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

Paul R. Krafft, MD; Orhan Altay, MD; William B. Rolland, BS; Kamil Duris, MD; Tim Lekic, MD, PhD; Jiping Tang, MD; John H. Zhang, MD, PhD

Background and Purpose—Perihematomal 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 perihematomal 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.)

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to aggravate the brain injury after experimental ischemic stroke and subarachnoid hemorrhage; however, the role of GSK-3β in ICH remains unexplored.

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 injection 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 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 induced, 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 consecutive 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 18 has been 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 Garcia 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 out of 10 to 15 trials, and a score was calculated as number of left turns/all turns × 100 (%). Reflexive motor ability of the animal’s contralateral (left) forelimb, to respond to a vibrissae-elicited excitation, was assessed through the forelimb placing test. The score was expressed as number of successful paw placements out of 10 consecutive vibrissae stimulations. Spatial exploratory and cognitive performances in mice were evaluated with the T-maze task as previously reported. This procedure relies on the rodent’s natural behavior to explore novelty and thus alternate between the right and the left arm of a T-maze. Spontaneous alternations were recorded as a percentage of all trials. All behavioral scores were converted as a percentage of average sham performance (100%).

Measurement of Brain Water Content

Brain water content was measured at 24 and 72 hours after surgery as previously reported. Briefly, mice were decapitated under lethal isoflurane anesthesia, and brains were quickly removed. 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 Instrument, 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 SDS-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-Akt, 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 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, Fluorescein, 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
considered statistically significant. All statistical analyses were performed using SigmaPlot version 10.0 for Windows.

Results

PH8-543613–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 α7nAChR 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).

**Discussion**

The first aim of the present study was to investigate whether the α7nAChR 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.22,23 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.8,18 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.19 During the T-maze assessment, rodents normally select alternating arms on consecutive trials (spontaneous alterations).20 A decreased number of those spontaneous alterations correlates well with the severity of the cognitive impairment in injured animals.24 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.19 Brain edema, defined as an increase in the water content of brain tissue, is observed in acute and delayed stages after ICH.25 Several studies suggest a close association between the degree of perihematomal brain edema and poor outcome in patients.25,26 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 alpha7nAChR 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 alpha7nAChR through PNU-282987 did not alter physiological parameters (blood pressure, heart rate) or plasma concentrations of Na+, K+, Cl−, and glucose in rodents.8 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 alpha7nAChR-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] alpha-bungarotoxin binding site of the alpha7nAChR (Ki=1.14 nm).27,28 MLA is quickly redistributed from the vasculature to the brain, where it extensively inhibits the receptor activation by PHA-543613 (Ki=8.8 nm).17,27,28 Wortmannin inhibits PI3K irreversibly and is commonly used to prevent downstream phosphorylation of Akt.8 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-

Figure 3. Representative Western blots and densitometric quantification of p-Akt/Akt (A), p-GSK-3β/GSK-3β (B), and CC3/β-Actin (C) at 24 hours after intracerebral hemorrhage induction or sham surgery. Data in bar graphs are expressed as mean±SEM. *P<0.05 compared with sham, †P<0.05 compared with vehicle, ‡P<0.05 compared with PHA, 12 mg; n=5 in each group.
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, 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.

References
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