Neuroscience 222 (2012) 10–19

α7 NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST ATTENUATES NEUROPATHOLOGICAL CHANGES ASSOCIATED WITH INTRACEREBRAL HEMORRHAGE IN MICE

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Abstract—We have demonstrated previously that nicotine affords neuroprotective and anti-inflammatory effects against intracerebral hemorrhage (ICH)-associated neuropathological changes. The present study was undertaken to clarify whether subtype-specific agonists of nicotinic acetylcholine receptors (nAChRs) could preserve tissue integrity in mouse ICH model *in vivo*. ICH was induced by unilateral injection of collagenase into the striatum of male C57BL/6 mice. Daily intraperitoneal injection of α7 nAChR agonist PNU-282987 (3–10 mg/kg) for 3 days, starting from 3 h after induction of ICH, significantly increased the number of surviving neurons in the central and the peripheral regions of hematoma at 3 days after ICH. In contrast, α4β2 nAChR agonist RJR-2403 (2–10 mg/kg) given in the same regimen showed no significant effect. PNU-282987 and RJR-2403 did not affect either the size of hemorrhage or the extent of brain edema associated with ICH. PNU-282987 decreased the number of activated microglia/macrophages accumulating in the perihematoma region at 3 days after ICH, in a dose-dependent manner. On the other hand, the number of microglia/macrophages in the central region of hematoma at early phase of pathology (6 h after ICH) was increased by 10 mg/kg PNU-282987. These results suggest that α7 nAChR agonist can provide neuroprotective effect on ICH-induced injury, independently of its anti-inflammatory actions.

Key words: hemorrhagic stroke, neuroprotection, neuroinflammation, basal ganglia, nicotinic receptor.

INTRODUCTION

Intracerebral hemorrhage (ICH) results from leakage of blood into brain parenchyma by rupture of blood vessels. This neurological disorder is characterized by high mortality and severe motor dysfunctions associated with brain edema, inflammation and neurodegeneration (Qureshi et al., 2009). Although several therapeutic interventions including regulation of osmotic pressure are in clinical practice, neuroprotective drug therapies have not been established to date (Katsuki, 2010). In the brain suffering ICH, inflammation and neurodegeneration are induced by several blood constituents such as thrombin and hemoglobin. These blood constituents triggers inflammatory responses accompanied by activation of microglia/macrophages (Möller et al., 2000; Fujimoto et al., 2006; Cai et al., 2011). Several lines of evidence indicate that suppression of activation of microglia/macrophages is a promising approach to improve ICH pathologies (Ohnishi et al., 2007; Wasserman and Schlichter, 2007). In fact, therapeutic agents that aim at suppressing microglia/macrophage activation and simultaneously preventing neuron loss are being explored (Lee et al., 2006; Matsushita et al., 2011).

Nicotinic acetylcholine receptors (nAChRs) are expressed not only in neurons but also in microglia/macrophages. Previous studies have demonstrated that stimulation of nAChRs by nicotine suppresses activation of microglia/macrophages (Suzuki et al., 2006; Ohnishi et al., 2008). In addition, stimulation of nAChRs may produce direct neuroprotective effect in various neurodegenerative disease models (Mudo et al., 2006). Neuroprotective effect of nicotine may be mediated by upregulation of an anti-apoptotic protein, B cell lymphoma-2 (Tait and Green, 2010), via phosphatidylinositol 3-kinase/Akt pathway that lies downstream of α7 nAChRs (Kihara et al., 2001). We have reported recently that daily intraperitoneal administration of nicotine in mice from 3 h after induction of ICH ameliorated neuropathological changes associated with hematoma (Hijioka et al., 2011). The effect of nicotine was featured by an increased number of surviving striatal neurons in the central region of hematoma, and by a decreased number of activated microglia/macrophages in the perihematoma region. On the other hand, nicotine showed no significant effect on expansion of hematoma and formation of brain edema (Hijioka et al., 2011).

