α7 Nicotinic Acetylcholine Receptor Agonist PNU-282987 Attenuates Early Brain Injury in a Perforation Model of Subarachnoid Hemorrhage in Rats

Kamil Duris, MD; Anatol Manaenko, PhD; Hidenori Suzuki, MD, PhD; William B. Rolland, BS; Paul R. Krafft, MD; John H. Zhang, MD, PhD

Background and Purpose—Early brain injury is an important pathological process after subarachnoid hemorrhage (SAH). The goal of this study was to evaluate whether the α7 nicotinic acetylcholine receptor (α7nAChR) agonist PNU-282987 attenuates early brain injury after SAH and whether α7nAChR stimulation is associated with down-regulation of caspase activity via phosphatidylinositol 3-kinase-Akt signaling.

Methods—The perforation model of SAH was performed, and neurological score, body weight loss, and brain water content were evaluated 24 and 72 hours after surgery. Western blot and immunohistochemistry were used for quantification and localization of phosphorylated Akt and cleaved caspase 3. Neuronal cell death was quantified with TUNEL staining. α7nAChR antagonist methylcaconitine and phosphatidylinositol 3-kinase inhibitor wortmannin were used to manipulate the proposed pathway, and results were quantified with Western blot.

Results—PNU-282987 improved neurological deficits both 24 and 72 hours after surgery and reduced brain water content in left hemispheres 24 hours after surgery. PNU-282987 significantly increased phosphorylated Akt levels and significantly decreased cleaved caspase 3 levels in ipsilateral hemispheres after SAH. Methylcaconitine and wortmannin reversed effects of treatment. Phosphorylated Akt and cleaved caspase 3 were colocalized to neurons in the ipsilateral basal cortex. Phosphorylated Akt was mainly localized in TUNEL-negative cells. PNU-282987 significantly reduced neuronal cell death in the ipsilateral basal cortex.

Conclusions—α7nAChR stimulation decreased neuronal cell death and brain edema and improved neurological status in a rat perforation model of SAH. α7nAChR stimulation is associated with increasing phosphorylation of Akt and decreasing cleaved caspase 3 levels in neurons. (Stroke. 2011;42:3530-3536.)

Key Words: α7 nicotinic acetylcholine receptor ■ PNU-282987 ■ subarachnoid hemorrhage ■ early brain injury ■ apoptosis ■ Akt ■ cleaved caspase 3

Subarachnoid hemorrhage (SAH), with an annual incidence of 7 per 100 000 and a case fatality rate of ~50%, is a severe condition caused by rupture of intracranial aneurysm. Early brain injury is a major contributor to the high mortality and morbidity after SAH, and apoptosis is a significant element of early brain injury. The phosphatidylinositol 3-kinase-Akt pathway is an important pathway in neuronal cells, decreasing activation of proapoptotic caspases.

α7 nicotinic acetylcholine receptors (α7nAChRs) are ion channels expressed mainly in neurons and immune and endothelial cells, where they are responsible for providing antiapoptotic and anti-inflammatory effects. The antiapoptotic effect of α7nAChR activation in neuronal cells was reported to involve PI3K-Akt. The protective role of the PI3K-Akt pathway in reducing cleaved caspase 3 (CC3) after SAH was described previously; however, references regarding α7nAChR and SAH are still missing.

In this study we tested 2 hypotheses. First, the α7nAChR agonist PNU-282987 attenuates early brain injury after SAH. Second, the α7nAChR stimulation is associated with the PI3K-Akt signaling pathway, which decreases proapoptotic caspase activation.

Materials and Methods

Animals and Drugs
All of the experiments were approved by the Loma Linda University Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (277–320 g) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were fed ad libitum. We used 134 animals in this study, assigned to the following groups, naïve (n=4), sham (n=24), vehicle (n=39), PNU-12 (n=36), PNU-4 (n=10), methylcaconitine (MLA; n=12), and wortmannin (WOR; n=9). All of the drugs were purchased from Sigma-Aldrich. Drugs were dissolved and administered according to data published previously.

