Therapeutic effect of nicotine in a mouse model of intracerebral hemorrhage

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ABBREVIATIONS:
Bcl-2, B cell lymphoma; CNS, central nervous system; ICH, intracerebral hemorrhage; MPO, myeloperoxidase; nAChR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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ABSTRACT

Intracerebral hemorrhage (ICH) resulting from leakage of blood into brain parenchyma triggers severe tissue damage involving neurodegeneration and inflammation. Increasing lines of evidence indicate that stimulation of central nicotinic acetylcholine receptors affords neuroprotection against various insults and also suppresses proinflammatory activation of microglia. Therefore, the present study was aimed to determine whether administration of nicotine modifies pathological consequences of ICH, using a mouse model of ICH induced by intrastriatal injection of collagenase. Daily intraperitoneal administration of nicotine (2 mg/kg), starting from 3 h after induction of ICH, inhibited occurrence of apoptosis as well as a decrease in the number of remaining striatal neurons at 3 days after the insult. We also found that nicotine administration increased relative expression level of an anti-apoptotic protein Bcl-2 against a pro-apoptotic protein Bax in the brain. In addition, nicotine administration attenuated activation of microglia/macrophages, infiltration of neutrophils and increment of oxidative stress associated with ICH, without affecting hematoma expansion and brain edema. Importantly, mice treated with nicotine exhibited improved sensorimotor performance and a marked increase in survival rate after ICH. These results indicate that nicotinic acetylcholine receptors may serve as a novel target for emergency therapy for ICH.
Introduction

Intracerebral hemorrhage (ICH) results from ruptures of blood vessels within the brain and leakage of blood constituents into the brain parenchyma. Brain edema and tissue damage, which frequently lead to enduring brain dysfunctions and poor prognosis, are principal characteristics of ICH pathology (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).

Brain tissue damage associated with ICH involves substantial neuron loss in the region invaded by hematoma. In addition to direct cytotoxicity of blood constituents on neurons, inflammatory reactions are considered to make important contribution to the induction of neuron death (Wang and Dore, 2007b; Wasserman and Schlichter, 2007). Inflammatory reactions in the brain are represented by activation of microglia/macrophages, which is found prominently in perihematomal region (Gong et al., 2000). In this context, our recent study has shown that retinoic acid receptor stimulation can prevent several pathological events associated with ICH, including neuron loss, activation of microglia/macrophages, and expansion of oxidative stress (Matsushita et al., 2011). Thus, drugs with neuroprotective and anti-inflammatory properties are promising candidates for ICH therapy.

Nicotinic acetylcholine receptors (nAChRs) are widely expressed in the central nervous system (CNS) and mediate fast synaptic transmission in brain cholinergic system (Taly et al., 2009). nAChRs are ionotropic receptors composed of five subunits, and their major subtypes in the CNS are homomeric α7 receptors and β2-containing heteromeric receptors (Hogg et al., 2003). Substantial lines of evidence indicate that stimulation of nAChRs protects neurons from insults associated with several neurodegenerative disorders (Mudo et
For example, in animal models of Parkinson disease, nicotine protects dopaminergic neurons from nigrostriatal damage induced by dopaminergic neurotoxins (Quik et al., 2009). Nicotine also protects cortical neurons in culture from Alzheimer disease-associated amyloid β protein (Kihara et al., 2001). In the latter case, the protective effect of nicotine may be mediated by up-regulation of B cell lymphoma-2 (Bcl-2), an anti-apoptotic protein (Tait and Green, 2010), via recruitment of phosphatidylinositol 3-kinase/Akt pathway (Kihara et al., 2001).

nAChRs are expressed not only in neurons but also in microglia/macrophages, and stimulation of nAChRs suppresses inflammatory activation of these cells (Wang et al., 2003; Shytle et al., 2004; Lee et al., 2009). Indeed, nicotine has been shown to protect dopaminergic neurons via anti-inflammatory action in inflammation models of neurodegeneration in vitro and in vivo (Park et al., 2007). Overall, these findings indicate that nicotine may act as both a neuroprotectant and an anti-inflammatory agent.

Previously we examined the effect of nicotine on thrombin-induced tissue injury in organotypic cortico-striatal cultures, and found that long-term treatment with nicotine prevented microglial activation and afforded neuroprotection (Ohnishi et al., 2009). Here we investigated potential therapeutic effect of nicotine in a mouse model of ICH in vivo.
Materials and methods

Induction of Intracerebral Hemorrhage and Administration of Nicotine. All procedures were approved by our institutional ethical 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 to 10 weeks of age weighing 22 to 28 g were used to produce collagenase-induced model of ICH, as described previously (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 and 20:00), with food and water available ad libitum. When surgery was performed, mice were placed in a stereotaxic frame after anesthesia with 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 depth below the skull). ICH was induced by injection of 0.025 U collagenase type VII (Sigma, St Louis, MO, USA) in 0.5 μL 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.