Our previous study (Hijioka et al., 2011) was focused on the effect of nicotine, and which subtype of nAChRs plays an important role in ameliorating ICH pathology remained undetermined. Therefore, the present study was aimed to clarify whether subtype-specific nAChR agonists can reproduce the effect of nicotine in mouse ICH model. For this purpose, we used PNU-282987, a specific α7 nAChR

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Abbreviations: ICH, intracerebral hemorrhage; iNOS, inducible isoform of nitric oxide synthase; MRI, magnetic resonance imaging; nAChR, nicotinic acetylcholine receptor; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.07.024

agonist, and RJR-2403, a specific α4β2 nAChR agonist (Papke et al., 2000; Walker et al., 2006). Both α7 and α4β2 are major subtypes of nAChRs distributed in the central nervous system, and these receptor subtypes are implicated in neuroprotection and regulation of inflammation (Suzuki et al., 2006; Ohnishi et al., 2008; Akaike et al., 2010).

EXPERIMENTAL PROCEDURES

Mouse model of ICH

All procedures were approved by our institutional ethics committee concerning animal experiments, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. Male C57BL/6J mice at 8–10 weeks of age weighing 22–28 g were used to produce collagenase-induced model of ICH, as described previously (Hijioka et al., 2011; Matsushita et al., 2011). Animals were maintained at constant ambient temperature (22 ± 1 °C) under a 12-h light/dark cycle (lights on between 8:00 AM and 8:00 PM) with food and water available *ad libitum*. When surgery was performed, mice were placed in a stereotaxic frame after anesthesia with an intraperitoneal injection of 50 mg/kg pentobarbital. A 30-gauge needle was inserted through a burr hole on the skull into the striatum (stereotaxic coordinates; 2.3 mm lateral to the midline, 0.2 mm anterior to the bregma, and 3.5 mm deep below the skull). ICH was induced by injection of 0.025 U of collagenase type VII (Sigma–Aldrich, St. Louis, MO, USA) in 0.5 μl of saline at a constant rate of 0.20 μl/min with a microinfusion pump. Sham-operated mice received injection of the same volume of physiological saline. Body temperature was maintained at 37 °C during surgery.

Administration of nAChR agonists

Nicotine tartrate dihydrate (Nacalai Tesque, Kyoto, Japan) was dissolved in 0.9% saline at a concentration of 0.2 mg/ml (as nicotine free base). PNU-282987 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and RJR-2403 oxalate (Santa Cruz) were also dissolved in 0.9% saline at concentrations of 0.1, 0.3, 1.0 mg/ml for PNU-282987 and 0.2, 0.3, 1.0 mg/ml for RJR-2403, respectively. These drugs were intraperitoneally administrated to mice at doses of 2 mg/kg (nicotine), 1, 3, 10 mg/kg (PNU-282987) or 2, 3, 10 mg/kg (RJR-2403), once per day. The first administration of nAChR agonists was performed 3 h after induction of ICH and then daily at a 24-h interval, unless otherwise indicated. Effect of 10 mg/kg PNU-282987, which was first administered 1 h before, 6 h after, or 24 h after induction of ICH and then daily at a 24-h interval, was also examined. Control animals received intraperitoneal administration of the same volume of saline.