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α7nAChR agonist PNU-282987 was dissolved in saline and was injected IP 1 hour after surgery in doses of 12 mg/kg (PNU-12, MLA, and WOR groups) or 4 mg/kg (PNU-4 group). In the naïve group, 12 mg/kg of PNU-282987 was injected IP 15 minutes after initiation of physiological parameter monitoring. α7nAChR antagonist methylcaco-

nitrine was dissolved in saline and injected IP at a dose of 6 mg/kg 15 minutes before administration of PNU-282987 (MLA group). PI3K inhibitor wortmannin (Sigma-Aldrich) was dissolved in saline and injected IV at a dose of 15 μg/kg 90 minutes before PNU-282987 administration. Sham or vehicle animals received equivalent volumes of saline given to animals in other groups (500 μL).

**Physiological Parameters**

Blood pressure/heart rate measurements and analysis of blood samples were performed in naïve animals. Animals were anesthetized, intubated, and kept on artificial ventilation using isoflurane 3% in 60/40% medical gas/oxygen mixture. The right femoral artery was cannulated for invasive blood pressure/heart rate monitoring (DIGIMED Blood Pressure Analyzer) and collection of blood samples for subsequent analysis (IL GEM 4000 Premiere automatic analyzer).

The following parameters were evaluated from the blood samples: pH, PO2, PCO2, Na+, K+, Cl–, and glucose. Monitoring was performed from 10 minutes before (3 measurements) and ≤60 minutes after (5 measurements) application of PNU-282987 (12 mg/kg). Mean values of physiological parameters before PNU-282987 administration were compared with the mean values after administration. Selected data are shown in Figure 1 at each time point.

**Surgery**

The endovascular perforation model of SAH was performed as described previously. Briefly, rats were anesthetized, intubated, and kept on artificial ventilation using isoflurane 3% in 60/40% medical-air/oxygen mixture. Body temperature was monitored by rectal probe, and normothermia was maintained by a heating lamp. A sharpened 4-O nylon suture was introduced into the left carotid artery; however, no perforation was performed. After suture removal, the incision was closed, and rats were individually housed in heated cages until recovery.

**Body Weight and SAH Grade**

Body weight was measured after induction of anesthesia before intubation and before euthanization. Results are expressed as a ratio of body weight after surgery:body weight before surgery. SAH grade was evaluated as described previously. Briefly, after removing the brain, a picture of the underside of the brain was taken, and pictures were divided into 6 parts (left and right frontal, left and right temporal, and upper and lower brain stem). Each part was scored according to occurrences of blood in the subarachnoid space and a total score (maximum: 18) was calculated as the sum of all of the subscores. Animals with an SAH score of ≤5 were excluded from the study for low SAH grade.

**Neurological Score**

Neurological scores were evaluated before euthanization in a blinded fashion using a Garcia scoring system. The following were evaluated: spontaneous activity, symmetrical movements of limbs, forelimbs outstretching, climbing a wall of a wire cage, axillary touch response, and vibrissae touch response. Minimal score was 0 (worst) and maximal 3 (the best) for each subtest, and total score was calculated as a sum of all of the subtests.

**Brain Water Content**

Brains were removed 24 or 72 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and after drying in 100°C for 72 hours (dry weight). The percentage of water content was calculated as [(wet weight−dry weight)/wet weight]×100%.