Nicotine tartrate dihydrate (Nacalai Tesque, Kyoto, Japan) was dissolved in 0.9% saline at 0.1 or 0.2 mg/mL (as nicotine free base) and intraperitoneally administrated to mice at 1 or 2 mg/kg, once per day. Administration of nicotine was first performed 3 h after induction of ICH by intrastriatal collagenase injection, and then daily at 24-h interval. Control animals received intraperitoneal administration of the same volume of saline.

Immunohistochemistry. Three days (72 h) after ICH, mice were anesthetized again
with pentobarbital and perfused transcardially with 30 mL of ice-cold phosphate-buffered saline (PBS) 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 freezing, they were cut into sections of 30 µm thickness, and four sections around the injection site were collected every 120 µm and mounted onto slides. Antigen retrieval was achieved by soaking specimens in 10 mM citrate buffer (pH 8.0 - 8.5) for 30 min at 80 °C followed by incubation for 1 h at 22 – 25 °C. After rinsing with PBS containing 0.3% Triton X-100, specimens were treated with PBS containing Triton X-100 and blocking serum for 1 h at 22 – 25 °C, then incubated with primary antibodies overnight at 4 °C. Primary antibodies were mouse anti-NeuN (1:500, Millipore, Bedford, MA, USA), rabbit anti-nitrotyrosine (1:500, Millipore), and rabbit anti-myeloperoxidase (MPO; 1:500, Dako, Glostrup, Denmark). After rinsing with PBS containing Triton X-100, specimens were incubated with corresponding secondary antibodies for 2 h at 22 – 25 °C. Biotinylated goat anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA) and biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories) were used as secondary antibodies. Microglia/macrophages were labeled by overnight incubation with biotinylated *Griffonia simplicifolia* isoelectin B4 (1:100, Vector Laboratories; Lee et al., 2006). After incubation with biotinylated conjugates, specimens were treated with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) and then peroxidase was visualized with diaminobenzidine and H2O2. The number of NeuN-positive cells per 230 × 340 µm2 was counted at the central and the peripheral regions of hematoma in the striatum as described (Ohnishi et al., 2007; Matsushita et al., 2011). Here, the central region of hematoma refers to the region adjacent to the collagenase injection site defined by a track of cannula.
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 from around the injection site in each mouse were examined for cell counting, and the averaged number of cells from these sections was taken as a value of each mouse. The number of MPO-positive cells per $230 \times 340$ µm$^2$ was counted in the central region of hematoma. The number of isolectin B$_4$ binding-positive cells per $230 \times 340$ µm$^2$ was counted in the peripheral region of hematoma. Concerning isolectin B$_4$ binding-positive cells, only cells exhibiting morphology of activated microglia/macrophages, such as amoeboid appearance with short and thick processes, were incorporated in cell counting. For nitrotyrosine immunoreactivity, threshold-based quantification of immunopositive area was conducted with ImageJ in a section containing a track of cannula (Matsushita et al., 2011). Nitrotyrosine-positive area was presented as a total value obtained from four fields of 0.25 mm$^2$.

Double immunofluorescence histochemistry was performed for combination of NeuN with Bax. Rabbit anti-Bax (1:200, Santa Cruz Biotech.) was used as a primary antibody along with mouse anti-NeuN (1:500). Alexa Fluor 594-conjugated donkey anti-rabbit IgG(H + L) (1:500, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-conjugated donkey anti-mouse IgG(H+L) (1:500, Molecular Probes) were used to detect localization. Confocal images were obtained with the usage of Fluoview FV300 system (Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL). TUNEL was carried out with a commercial kit (TAKARA BIO Inc., Shiga, Japan). Twenty-four h after induction of ICH, mice were decapitated under deep anesthesia, brains were isolated and frozen, and coronal brain sections of 30 µm thickness were prepared and mounted onto slides. Specimens were then fixed with 4% paraformaldehyde for 15 min
at 22 – 25 °C. Epitope was retrieved by treatment with 20 μg/ml proteinase K (Sigma) for 20 min at 22 – 25 °C. Intrinsic peroxidase was inactivated by 3% H₂O₂ applied for 10 min, and after permeabilization, specimens were incubated with constructed labeling reaction solution containing terminal deoxynucleotidyl transferase (1:10) for 2 h at 37 °C. After rinsing with PBS, specimens were incubated with anti-fluorescein isothiocyanate horseradish peroxidase conjugate overnight at 37 °C, and then, with biotinylated anti-rabbit IgG (1:200) for 2 h at 22 – 25 °C. After rinsing with PBS, specimens were treated with avidin–biotinylated horseradish peroxidase complex. Peroxidase was visualized with diaminobenzidine and H₂O₂.

