actin, and all secondary antibodies were purchased from Santa Cruz Biotechnology. Immunoblots were visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and densitometrically quantified, using the software ImageJ (National Institutes of Health). Results are expressed as relative density ratio, normalized to the mean value of the sham group.

**Immunofluorescence Staining**

Mice were euthanized at 24 hours after surgery, and brain specimens were processed as previously described with minor modifications. Triple immunofluorescence staining was performed using the neuronal marker anti-NeuN (1:100, Millipore, Temecula, CA) and anti–p–GSK-3β (Tyr216, 1:100) in combination with terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) (in situ Cell Death Detection Kit, Fluorescin, Roche Inc, Mannheim, Germany). Double immunofluorescence staining was performed with anti–NeuN and anti-CC-3 (1:1000). Microphotographs were analyzed with the use of a fluorescent microscope and Magna Fire SP system (Olympus). TUNEL-positive neurons were counted at ×400 magnification in 4 perihematomal areas (500×500-µm grids), and the data were expressed as cells/mm².

**Statistical Analysis**

Data were expressed as mean ± SEM and statistically analyzed with 1-way ANOVA followed by Tukey post hoc test. All behavior data were expressed as mean ± SEM of sham performance and analyzed with Kruskal-Wallis 1-way ANOVA on ranks, followed by the Student-Newman-Keuls method. A probability value of <0.05 was
Results

**PHA-543613–Mediated Attenuation of Behavioral Deficits and Brain Edema at 24 Hours After ICH Is Dependent on the PI3K-Akt Signaling Pathway**

Behavioral deficits were evaluated at 24 hours after surgery (n=6 per group). Mice subjected to ICH presented a significantly worse Garcia test performance than sham-operated animals (P<0.05, Figure 1A); however, treatment with α7nAChR agonists PHA-543613 (PHA-4 mg, PHA-12 mg) or PNU-282987 (PNU-12 mg) improved the outcome significantly (P<0.05, compared with vehicle). Mice within the pharmacological intervention groups additionally received an injection of the α7nAChR antagonist MLA (PHA+MLA) or the PI3K inhibitor wortmannin (PHA+Wort) before PHA-543613 (12 mg/kg) administration to assess whether the observed behavioral improvements are dependent on α7nAChR-induced activation of the PI3K-Akt signaling pathway. Wortmannin, as anticipated, reversed the initial attenuation of behavioral deficits observed with PHA-543613. The 2 applied inhibitors, MLA (6 mg/kg) and wortmannin (15 μg/kg), did not worsen the behavioral outcome of the Garcia test when administered alone (P>0.05, compared with vehicle). After experimental right-sided ICH, mice turned less frequently to the impaired (left) side while performing the corner turn test (P<0.05 compared with sham); however, no differences were found between treated and untreated ICH animals (P>0.05). Furthermore, vehicle animals demonstrated significantly impaired contralateral (left) forelimb function, evaluated through the forelimb placing test, as well as a reduced number of spontaneous alterations during the T-maze assessment (P<0.05 compared with sham). Treatments of PHA-543613 (PHA-12 mg) or PNU-282987 (PNU-12 mg) improved the forelimb placing
ability and increased the number of spontaneous alterations in ICH animals ($P<0.05$ compared with vehicle). In contrast, mice receiving the $\alpha$7nAChR agonist PHA-543613 (12 mg/kg) combined with MLA (PHA+MLA) or wortmannin (PHA+Wort) showed no significant differences compared with the respective vehicle group ($P>0.05$).

Brain edema was evaluated at 24 hours after surgery (n=6 per group). Treated mice (PHA-12 mg) showed a significantly reduced brain water content in the ipsilateral basal ganglia ($P<0.05$, compared with vehicle, Figure 1B); however, coadministration of MLA (PHA+MLA) or wortmannin (PHA+Wort) reversed this effect entirely ($P<0.05$ compared with PHA-12 mg). The latter compounds did not increase brain edema compared with the vehicle ($P>0.05$). No significant differences were evident between all groups, in contralateral and ipsilateral cortex, contralateral basal ganglia, or in the cerebellum ($P>0.05$).

### PHA-543613 Attenuates Behavioral Deficits and Brain Edema at 72 Hours After ICH

Sham-operated and vehicle and treated (PHA-12 mg) animals (n=6 per group) were used to evaluate behavioral outcomes and brain edema at 72 hours after surgery (Figure 2). PHA-543613 treatment significantly improved performances of Garcia, corner turn, forelimb placing, and T-maze tests as compared with the vehicle group ($P<0.05$).