**Western Blot**

Animals were euthanized 24 hours after surgery, and ipsilateral hemispheres were processed for Western blot as described previously. Equal amounts (50 μg) of total protein were separated in 12% SDS-PAGE and transferred onto nitrocellulose membranes. As primary antibodies we used anti–phospho-Akt (Ser 473; 1:1000; Cell Signaling, No. 9271), anti-CC3 (1:1000; Cell Signaling, No. 9661), primary antibodies we used anti–phospho-Akt (Ser 473; 1:1000; Cell

Signaling, No. 9271), anti-CC3 (1:1000; Cell Signaling, No. 9661), and anti–β-actin (1:1000; Santa Cruz Biotechnology, SC-1616), and appropriate secondary antibodies (Santa Cruz Biotechnology) were
used in dilution 1:1000. Bands were visualized using the enhanced chemiluminescence reagent kit (Amersham ECL plus kit, GE Healthcare UK Limited), and quantification was performed by optical density methods using ImageJ software (National Institutes of Health). Results are expressed as a relative density to β-actin subsequently normalized to the mean value of the sham group.

**Immunohistochemistry and TUNEL Staining**

Animals were euthanized 24 hours after surgery and brains were processed as described previously.14 Double-fluorescence labeling for NeuN (1:100; Millipore, MAB377) and CC3 (1:1000; Cell Signaling, No. 9271) or NeuN and phosphorylated Akt (p-Akt; Ser473; 1:25; Cell Signaling, No. 9661) or NeuN and NeuN was performed as described previously.14 TUNEL staining (In Situ Cell Death Detection kit, Roche Inc) either with NeuN and p-Akt for neuronal localization or with NeuN alone for cell counting was performed as described previously.1 For negative controls, the same staining without primary antibody was performed. The stained sections were processed using a fluorescent microscope and Magna Fire SP system (Olympus). The TUNEL-positive cells were counted in the ipsilateral basal cortex in 3 fields per animal at 400 magnification, and data were expressed as the number of TUNEL-positive neurons per millimeter squared.15

**Statistical Analysis**

Data are expressed as a mean and SEM. Mortality data were analyzed by Fisher exact test. SAH grading data in the 72-hour study were evaluated by unpaired t test. Physiological parameters were evaluated by paired t test. All of the other data were analyzed by 1-way ANOVA followed by Tukey post-test. A P value of <0.05 was considered statistically significant. All of the statistical analyses were performed using GraphPad Prism version 5.02 for Windows.

**Results**

We performed 134 surgeries, 29 animals died because of severe SAH, and 11 animals were excluded because of low SAH grade, 5 animals in the vehicle group, 3 in the PNU-12 group, 1 in the PNU-4 group, and 2 in the MLA group. Mortality rates were as follows: sham 0% (0 of 24 animals), naïve + PNU-12 0% (0 of 4 animals), vehicle 29% (10 of 34 animals), PNU-12 27% (9 of 33 animals), PNU-4 33% (3 of 9 animals), MLA 40% (4 of 10 animals), and WOR 33% (3 of 9 animals). Mortality occurred within 6 hours after surgery, and there were no statistically significant differences between the vehicle and PNU-12 groups (P value not significant [NS], Fisher exact test).

**Effect of PNU-282987 Administration on Physiological Parameters**

Blood pressure/heart rate monitoring and blood sample analysis (pH, Po2, PCO2, Na+, K+, Cl-, and glucose) were performed in naïve animals (n=4) from 10 minutes before and ≤60 minutes after application of PNU-282987 (12 mg/kg).

There were no significant differences in any measured parameters between mean value before application and mean value after PNU application (P=NS; Table). Selected data (blood pressure, heart rate, pH, Po2, and PCO2) are also shown in Figure 1 at each time point.