Histochemical examinations

Six or 72 h after induction of ICH, mice were anesthetized again with pentobarbital and perfused transcardially with 30 ml of ice-cold phosphate-buffered saline followed by 30 ml of 4% paraformaldehyde. Brains were isolated and fixed in 4% paraformaldehyde overnight and then soaked in 15% sucrose overnight at 4 °C. After being frozen, they were cut into sections of 30 μm in thickness, and four sections around the injection site were collected every 120 μm and mounted onto slides. In several sets of experiments (Figs. 2B, E and 4C), 8–10 sections were collected every 120 μm. Antigen retrieval was achieved by soaking specimens in 10 mM citrate buffer (pH 8.0–8.5) for 30 min at 85 °C, followed by incubation for 1 h at 22–25 °C. After being rinsed with phosphate-buffered saline containing 0.3% Triton X-100, specimens were treated with phosphate-buffered saline containing Triton X-100 and blocking serum for 1 h at 22–25 °C and then incubated with primary antibody overnight at 4 °C. For immunostaining of a neuron-specific nuclear protein NeuN (Mullen et al., 1992), mouse anti-NeuN (1:500; Millipore Corporation, Billerica, MA, USA) and biotinylated goat anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA, USA) were used as a primary antibody and a secondary antibody, respectively. Then specimens were treated with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories), and peroxidase was visualized by diaminobenzidine and H_2O_2. The number of NeuN-positive cells per $230 \times 340\ \mu m^2$ was counted at the central and the peripheral regions of hematoma in the striatum as described (Matsushita et al., 2011). The central region of hematoma refers to the region adjacent to the collagenase injection site defined by the track of the cannula, and the peripheral region of hematoma is the region adjacent to the edge of hematoma defined by low NeuN immunoreactivity. Four coronal sections collected every 120 μm around the injection site in each mouse were examined for cell counting, and the averaged number of cells from these sections was taken as the value for each mouse. In experiments shown in Fig. 2B, E, 8–10 coronal sections collected every 120 μm were examined to obtain the number of cells for each mouse, and the obtained values were quantitatively similar to those derived from four sections.

Activated microgila/macrophages were identified by double fluorescence staining with biotinylated *Griffonia simplicifolia* isolectin-B_4 (1:100; Vector Laboratories; Lee et al., 2006) and rabbit anti-Iba-1 antibody (1:1000; Wako Chemicals, Osaka, Japan) in experiments shown in Fig. 4A, B. Isolectin-B_4 binding was detected by Alexa Fluor 488-conjugated streptavidin (1:2000; Invitrogen, Carlsbad, CA, USA), and Iba-1 immunoreactivity was detected by Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H + L) (1:500; Invitrogen). Morphological criteria such as ameboid appearance with short and thick processes, in addition to isolectin-B_4 binding positivity, were used for identification of activated microgila/macrophages in experiments shown in Fig. 4C. Double immunofluorescence staining was also performed for NeuN and Iba-1 on brain sections obtained 6 h after induction of ICH. In this experiment, Alexa Fluor 488-conjugated donkey anti-mouse IgG (H + L) (1:500; Invitrogen) was used as a secondary antibody to detect NeuN immunoreactivity. Confocal images were obtained with the Fluoview FV300 system (Olympus, Tokyo, Japan). Iba-1 positive area in images of $725 \times 546\ \mu m^2$ was quantified with the use of Image J software (National Institutes of Health, Bethesda, MA, USA). The number of NeuN-positive cells per $725 \times 456\ \mu m^2$ was counted at the central region of the hematoma.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and immunostaining for cleaved caspase-3 were performed in combination with NeuN immunostaining to examine whether NeuN-positive cells were undergoing apoptotic cell death. TUNEL was based on a commercial kit (TAKARA BIO Inc., Shiga, Japan), and signals were detected with Alexa Fluor 488-conjugated streptavidin (1:2000; Invitrogen). When combined with TUNEL, NeuN-immunopositive signals were detected with Alexa Fluor 594-conjugated donkey anti-mouse IgG (H + L) (1:500; Invitrogen). In immunostaining for cleaved caspase-3, rabbit anti-cleaved caspase-3 (1:200; Cell Signaling Technology, Danvers, MA, USA) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H + L) (1:500; Invitrogen) were used as a primary antibody and a secondary antibody, respectively. When combined with cleaved caspase-3 immunostaining, NeuN-immunopositive signals were detected with Alexa Fluor 488-conjugated donkey anti-mouse IgG (H + L) (1:500; Invitrogen).