Brain water content of the ipsilateral basal ganglia was significantly higher in the vehicle group than in sham animals ($P<0.05$, Figure 2B), and PHA-543613 treatment (PHA-12 mg) reduced the amount of brain edema significantly ($P<0.05$, compared with vehicle). No significant differences were evident between all groups, in contralateral and ipsilateral cortex, contralateral basal ganglia, or in the cerebellum ($P>0.05$).

### PHA-543613 Activates the PI3K-Akt Signaling Pathway and Decreases Protein Expressions of g-GSK-3β and CC-3 at 24 Hours After ICH

To further confirm that PHA-543613–mediated neuroprotection is dependent on the antiapoptotic PI3K-Akt signaling pathway, Western blot analysis was conducted and the changes in protein levels of phosphorylated Akt (p-Akt, Ser473), phosphorylated GSK-3β (p-GSK-3β, Tyr216), and CC3 were quantified. The treatment (PHA-12 mg) increased phosphorylation of Akt significantly, compared with both the sham and the vehicle group ($P<0.05$, n=5 per group, Figure 3A). MLA and wortmannin (PHA+MLA, PHA+Wort) significantly decreased the level of p-Akt at 24 hours after ICH ($P<0.05$, compared with PHA-12 mg). GSK-3β phosphorylation was significantly reduced by PHA-543613 ($P<0.05$, compared with vehicle, Figure 3B); however, MLA and wortmannin reversed this effect entirely. PHA-12 mg, brain specimens contained significantly decreased quantities of CC3 ($P<0.05$, compared with vehicle, Figure 1C), and wortmannin (PHA+Wort) abolished the antiapoptotic effect of the treatment ($P<0.05$, compared with PHA-12 mg).

### PHA-543613 Reduces Neuronal Apoptosis at 24 Hours After ICH

Immunofluorescence staining and neuronal cell death quantification was performed in sham, vehicle, and PHA-12 mg, animals (n=4 per group). All mice were euthanized at 24 hours after ICH or sham surgery, and immunoreactivities of NeuN, p-GSK-3β, TUNEL, and CC3 were evaluated in the perihematomal area or near the needle tract in sham animals. Consistent with the Western blot results, the immunofluorescence analysis revealed decreased expressions of p-GSK-3β (Figure 4A) and CC3 (Figure 4B) in the treatment group (PHA-12 mg). TUNEL-positive cells (Figure 4A) were less apparent after PHA-543613 treatment. Immunoreactivities of p-GSK-3β, TUNEL, and CC3 were generally colocalized within NeuN-positive cells (neurons).

The total number of TUNEL and NeuN double-stained cells (TUNEL+neurons) in the perihematomal area was significantly decreased after PHA-543613 treatment compared with the vehicle group at 24 hours after ICH ($P<0.05$, Figure 4C).

### Discussion

The first aim of the present study was to investigate whether the $\alpha$7nAChR agonists PHA-543613 and PNU-282987 ameliorate behavioral deficits and brain edema after experimental ICH in mice. Hemispheric hemorrhage, affecting the basal
ganglia, has been reported to cause debilitating sensorimotor as well as cognitive deficits in humans. Intending to achieve a translational perspective, we included sensorimotor (Garcia, corner turn, and forelimb placing test) and cognitive (T-maze) assessments in our experiment. The composite Garcia test sensitively detects sensorimotor consequences of unilateral hemorrhagic or ischemic brain injury. Both the corner turn and the forelimb placing test have been widely used to evaluate lateralizing behaviors as well as sensorimotor impairments in preclinical ICH studies. During the T-maze assessment, rodents normally select alternating arms on consecutive trials (spontaneous alterations). A decreased number of those spontaneous alterations correlates well with the severity of the cognitive impairment in injured animals. High-dose treatment (12 mg/kg) of PHA-543613 or PNU-282987 significantly improved the outcome of all conducted behavioral tests, with the exception of the corner turn test performance at 24 hours after surgery (compared with vehicle). Even mild unilateral brain injuries may cause a lateralizing behavior in rodents, and the outcome of the corner turn test after experimental ICH was previously reported to improve to a lesser extent than the outcome of the forelimb placing test. Brain edema, defined as an increase in the water content of brain tissue, is observed in acute and delayed stages after ICH. Several studies suggest a close association between the degree of perihematomal brain edema and poor outcome in patients. Our results showed significantly reduced brain water content of the ipsilateral basal ganglia in the PHA-12 mg, group, at 24 and 72 hours after ICH induction (compared with vehicle). The aforementioned findings support our first hypothesis that the α7nAChR agonists PHA-543613 and PNU-282987 ameliorate behavioral and morphological outcomes (brain edema) after ICH in mice. PHA-543613, in a concentration of 12 mg/kg, ameliorated the brain injury to a greater extent than did 4 mg/kg of PHA-543613 and 12 mg/kg of PNU-282987 (Figure 1) and was therefore exclusively applied in the following experiments (Figures 2, 3, and 4). We previously reported that short-term activation of the α7nAChR through PNU-282987 did not alter physiological parameters (blood pressure, heart rate) or plasma concentrations of Na⁺, K⁺, Cl⁻, and glucose in rodents. The observed amelioration of the brain injury in the treatment groups (PHA-4 mg, PHA-12 mg, and PNU-12 mg) were unlikely to be caused by changes of physiological parameters.