**Western Blot Analysis.** Mice were deeply anesthetized and perfused transcardially with ice-cold PBS. The whole brain tissue except the cerebellum and the olfactory bulb was homogenized in ice-cold lysis buffer consisting of 150 mM NaCl, 50 mM Tris HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate. After incubation at 4 °C for 30 min, lysates were centrifuged at 14,000 g at 4 °C for 30 min, and the protein concentration in each sample was determined by Bicinchoninate method. With added sample buffer containing 0.5 M Tris HCl (pH 6.8), 10% SDS, 2-mercaptoethanol, glycerol and 1% bromophenol blue, each sample was heated at 99 °C for 5 min. SDS polyacrylamide gel electrophoresis was performed on a 5.4% stacking gel with 12% separating gel. After gel electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes. The blots were washed with Tris-buffered saline containing 0.1% Tween 20 and blocked with Blocking One (Nacalai Tesque) at 22 – 25 °C for 1 h. The membrane was incubated with rabbit anti-Bcl-2 antibody (1:1000, Santa Cruz Biotech), rabbit ant-Bax antibody (1:1000, Santa Cruz Biotech) and mouse anti-β-actin antibody (1:1000, Sigma) overnight at 4 °C.
After incubation with horseradish peroxidase-conjugated secondary antibodies at 22 – 25 °C for 1 h, bands were detected with ECL Advance western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA) on a lumino-imaging analyzer (LAS-3000mini, Fuji Film, Tokyo, Japan).

**Estimation of Lesion Size.** Lesion volume, which reflected the extent of hematoma expansion, was estimated by Nissl staining with Cresyl violet (Wang and Dore, 2007a; Matsushita et al., 2011) of 30 µm coronal frozen brain sections obtained every 210 µm. Injured hemorrhagic areas in sections spanning the entire hematoma were measured by ImageJ software. Lesion volume (in mm³) was determined by integration of the injured area in each section over the section depth.

**Measurement of Brain Water Content.** Three days after ICH, mice were decapitated under deep anesthesia with pentobarbital. Brain was removed from the skull and divided into ipsilateral and contralateral hemispheres along the midline, and then the olfactory bulb and the cerebellum were removed. After wet weight of the tissues was obtained, tissues were desiccated at 75 °C for 12 h to give the dry weight. The water content was calculated by the following formula: \[
\frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100 \%
\].

**Behavioral Tests.** Sensorimotor functions of mice were evaluated by means of beam walking test (Matsushita et al., 2011), modified limb-placing test (Jeong et al., 2003; Song et al., 2003), and adhesive removal test (Bouët et al., 2007; Beray-Berthat et al., 2010) at 1, 3, 7, and 14 days after surgery. These tests were conducted by an experimenter blinded to the treatments. Mice were trained once daily for 3 days before surgery.

In the beam walking test, mouse was placed on a beam (1.2 m long, 1.5 cm wide, and 50 cm high), and usage of hindlimb during beam crossing was analyzed on the basis of an
eight-point scale as well as a fault rate. A score of 0 was given when mouse could not balance on the beam (< 5 s); 1 was given when mouse remained on the beam for > 5 s but could not cross the beam; 2 was given when mouse could balance on the beam but not traverse it; 3 was given when mouse traversed the beam with the affected limb extended and not reaching the surface of the beam, or when mouse made a turn on the beam; 4 was given when mouse traversed the beam with 100% footslips; 5 was given when mouse traversed the beam with > 50% but < 100% footslips; 6 was given when mouse traversed the beam with < 50% footslips; 7 was given when mouse traversed the beam with two or less footslips.

Performance on each day was expressed as a summated score of three trials. Fault rate was presented as an average from three trials.

The modified limb-placing test consisted of two limb-placing tasks that assess the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimuli. First, mouse was suspended 10 cm over a table, and the stretch of the forelimbs toward the table was observed and evaluated: normal stretch, 0 points; abnormal flexion, 1 point. Next, the mouse was positioned along the edge of the table, with its forelimbs suspended over the edge and allowed to move freely. Each limb (forelimb, hind limb) was gently pulled down, and retrieval and placement were checked. Finally, the mouse was placed toward the table edge to check for lateral placement of the forelimb. Total of 5 points means maximal neurological deficits, and 0 point means normal performance.

