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Administration of S-methyl-L-thiocitrulline protects against brain injuries after intracerebral hemorrhage

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Abstract

Although intracerebral hemorrhage (ICH) increases the level of glutamate in the perihematomal area and cerebral spinal fluid (CSF) in the ICH acute phase, it is unclear whether elevated glutamate activates neuronal nitric oxide synthase (nNOS) in the ICH brain and whether nNOS is an important target for ICH treatment. Here, we assessed the role of the nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) in the activity of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and ICH-induced brain injuries. An autologous blood intracerebral infusion model in male rats was used. All of the rats were sacrificed 24 hours after ICH. ICH increased NADPH-d activity in the striatum. Administering SMTC 3 h after ICH decreased the activity of NADH-d ($p < 0.05$ vs. the ICH group). The activation of gelatinolytic enzymes in the perihematomal region of the striatum was reduced by SMTC treatment ($p < 0.01$, vs. the ICH group). The loss of laminin- and occludin-stained vessels was significant in perihematomal regions after 24 h of ICH and was significantly attenuated by the administration of SMTC ($p < 0.01$ for laminin, $p < 0.05$ for occluding, compared with the ICH group). Neuronal death and neurological deficits after ICH were also decreased in SMTC treatment rats ($p < 0.01$, vs. the ICH group). The results suggest that the administration of the nNOS inhibitor SMTC after ICH protects against ICH-induced brain injuries and improves neurological function.

Keywords

intracerebral hemorrhage; S-methyl-L-thiocitrulline; brain injury; rats

Introduction

It is known that intracerebral hemorrhage (ICH) increases the level of glutamate in the perihematomal area and in cerebral spinal fluid (CSF) during the acute phase of ICH (Chiang et al., 2006, Nilsson et al., 2006). In addition, blood and CSF glutamate concentrations independently predict a poor clinical outcome in ICH patients (Castillo et al., 2002, Chiang et al., 2006); however, whether elevated glutamate activates neuronal nitric

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oxide synthase (nNOS) in the ICH brain and whether nNOS is an important target for ICH treatment have not been previously investigated. nNOS is the main subtype of nitric oxide synthase in the brain (Zhou and Zhu, 2009). Pathological stimulation by glutamate leads to unregulated nNOS activation and to the production of toxic levels of nitric oxide (NO) (Dawson and Dawson, 1996). Following ICH, non-heme iron and unconjugated bilirubin increase and contribute to ICH-induced secondary brain injuries (Xi et al., 2006, Loftspring et al., 2011). It has been shown that iron and bilirubin lead to neurotoxicity, partially through activating the NMDA-nNOS signaling cascade (Gulturk et al., 2008, Brito et al., 2010). Therefore, it is possible that nNOS is activated in the acute phase of ICH and contributes to early ICH-induced brain injuries.

In the current study, an autologous blood intracerebral infusion model in rats was used to simulate ICH. The selective nNOS inhibitor SMTC was administered 3 h after ICH. We assessed changes in NADPH-d activity, the activation of gelatinolytic enzymes, the levels of basal lamina and tight junction proteins, neuronal death and neurological functions.

Experimental procedures

Animals

The University of Cincinnati Animal Care Committee approved all experimental procedures. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male Sprague–Dawley rats weighing 250 to 300 g were used for this study. The animals were allowed to acclimate in the animal housing facility for at least 3 days before entry into the study.

Intracerebral hemorrhage model and experimental groups

This study used an intrastriatal blood infusion ICH model similar to our previously published method, with some modifications (Lu et al., 2006). Briefly, rats were deeply anesthetized with isoflurane (3% in 21% oxygen and 76% nitrogen) and placed into a Kopf stereotaxic frame. After removing the hair and cleaning the scalp, the skin was incised. A burr hole was drilled 0.5 mm anterior and 3.2 mm lateral left of the bregma. A 27 G needle was lowered to a depth of 4 mm below the dural surface into the striatum. Thirty microliters (for 1 d of observation) or fifty microliters (for 3 d of observation) of autologous arterial blood that was drawn from the femoral artery was infused using a minipump at a rate of 5 μ l/min. The core temperature of each animal was maintained at 37°C, with a rectal thermistor that was connected to a feedback-controller-driven heating pad. The needle was removed, the wound cleaned and the scalp sutured. The animals were allowed to recover in their home cages. During the recovery period, the animals had free access to food and water and were maintained on a 12 h light–dark cycle.