Aiming to further examine the dependence between α7nAChR-induced neuroprotection and the PI3K-Akt signaling pathway, we administered either MLA or wortmannin before PHA-543613 (12 mg/kg). MLA is a potent competitor, interfering with the [125I]-bungarotoxin binding site of the α7nAChR (Kᵢ=1.4 nm). MLA is quickly redistributed from the vasculature to the brain, where it extensively inhibits the receptor activation by PHA-543613 (Kᵢ=8.8 nm). Wortmannin inhibits PI3K irreversibly and is commonly used to prevent downstream phosphorylation of Akt. Animals receiving these interventions in addition to the treatment did not show functional or morphological improvements at 24 hours after surgery. Furthermore, MLA or wortmannin ad-
ministered alone did not worsen the injury compared with the vehicle. These results indicate that α7nAChR agonists exert their neuroprotective effects through the PI3K-Akt signaling pathway. Consistent with these findings, earlier studies demonstrated the ability of α7nAChR agonists to trigger PI3K activation, possibly through stimulation of the receptor’s catalytic intracellular domain; however, the exact mechanism of this process remains yet to be discovered. Akt, a serine/threonine kinase, which is directly activated by PI3K-mediated phosphorylation, stimulates several antiapoptotic mechanisms, among them the inhibition of GSK-3β. Belonging also to the serine/threonine kinase family, GSK-3β has been described to activate proapoptotic caspase-3, thus aggravating neuronal injury after experimental ischemic stroke and subarachnoid hemorrhage.

In the present study, we additionally intended to show the protective effect of GSK-3β inhibition. PHA-543613 treatment significantly increased the protein expression of activated Akt (p-Akt, Ser473) in the ipsilateral hemisphere at 24 hours after surgery, which successively reduced the expression of activated GSK-3β (p-GSK-3β, Tyr216) and CC3. MLA or wortmannin, in combination with PHA-543613, showed similar levels of the respective proteins as the vehicle group. These results support the proposed antiapoptotic effect of α7nAChR agonism through PI3K-Akt activation and resulting GSK-3β-inhibition.

Given the fact that α7nAChR are not only expressed in neurons but also in neuroglia and endothelial cells of the mammalian brain, we used immunofluorescence staining to show colocalizations of p-GSK-3β and CC3 with NeuN (neuronal marker). Both target proteins were generally localized in neurons, and PHA-543613 treatment reduced the intensity of p-GSK-3β and CC3 immunofluorescence in the perihematomal area. We furthermore used TUNEL to detect apoptotic cell death and observed a significantly reduced density of apoptotic neurons in the treatment group (compared with vehicle). Added together, these findings support our second hypothesis, suggesting that α7nAChR stimulation reduces activated GSK-3β through the PI3K-Akt signaling pathway, thus decreasing the incidence of neuronal apoptosis after ICH in mice.

In the present study, we investigated the antiapoptotic effects of α7nAChR; however, previously reported anti-inflammatory properties of this receptor subtype may also participate in neuroprotection after hemorrhagic stroke. Inflammatory mechanisms, mediated by cellular components such as resident immune cells of the brain (microglia), are involved in the progression of the ICH-induced brain injury. Activation of α7nAChR in microglial cells inhibits the production of proinflammatory cytokines but not the production of anti-inflammatory cytokines, thereby reducing the local inflammatory response. These anti-inflammatory effects of α7nAChR may additionally protect the blood-brain barrier integrity after experimental ICH. Inhibition of GSK-3β will also increase the expression of its downstream protein β-catenin, an important component of the blood-brain barrier. Furthermore, α7nAChR stimulation resulted in enhanced cognition and attention in animals as well as humans, and α7nAChR agonists have been represented as promising treatments for schizophrenia and Alzheimer disease.

In conclusion, the present study found that α7nAChR stimulation improved functional and morphological outcomes after experimental ICH in mice. We demonstrated that PHA-543613 treatment reduced the expression of proapoptotic GSK-3β through the PI3K-Akt signaling pathway, and the pharmacological reversal thereof obliterated this effect. Additional preclinical studies are needed to investigate potential anti-inflammatory effects of α7nAChR agonists, and it is also essential to elucidate further mechanisms on how α7nAChR...
stimulation reduces brain edema and neuronal apoptosis after experimental ICH, as shown in the present study.

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Disclosures
None.

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