In the adhesive removal test, mouse was placed into a transparent box (345 mm × 403 mm × 177 mm) for 1 min to get used to the environment. Thereafter, adhesive tape (3 mm × 4 mm) was applied on palm of the impaired forepaw. The time when the mouse gave the first
touch to the tape (contact time) and the time when the mouse removed the tape (removal time) was recorded, with a cut-off of 120 sec for the removal time. The performance was expressed as the removal time after subtracting the contact time.

**Statistical Analysis.** All data are presented as means ± S.E.M. Data were statistically analyzed by unpaired $t$ test for two group comparisons (data in Fig. 2). When data sets included more than two groups (data in Figs. 1, 3, 4, and 5), one-way analysis of variance followed by post hoc comparisons by Tukey-Kramer multiple comparisons test was used. Behavioral data (Fig. 6) were analyzed by two-way analysis of variance with repeated measures, followed by post hoc comparisons with Bonferroni method. Survival rate (Fig. 7) was analyzed by Log-rank test. Two-tailed probability values less than 0.05 were considered significant.
Results

**Nicotine Inhibits ICH-Induced Neuron Loss.** Neuropathological changes within the hematoma were assessed by immunohistochemistry against a neuronal marker NeuN, at 3 days after injection of collagenase into the striatum of the right hemisphere of mice. At macroscopic level, NeuN-positive signals were much lower in the hematoma region than the surrounding intact area of immunostained sections (Fig. 1A). In a close view, the number of NeuN-positive cells in the center of hematoma (central region) and in the region adjacent to the intact area (peripheral region) was found to decrease substantially (Fig. 1B, 1C, 1F and 1G), which was consistent with our previous findings (Matsushita et al., 2011). Daily administration of nicotine (1 and 2 mg/kg), starting from 3 h after collagenase injection, prevented the decrease in the number of NeuN-positive cells in the central region (Fig. 1D, 1E and 1F). The effect of nicotine was dose-dependent and reached statistical significance at a dose of 2 mg/kg. In the peripheral region, the neuroprotective effect of nicotine was modest and did not reach statistical significance (Fig. 1G).

To confirm the protective effect of 2 mg/kg nicotine in the central region of hematoma, we next performed TUNEL staining. TUNEL detects apoptotic cell death associated with DNA fragmentation. We found that a substantial number of TUNEL-positive cells appeared in the central region of hematoma at 1 day after induction of ICH (Fig. 2A). Treatment with 2 mg/kg nicotine partially but significantly decreased the number of TUNEL-positive cells (Fig. 2B and 2C).

**Nicotine Reduces Expression of Pro-apoptotic Protein Bax.** Results above suggested that nicotine interfered with occurrence of apoptotic neuron death. In this context, nicotine has been reported to upregulate expression of Bcl-2 via stimulation of α7 nAChRs (Kihara et
al, 2001; Akaike et al., 2009). Bcl-2 is an anti-apoptotic protein localized in mitochondria that interrupt mitochondria-dependent pathway of apoptosis by preventing cytochrome c release (Tsujimoto, 2003). Accordingly, we examined whether the same mechanisms could be involved in the protective effect of nicotine in vivo. When we examined the level of Bcl-2 protein expression in mice without ICH by western blot analysis, we found that Bcl-2 in the brain tissue tended to increase at 3 h after intraperitoneal administration of 2 mg/kg nicotine. Concomitantly, the expression level of a pro-apoptotic protein Bax was decreased significantly by 2 mg/kg nicotine. Consequently, the expression ratio of Bcl-2/Bax was significantly increased after nicotine treatment (Fig. 3A and 3B). Moreover, double immunofluorescence histochemistry at 3 days after ICH revealed Bax immunoreactivity in NeuN-positive cells within the hematoma region of vehicle-treated mice (Fig. 3C-E). Daily treatment with 2 mg/kg nicotine decreased neuronal Bax immunoreactivity (Fig. 3F-H).