Three groups of animals were studied: (1) the sham group, a sham operation with needle insertion without infusion; (2) the ICH group, which was given an infusion of 30 μ l or 50 μ l autologous blood into the striatum, and (3) the SMTC group, which was given an infusion of 30 μ l or 50 μ l autologous blood into the striatum. Animals were then injected with SMTC in

a 0.9% saline solution (3 mg/kg, 0.5 ml/100 g, i.p.) 3 h after ICH and then every 24 h for 3 d. The SMTC dose is from a previous publication (Wakefield et al., 2003). Total twenty seven rats were used. The number of animals for each group was listed in the figure legends. All animals survived. The animals that were given the 30 μ l blood infusion were sacrificed on day 1, and those animals that were given the 50 μ l blood infusion were sacrificed on day 3. In the sham and ICH groups, animals were injected with a 0.9% saline solution (0.5 ml/100 g, i.p.) 3 h after the blood infusion and then every 24 h for 3 d as a vehicle control.

NADPH-d histochemistry

NADPH-d staining was performed as described previously, with minor modifications (Morris et al., 1997, Hoque et al., 2010). At designated time points, rats were deeply anesthetized with isoflurane and sacrificed. The brains were removed and frozen in 2-methylbutane. Coronal sections (20 μ m) were cut in a cryostat (-20°C) and stored at -80°C . Slide-mounted sections were fixed with 4% PFA for 10 min, followed by 3 washes with 0.1 M PBS (pH 7.4, 5 min each). Then, the sections were incubated with nitroblue tetrazolium (0.25 mg/mL) and NADPH (0.2 mg/mL) in 0.1 M PBS (pH 7.4), which contained 0.25% Triton X-100, at 37°C for 60 min, followed by 3 PBS washes (5 min each). Slides were then rinsed and coverslipped. The images were taken with a constant exposure, and the change in stain intensity across different groups was quantified using an MCID digital image analysis system (Imaging Research, Inc., St Catherines, Ontario, Canada).

In situ zymography

Gelatin *in situ* zymography was used to locate the sites of net gelatinolytic activity (mainly MMP-2 and MMP-9) as previously described (Wang and Tsirka, 2005, Lua et al., 2008). Briefly, the frozen sections were thawed and incubated with reaction buffer, which contained 100 μ g/ml FITC-labeled DQ gelatin, for 10 h at room temperature and 2 h at 37°C . The sections were rinsed in PBS and then mounted in ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA, USA). The images were taken using fluorescence microscopy. Gelatinolytic activity-positive cells and blood vessels were counted in four fields that were immediately adjacent to the hematoma and were expressed as number/ mm^2 areas (Xue et al., 2006). The number of gelatinolytic-positive blood vessels and cells were quantified.

Immunohistochemistry

Immunohistochemistry analysis was performed as previously described (Lu et al., 2008). Briefly, frozen coronal sections (20 μ m) were used. After fixing in 2% paraformaldehyde for 10 min, the sections were blocked for 1.5 h in blocking buffer (2% serum, 0.2% Triton X-100, 0.1% bovine serum albumin in 0.1 M PBS). Following incubation with rabbit anti-laminin (1:4000, Sigma, St. Louis, MO) and rabbit-anti-occludin (C-terminal; 1:100 for immunohistochemistry, Invitrogen, Camarillo, CA), the sections were washed in PBS and incubated with biotinylated secondary antibodies for 1.5 h (Vector Laboratories, Burlingame, CA, USA). Next, ABC reagent (Vector Laboratories) was applied to sections after three 5-min PBS washes. Lastly, diaminobenzidine was used to visualize the horseradish peroxidase. Four images were taken in the striatum immediately adjacent to the

hematoma. The percentage of laminin- and occluding-positive microvascular area in the measured image area (% of stained area) was measured and calculated using the MCID imaging system.