**Outcome Study**

**Neurological Score, Body Weight, and Brain Water Content 24 Hours After Surgery**

For the 24-hour outcome study we used sham, vehicle, PNU-12, and PNU-4 groups (n=6 in each group). There were no significant differences in SAH grade among vehicle (12.33±0.71), PNU-12 (11.83±1.01), and PNU-4 (11.67±1.09) groups (P=NS). Neurological score (Figure 2A) was significantly higher in sham group (17.50±0.22) compared with vehicle (13.67±0.71), PNU-12 (15.83±0.31), and PNU-4 (15.00±0.45) groups 24 hours after surgery.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MAP, mm Hg</th>
<th>SYST, mm Hg</th>
<th>DIAST, mm Hg</th>
<th>HR, /s</th>
<th>Po2, mm Hg</th>
<th>PCO2, mm Hg</th>
<th>pH</th>
<th>Na+, mmol/L</th>
<th>K+, mmol/L</th>
<th>Cl-, mmol/L</th>
<th>Glu, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before PNU</td>
<td>87.9 (±2.90)</td>
<td>113.5 (±3.87)</td>
<td>75.1 (±3.19)</td>
<td>325.3 (±13.76)</td>
<td>114.2 (±10.11)</td>
<td>49.17 (±5.17)</td>
<td>7.36 (±0.042)</td>
<td>132.8 (±0.854)</td>
<td>4.89 (±0.038)</td>
<td>103.4 (±0.84)</td>
<td>185.3 (±12.53)</td>
</tr>
<tr>
<td>After PNU</td>
<td>86.1 (±2.91)</td>
<td>112.2 (±3.70)</td>
<td>73.0 (±5.07)</td>
<td>329.9 (±20.26)</td>
<td>110.1 (±10.03)</td>
<td>55.40 (±6.237)</td>
<td>7.33 (±0.038)</td>
<td>134.3 (±0.919)</td>
<td>5.03 (±0.043)</td>
<td>102.6 (±0.58)</td>
<td>183.7 (±10.47)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (±SEM). MAP indicates mean arterial pressure; SYST, systolic pressure; DIAST, diastolic pressure; HR, heart rate.
(P<0.05). Compared with vehicle, neurological score was significantly higher in the PNU-12 group (P<0.05); however, there was no significant difference between the vehicle and PNU-4 group (P=NS). Body weight ratio 24 hours after surgery (Figure 2B) was not significantly different between groups (P=NS). Brain water content (Figure 2C) was significantly higher in the left hemisphere of vehicle animals (79.54±0.12) compared with the sham (78.86±0.11) and PNU-12 (79.00±0.12) groups (P<0.05). There was no significant difference in brain water content in the left hemisphere between the PNU-4 group (79.16±0.17) and vehicle group (P=NS). There were no significant differences between groups in brain water content in the right hemisphere, cerebellum, and brain stem (P=NS).

Neurological Score, Body Weight, and Brain Water Content 72 Hours After Surgery
At 72 hours we evaluated the same parameters as in the 24-hour outcome study in sham, vehicle, and PNU-12 groups (n=6 in each group). We did not use the 4-mg/kg dose of PNU-282987, because this dosage did not show significant effects after 24 hours.

There was no significant difference in SAH grade between vehicle (10.33±0.92) and PNU-12 (11.50±0.99) groups (P=NS). Neurological score (Figure 3A) was significantly lower in the vehicle group (14.50±0.43) compared with the sham (17.67±0.21) and PNU-12 (16.33±0.33) groups (P<0.05). There was a significant difference in neurological score between the sham and PNU-12 groups (P=NS). Body weight ratio (Figure 3B) was significantly lower in the vehicle (0.87±0.014) compared with the sham (0.97±0.022) group (P<0.05). There were no significant differences in body weight ratio of PNU-12 (0.92±0.012) compared with the sham or vehicle groups (P=NS). Brain water content (Figure 3C) was not significantly different between groups in any other part of the brain 72 hours after surgery (P=NS).