**Nicotine Decreases Activated Microglia/Macrophages, Neutrophils and Areas Affected by Oxidative Stress.** Pathogenic events in ICH include recruitment of activated microglia/macrophages and neutrophils, which is intimately associated with increased oxidative stress (Wang and Dore, 2007a, 2007b). In the next set of experiments, we examined the effect of nicotine on these inflammatory processes, because nicotine has been reported to suppress microglial activation (Suzuki et al., 2006; Lee et al., 2009). To evaluate ICH-induced inflammation, activated microglia/macrophages, neutrophils and oxidative stress were probed by isolectin B4 binding, MPO immunoreactivity, and nitrotyrosine immunoreactivity, respectively. Since isolectin B4 binding also detected endothelial cells and neutrophils, morphological criteria were applied to identify activated microglia/macrophages (Matsushita et al., 2011). At 3 days after induction of ICH, activated
microglia/macrophages accumulated mainly in the peripheral region of hematoma. Daily administration of nicotine (1 and 2 mg/kg) resulted in a significant decrease in the number of activated microglia/macrophages, in a dose-dependent manner (Fig. 4A, 4D and 4G). MPO-positive infiltrating neutrophils were distributed diffusely within the entire region of hematoma. Nicotine at 2 mg/kg partially but significantly decreased the number of infiltrating neutrophils (Fig. 4B, 4E and 4H). Expansion of the area affected by oxidative stress as revealed by nitrotyrosine immunoreactivity was also significantly suppressed by daily treatment with 2 mg/kg nicotine (Fig. 4C, 4F and 4I). These results suggested that nicotine attenuated inflammatory reactions in response to ICH.

Nicotine Does Not Affect Hematoma Expansion or Brain Edema. We assessed lesion volume and brain water content, to clarify whether nicotine suppressed hematoma expansion and brain edema. According to the procedures by Matsushita et al. (2011), lesion volume at 3 days after ICH induction was assessed by Nissl staining. Obtained values were not different between vehicle-treated mice and nicotine-treated mice (Fig. 5A-C). Brain water content at 3 days after ICH induction was increased in the hemisphere ipsilateral to the hemorrhage, compared with the sham-operated group, indicating edema formation (Fig. 5D). Treatment with nicotine (1 and 2 mg/kg) had no significant effect on the level of brain water content.

Nicotine Improves Neurological Deficits after Intracerebral Hemorrhage. We conducted several sets of behavioral experiments to verify whether nicotine treatment improved neurological deficits. Behavioral assessments were performed before, and 1, 3, 7 and 14 days after induction of ICH (Fig. 6A). Following induction of ICH, foot fault rate in the beam walking test was substantially increased. Mice treated with 2 mg/kg nicotine, once
daily for 3 days, showed significant improvement in recovery from initial deficits (Fig. 6B).

With regard to performance score in the same test, nicotine treatment produced tendency to improve recovery from ICH-induced decrease, although the effect did not reach statistical significance (Fig. 6C).

In the modified limb placing test, neurological score was increased after induction of ICH and remained elevated during 14 days of observations. Treatment with 2 mg/kg nicotine significantly lowered the score throughout the entire period (Fig. 6D). Nicotine was also effective in alleviating the deficit in performance in the adhesive removal test at 1 and 3 days after induction of ICH (Fig. 6E). In these sets of behavioral experiments, sham-operated mice treated with 2 mg/kg nicotine showed performance indistinguishable to that of vehicle-treated sham-operated mice (data not shown).

**Nicotine Increases Survival Rate of Mice after ICH.** Compared to sham-operated mice that showed 100% survival at 3 days after surgery, mice with ICH exhibited decline in the survival rate, which reached 67.2% at 3 days after induction of ICH. Notably, daily treatment with 2 mg/kg of nicotine significantly improved the survival rate of mice after ICH to 87.5% (Fig. 7). Nicotine-treated sham-operated mice retained 100% survival (7 of 7 mice) at 3 days after surgery.
Discussion

The effect of nicotine on ‘ischemic’ brain injury has been a subject of several studies. Acute pretreatment with nicotine attenuated ischemia-reperfusion injury in gerbil hippocampus (Nanri et al., 1998). Similarly, nicotine administered 5 min before occlusion prevented delayed death of hippocampal neurons in rats (Kagitani et al., 2000), but this effect was attributed to enhanced regional blood flow in the hippocampus by nicotine. In a chronic treatment regimen, two daily injections of nicotine for 12 days in rats, from 48 h after unilateral devascularization of the motor cortex, facilitated recovery of motor performance (Gonzalez et al., 2006). In contrast, continuous delivery of nicotine for 14 days by osmotic minipumps has been reported to exacerbate focal ischemia-induced injury (Wang et al., 1997). Therefore, depending on treatment regimens, nicotine exerts divergent influences on pathological consequences in ischemic brain injury. Despite a wealth of these findings on brain ischemia, there are no reports examining the effect of nicotine or nAChR agonists on hemorrhagic brain injury. Here we for the first time addressed this issue and found that nicotine exerted therapeutic effects on ICH model in mice.

Intraperitoneal nicotine administration, starting from 3 h after induction of ICH, significantly inhibited neuron loss. This effect was unexpected because, in organotypic slice culture model of hemorrhagic injury, long-term pretreatment with nicotine was required to produce significant neuroprotective effect (Ohnishi et al., 2009). In this context, our preliminary examinations in ICH model in vivo revealed that pretreatment with nicotine from 1 day or 7 days before induction of ICH gave essentially similar results with those of the post-treatment regimen shown in the present study, although the degree of neuroprotection was somewhat greater in pretreatment regimens (data not shown). Reasons for discrepancy
of results between in vitro and in vivo remain to be clarified, but the present results suggest that nAChRs are promising targets for emergency therapy for ICH.

Neuroprotective effect of nicotine was significant in the central region of hematoma, where ICH-associated inflammatory reactions such as accumulation of activated microglia/macrophages and increased oxidative stress (Matsushita et al., 2011) were less prominent than in the peripheral region. This fact implies that direct actions of nicotine onto neurons (Akaike et al., 2009) contributed to neuroprotection. Previous studies have shown that neuroprotection by stimulation of α7 nAChRs may be mediated by up-regulation of Bcl-2, an anti-apoptotic protein (Kihara et al., 2001). Bcl-2 antagonizes the action of Bax, a pro-apoptotic protein involved in mitochondrial outer membrane permeabilization (Tait and Green, 2010), and increased Bcl-2/Bax ratio is generally considered to reflect that the cells are protected from cell death program. Unfortunately, the expression levels of Bcl-2 and Bax proteins within the hematoma could not be evaluated precisely, because of inevitable contamination of blood constituents. However, examinations in animals without ICH revealed that systemic nicotine treatment promptly increased Bcl-2/Bax ratio in the brain. Particulaly, Bax protein level was significantly decreased by nicotine treatment. Moreover, immunohistochemical examinations showed that Bax immunoreactivity in neurons after ICH induction was lowered by nicotine treatment. Down-regulation of neuronal Bax by nicotine has not been reported so far, although in lung cancer cell lines nicotine inactivates Bax by promoting its phosphorylation (Xin and Deng, 2005). Precise molecular mechanisms leading to down-regulation of Bax should be explored in future investigations, but the effect of nicotine on Bcl-2/Bax may be closely related to the therapeutic effect. The observation that ICH-induced increase in TUNEL-positive cells was significantly attenuated by nicotine is
also consistent with this proposal.

Stimulation of α7 nAChRs suppresses activation of microglia/macrophages and resultant release of cytotoxic mediators such as nitric oxide and tumor necrosis factor α (Wang et al., 2003; Suzuki et al., 2006). Results of our histochemical examinations also indicated that ICH-associated activation of microglia in the peripheral region of hematoma was suppressed by nicotine treatment. Significant attenuation by nicotine of the increase in oxidative stress in the peripheral region may have resulted from suppressed activation of microglia/macrophages. Nicotinic receptor subtypes mediating neuroprotective and anti-inflammatory actions of nicotine in hemorrhagic brain remain to be identified. In this context, our previous study has demonstrated that both α7- and β2-containing nAChRs are involved in counteraction by nicotine of thrombin-induced increase in activated microglia in cortico-striatal slice cultures (Ohnishi et al., 2009).

A finding that deserves consideration is apparent discrepancy between neuroprotective action and anti-inflammatory action of nicotine. That is, anti-inflammatory action of nicotine was mainly observed in the peripheral region where activated microglia/macrophages accumulated, but neuronal survival in the same region was not clearly promoted by nicotine. At present we do not have clear explanations for this discrepancy, but in any case, these results propose the possibility that neuroprotective action of nicotine has minimal relation with its action onto microglia/macrophages. On the other hand, nicotine-induced attenuation of neutrophil infiltration, though its degree was small, may contribute to neuroprotection by nicotine, because infiltrated neutrophils were distributed in the central region of hematoma. A recent study has demonstrated important roles of neutrophils in the pathogenesis of experimental ICH (Moxon-Emre and Schlichter, 2011).
Nicotine administration under the present experimental conditions produced no significant influences on either lesion volume (that reflected the extent of hematoma expansion) or edema formation. In contrast, a recent study on brain ischemia model in vitro and in vivo has shown that nicotine exacerbates brain edema (Paulson et al., 2010). The difference between our study and that of Pauson et al. (2010) may be explained by different experimental conditions such as dosing regimens and also by different events involved in formation of edema under hemorrhagic and ischemic conditions. In any case, the present results indicate that neuroprotective effect of nicotine was independent of physical damage-related factors such as hematoma expansion and brain edema.