Mechanism Study
Quantification of p-Akt and CC3 at 24 Hours After Surgery
Western Blot analysis was used for quantification of p-Akt and CC3 levels in ipsilateral hemispheres 24 hours after surgery in sham, vehicle, PNU-12, MLA, and WOR animals (n=6 in each group). There was no significant difference in SAH grade among vehicle (12.67±0.56), PNU-12 (12.00±0.73), MLA (11.33±0.61), and WOR (11.17±0.48) groups (P=NS). The levels of p-Akt (Figure 4A and 4B) in the ipsilateral hemisphere were significantly higher in the PNU-12 group (1.95±0.31) compared with both vehicle (0.58±0.11) and sham (1.00±0.17) groups (P<0.05), whereas there was no significant difference between sham and vehicle groups (P=NS). P-Akt level was significantly lower in both MLA (1.08±0.19) and WOR (0.95±0.16) groups compared with the PNU-12 group (P<0.05). CC3 levels (Figure 4A and 4C) were significantly higher in the vehicle (2.62±0.31) compared with the sham (1.00±0.10) and PNU-12 (1.41±0.14) groups (P<0.05), whereas there was no significant difference between sham and PNU-12 groups (P=NS). CC3 levels in both MLA (2.16±0.26) and WOR (2.41±0.33) groups were significantly higher compared with sham (P<0.05); however, there was no significant difference between these and the PNU-12 group (P=NS).

Neuronal Colocalization of p-Akt and CC3 Immunoreactivity and Neuronal Cell Death Quantification
Localization of previously described changes was performed by immunohistochemistry in sham, vehicle, and PNU-12 groups (n=2 in each group). Neuronal cell death was quantified by TUNEL cell counting in the above groups (n=6 in each group). Ipsilateral basal cortex was the area of interest in all of the cases.

CC3 immunoreactivity (Figure 5A) was enhanced in the vehicle group compared with both PNU-12 and sham groups with good colocalization to neurons. P-Akt immunoreactivity (Figure 5B) in the PNU-12 group tended to be higher than the sham group and even higher when compared with the vehicle group. P-Akt signal was mainly colocalized to neurons, and TUNEL-positive cells did not generally colocalize with p-Akt-positive cells. The number of TUNEL-positive neurons in the ipsilateral basal cortex was significantly lower in sham (29.67±7.2) compared with both the vehicle (38.79±50.6) and PNU-12 (22.51±29.6) groups. There was statistically a signifi-
icant difference in TUNEL-positive neuron counts between the vehicle and PNU-12 groups ($P < 0.05$; Figure 5C).

**Discussion**

In this study we tested 2 hypotheses. First, the $\alpha_7nAChR$ agonist PNU-282987 attenuates early brain injury after SAH. Second, the $\alpha_7nAChR$ stimulation is associated with the PI3K-Akt signaling pathway, which decreases proapoptotic caspase activation. In the descriptive part (the first part), we focused on brain water content, neurological score, and body weight loss; whereas in the mechanistic part (the second part), we focused on the involvement of p-Akt–mediated decrease in caspase activation and neuronal cell death after $\alpha_7nAChR$ stimulation.

SAH grade, at each time point, was comparable among all of the groups. This precaution was taken to allow comparison of measured parameters. All of the cases of mortality experienced severe SAH. The mortality rate was not improved by treatment with PNU-282987 compared with vehicle possibly because of SAH grade.

Basic physiological parameters were not affected by the application of PNU-282987 in our study. We performed monitoring for 60 minutes after PNU-282987, because this procedure is invasive, and a longer exposure to anesthesia could affect physiological parameters. Physiological parameters during our experiment were comparable with previous work from our laboratory.10,14 PNU-282987 was reported previously to inhibit human ERG potassium channels; however, this adverse effect is associated with the structure of this drug, not with specific $\alpha_7nAChR$ stimulation. It should be noted that a newer $\alpha_7nAChR$ agonist with reduced human ERG has been developed.16

Neurological dysfunction is an important clinical and experimental outcome present after SAH. Neurological deficits follow the peak of brain edema after SAH.17 The Garcia test is widely used in animal models of SAH.10–12 In our experiment, PNU-282987 improved neurological deficit at 24 hours in a dose-dependent manner and significantly improved neurological function 72 hours after surgery.

Body weight can be considered as a nonspecific marker, providing information about the animal’s overall condition. In our experiment, body weight was not significantly different between groups 24 hours after surgery. Body weight at 72 hours was significantly lower in vehicle compared with sham, and PNU-282987 groups showed a tendency to decrease body weight loss. Given these results, we believe that body weight is influenced more by surgery than by SAH at 24 hours, and this effect is not present at 72 hours.