The most notable findings were that daily nicotine administration for 3 days enhanced recovery of sensorimotor functions of mice and also improved survival rate of mice after ICH. Although various receptors, enzymes and signaling molecules have been proposed as potential targets for ICH therapy (Katsuki, 2010), to our knowledge, none of them has been shown to decrease the mortality rate of experimental animals. Overall, multiple beneficial effects of nicotine demonstrated in the present study may provide a basis for establishing novel therapeutic strategies for ICH.
Authorship Contributions

Participated in research design: Hijioka, Matsushita, Hisatsune, Isohama, and Katsuki

Conducted experiments: Hijioka and Matsushita

Performed data analysis: Hijioka, Hisatsune, Isohama, and Katsuki

Wrote or contributed to the writing of the manuscript: Hijioka and Katsuki

Other: Katsuki acquired funding for the research.
References


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Footnotes

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Figure legends

**Fig. 1.** Effect of nicotine on ICH-induced neuronal damage.  (A) A representative image of a coronal section immunostained with anti-NeuN antibody.  The central region and the peripheral region for cell counts were denoted by rectangles with and without an asterisk, respectively.  Scale bar = 1 mm.  (B-E) Representative images of the central region of hematoma in NeuN-immunostained coronal sections obtained 3 days after induction of ICH.  Mice received intrastriatal injection of saline (B) or collagenase (C–E).  Administration of vehicle (saline; B and C) or nicotine at indicated doses (D and E) was performed once daily for 3 days, starting from 3 h after induction of ICH.  Scale bar = 50 µm.  (F, G) Quantitative results on the number of NeuN-positive cells in the central (F) and the peripheral (G) regions of hematoma.  *p < 0.05 versus sham group; †††p < 0.001 versus vehicle group.

**Fig. 2.** Nicotine inhibits cell death within the hematoma.  (A, B) Representative TUNEL images of the central region of hematoma in coronal sections obtained 24 h after induction of ICH.  Mouse received intraperitoneal administration of vehicle (A) or 2 mg/kg nicotine (B) at 3 h after induction of ICH.  Scale bar = 50 µm.  (C) The number of TUNEL-positive cells in the central region of hematoma was quantified.  n = 5 mice for each condition.  *p < 0.05 versus vehicle group.

**Fig. 3.** Nicotine reduces expression of neuronal Bax.  (A) Representative images of immunoblots showing expression levels of Bcl-2, Bax and β-actin, at 3 h after administration of vehicle or 2 mg/kg nicotine.  (B) Results of densitometrical quantification of Bcl-2, Bax
and Bcl-2/Bax, expressed as fold of vehicle group. The protein levels of Bcl-2 and Bax were obtained after normalization with the band intensity of β-actin. \( n = 4 \). \( * p < 0.05 \) versus vehicle group. (C-H) Double immunohistochemical localization of Bax and NeuN, at 3 days after induction of ICH. Mouse received daily intraperitoneal administration of vehicle (C-E) or 2 mg/kg nicotine (F-H). Arrowheads denote doubly positive cells. Scale bar = 50 μm.

**Fig. 4.** Nicotine reduces the number of microglia/macrophages, neutrophils and the level of oxidative stress. (A-F) Representative images of cells positive for isolectin B4 binding in the peripheral region of hematoma (A, D), myeloperoxidase in the central region of hematoma (B, E) and nitrotyrosine (C, F), at 3 days after induction of ICH. Mouse received daily intraperitoneal administration of vehicle (A-C) or 2 mg/kg nicotine (D-F). Scale bar = 50 μm (A, D), 20 μm (B, E), 500 μm (C, F). (G-I) Vehicle or nicotine at indicated doses was intraperitoneally administered, once daily for 3 days starting from 3 h after induction of ICH by intrastriatal collagenase injection. Shown are quantitative results on the number of isolectin B4 binding-positive cells (G), the number of myeloperoxidase-positive cells (H), and nitrotyrosine-positive area (I). \( n = 5 – 8 \) mice for each condition. \( * P < 0.05, ** P < 0.01, *** P < 0.001 \) versus vehicle group.

**Fig. 5.** Nicotine does not affect lesion volume and brain edema. (A, B) Representative images of Nissl stained sections showing the area invaded by hematoma in vehicle-treated (A) and nicotine-treated (B) mice, obtained 3 days after induction of ICH. Scale bar = 1 mm. (C) Results of quantification of lesion volume in vehicle-treated and nicotine-treated mice. \( n = 6 – 7 \) mice for each condition. (D) Results of quantification of brain water content.
Water content of hemispheres ipsilateral and contralateral to ICH was quantified at 3 days after induction of ICH. Mice received daily intraperitoneal administration of vehicle or nicotine at indicated doses, starting from 3 h after intrastriatal collagenase injection. \( n = 5 – 6 \) mice for each condition.

**Fig. 6.** Nicotine alleviates neurological deficits after ICH. (A) Schematic representation of the experimental schedule. Vehicle or nicotine (2 mg/kg, i.p.) was administered once daily for 3 days at 24-h interval, starting from 3 h after induction of ICH. Behavioral tests were performed at 1 day (24 h), 3 days (72 h), 7 days and 14 days after surgery. That means, tests at 1 day were performed 21 h after the first injection of vehicle or nicotine, and tests at 3 days were performed 21 h after the last injection of vehicle or nicotine. (B and C) Performance of mice in the beam-walking test, evaluated by foot fault rate (B) and performance score (C). (D and E) Results of performance in the modified limb-placing test (D) and the adhesive removal test (E). \( n = 14 – 19 \) mice for each condition. *\( p < 0.05, *** p < 0.001 \) versus vehicle group.

**Fig. 7.** Nicotine improves survival rate of mice after ICH. Vehicle or nicotine (2 mg/kg, i.p.) was administered once daily for 3 days, starting from 3 h after induction of ICH. *\( p < 0.05 \) versus sham group, †† \( p < 0.01 \) versus vehicle group.
Fig. 1

A

B  C

D  E

F  G

Sham  Vehicle  1  2

Nicotine (mg/kg)

Collagenase

Center

Periphery

Number of NeuN positive cells

Sham  Vehicle  1  2

Nicotine (mg/kg)

Collagenase
Fig. 2

A  Vehicle  B  Nicotine

C

Number of TUNEL positive Cells

Vehicle  Nicotine

*
Fig. 3

(A) Western blot analysis showing the expression of Bcl-2, Bax, and β-actin in Vehicle and Nicotine treated groups. The molecular weights are indicated in kDa for each protein.

(B) Bar graph depicting the fold change of Bcl-2, Bax, and Bcl-2/Bax ratios in Vehicle and Nicotine treated groups. The error bars represent the standard error of the mean (SEM).

(C, D, E) Confocal microscopy images showing Bax, NeuN, and merged images in Vehicle and Nicotine treated groups. The arrows indicate the localization of proteins.

(F, G, H) Scale bar: 30 μm.
Figure 4

**Isolectin B$_4$**

- A: Vehicle
- D: Nicotine

**Myeloperoxidase**

- B: Vehicle
- E: Nicotine

**Nitrotyrosine**

- C: Vehicle
- F: Nicotine

**Graphs**

- **G**: Number of Isolectin B$_4$-positive cells
  - Vehicle
  - 1 mg/kg Nicotine
  - 2 mg/kg Nicotine

- **H**: Number of Myeloperoxidase positive cells
  - Vehicle
  - 1 mg/kg Nicotine
  - 2 mg/kg Nicotine

- **I**: Nitrotyrosine positive area (mm$^2$)
  - Vehicle
  - 1 mg/kg Nicotine
  - 2 mg/kg Nicotine

**Note:** The images and graphs are not labeled with specific data points or statistical significance indicators (e.g., * or **).
Fig. 5

A and B: Images showing the effect of nicotine on lesion volume compared to vehicle control.

C: Bar graph showing lesion volume (mm³) with different nicotine treatments.

D: Bar graph showing water content (%) with different nicotine and collagenase treatments.
Fig. 6

A
ICH Induction

Vehicle or Nicotine

0 1 3 7 14 (day)

Behavioral Test

B
Beam Walking Test (Fault rate)

Fault rate (%)

0 20 40 60 80 100

0 2 4 6 8 10 12 14

Days after ICH

***

C
Beam Walking Test (Score)

Score

0 5 10 15 20

0 2 4 6 8 10 12 14

Days after ICH

D
Modified Limb Placing Test

Score

0 1 2 3 4 5

0 2 4 6 8 10 12 14

Days after ICH

***

E
Adhesive Removal Test

Removal time – Adhesive time (sec)

0 20 40 60 80 120

0 2 4 6 8 10 12 14

Days after ICH

- Sham+Vehicle
- ICH+Vehicle
- ICH+Nicotine
(2 mg/kg)

***

*
Fig. 7

Survival rate (%) vs. Days after ICH

- Sham+Vehicle: 33/33
- ICH+Nicotine: 42/48
- ICH+Vehicle: 41/61

* ††