Brain edema is an important element of early brain injury, the etiology of which is cytotoxic and vasogenic.2 Brain edema peaks 24 hours after surgery in the rat perforation model of SAH.17 Previous studies show increased brain water content after SAH in the left hemisphere only,17 as well as in all parts of the brain.14 In our study, PNU-282987 reduced brain water content in left hemispheres 24 hours after surgery in a dose-dependent manner, whereas no significant differences in brain water content were found 72 hours after surgery.

The PI3K-Akt signaling pathway plays important roles in preventing apoptosis.18 Akt is a member of serine/threonine kinase family, which is activated directly by PI3K-mediated phosphorylation. p-Akt activates several antiapoptotic mechanisms; among them is the regulation of BCL-2 family proteins, which control proapoptotic caspase activation.3,18 The antiapoptotic role of the PI3K-Akt pathway in neurons after SAH was reported previously,6 and the neuroprotective effects of $\alpha_7nAChR$ stimulation via the PI3K-Akt pathway was also reported.5 In our study we evaluated p-Akt and CC3 levels 24 hours after surgery in ipsilateral hemispheres, and we used methylcaconitine and wortmannin to manipulate this antiapoptotic pathway. Methylcaconitine is a potent competitive antagonist of $\alpha_7nAChR$,8 whereas wortmannin is an irreversible inhibitor of PI3K, which is widely used to inhibit downstream Akt phosphorylation.10 We did not evaluate total levels of Akt, because expression of Akt was reported not to be changed after SAH8 or after $\alpha_7nAChR$ stimulation/inhibition.19,20 In our study, p-Akt levels were increased in ipsilateral hemispheres after PNU-282987 treatment compared with vehicle, and both methylcaconitine and wortmannin reversed the effect of treatment. CC3 was increased in ipsilateral hemispheres in the
vehicle group compared with the PNU-282987-treated group, and both methylcaconitine and wortmannin showed tendencies to reverse the effect of PNU-282987 treatment.

α7nAChR is reported in endothelial cells and immune cells, as well as neurons. Our focus centered around neuronal apoptosis. Therefore, we used immunohistochemistry to localize our findings and found that both p-Akt and CC3 were colocalized to neurons in the ipsilateral basal cortex. Additionally, we used TUNEL staining, a marker for cells undergoing DNA fragmentation in the final phase of apoptotic cell death.

Apoptosis is an important component of early brain injury after SAH, which occurs both in neurons and endothelial cells. In our study we used TUNEL staining for quantification of neuronal cell death in the ipsilateral basal cortex, and PNU-282987 significantly reduced neuronal cell death in this area 24 hours after SAH.

In this study we focused on PI3K-Akt signaling, which plays a significant role in apoptosis, a major contributor to early brain injury after SAH. However, there are also other mechanisms of α7nAChR stimulation that could have a beneficial effect after SAH. α7nAChR is a part of the cholinergic anti-inflammatory pathway that attenuates the inflammatory response by decreasing both endothelial cell and macrophage activation. Inflammation is reported to be involved after SAH, both in early brain injury and vasospasm. The cholinergic anti-inflammatory pathway is mostly associated with the parasympathetic nervous system, but α7nAChR stimulation is also reported to induce NO-dependent dilation of the basilar artery associated with the sympathetic nervous system. Taken together, the antiapoptotic effect of α7nAChR stimulation in combination with the anti-inflammatory and antivasospatic effects make this a promising approach for treatment after SAH; however, further studies are required to elucidate those beneficial mechanisms.

Conclusions
α7nAChR stimulation decreased neuronal cell death, brain edema, and improved neurological status in a rat perforation model of SAH. α7nAChR stimulation is associated with increasing phosphorylation of Akt and decreasing CC3 levels in neurons. These results indicate that α7nAChR agonists are promising treatments after SAH.

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Disclosures
None.
References


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