Fluoro-Jade staining

Fluoro-Jade staining was used to reveal dying neurons as previously described (Xue et al., 2006). Briefly, sections were incubated and gently shaken in 0.06% potassium permanganate for 15 min. Fluoro-Jade (0.001%; Histo-Chem, Jefferson, AR) staining solution was applied for 30 min, followed by a PBS wash, drying, and coverslip application. The images were taken at high magnification (20X objective magnification). Fluoro-Jade-positive neurons were counted in four fields that were immediately adjacent to the needle insertion/damage site. Fluoro-Jade-positive cells per mm² were quantified.

Corner test

A corner test was performed to identify and quantify the changes in sensorimotor and postural asymmetries as described in previous publications (Hua et al., 2002, Schaar et al., 2010). An animal was placed between the boards facing the corner (30°). As the animal approached the corner, both sides of the vibrissae were simultaneously stimulated. The animal reared and turned 180 degrees. The test was repeated 10 times, with at least 30 seconds between trials, and the percentage of left turns was calculated. Only turns that involved full rearing along either wall were included (i.e., ventral tucks or horizontal turns were excluded). The rats were not picked up immediately after each turn so that the animals did not develop an aversion for their prepotent turning response.

Statistical analysis

Quantitative data were expressed as the mean $\pm$ SD. Statistical comparisons were conducted using a t-test or an ANOVA for group comparisons. Differences with $p < 0.05$ were considered statistically significant.

Results

ICH increased NADPH-d activity in the striatum 24 h after ICH. Administering SMTC 3 h after ICH decreased the activity of NADH-d

As shown in NADPH-d histochemistry staining in Fig. 1, ICH significantly increased the intensity of NADPH-d staining in the striatum 24 h after ICH (0.55 ± 0.04 in the ICH group vs. 0.36 ± 0.02 in the sham group, $p < 0.01$). When SMTC (3 mg/kg, i.p.) was administered 3 h after ICH, the intensity of NADPH-d staining significantly decreased (0.49 ± 0.02 in the SMTC group vs. 0.55 ± 0.04 in the ICH group, $p < 0.05$). There were many weak stains in the ICH brain due to the low sensitivity of the histochemistry staining, which was also reduced by SMTC administration (Fig. 1b, c).

ICH-induced activity of gelatinolytic enzymes was decreased by SMTC treatment

As shown in gelatin *in situ* zymography in Fig. 2, only a few blood vessels and cells with weak fluorescence were observed in the striatum of the sham group. ICH for 24 h caused the

obvious activation of gelatinolytic enzymes in the perihematomal region of the striatum, with increased gelatinolytic-positive cells and vessels (no./mm²; $p < 0.01$, compared with the sham group). The administration of SMTC (3 mg/kg, i.p.) 3 h after ICH reduced the gelatinolytic activity and the number of gelatinolytic-positive cells and vessels (no./mm²; $p < 0.01$, compared with the ICH group).

The ICH-induced injuries to cerebral microvascular basal lamina and tight junctions were decreased by SMTC treatment

The injuries to the basal lamina and tight junction in the cerebral blood vessels were assessed using laminin and occludin immunohistochemistry. As shown in Fig. 3 and Fig. 4, the loss of laminin and occludin was significant in perihematomal regions after 24 h of ICH, as determined by the percentage of laminin- and occludin-stained vessels compared with sham controls (Fig. 3, 4; $p < 0.01$). The laminin- and occludin-positive microvessels were degraded to short segments. After the administration of SMTC (3 mg/kg, i.p.) 3 h after ICH, the loss of laminin and occludin was significantly attenuated (Fig. 3; $p < 0.01$ for laminin, Fig. 4; $p < 0.05$ for occludin.), and the integrity of microvasculature (microvascular size) was improved.

Neuronal death after ICH decreased by SMTC treatment

The dying neurons after ICH were revealed using Fluoro-Jade staining. As shown in Fig. 5, ICH resulted in neuronal death in the perihematomal region of the striatum 24 h after ICH. The administration of SMTC (3 mg/kg, i.p.) 3 h after ICH significantly reduced neuronal death compared with the ICH group ($p < 0.01$).

Neurological deficits following ICH were improved by SMTC treatment

As shown in the corner turn test in Fig. 6, ICH rats exhibited significant behavioral deficits at all time points after ICH (Fig. 6, $p < 0.01$). The administration of SMTC (3 mg/kg, i.p.) 3 h after ICH significantly improved the neurological deficits at 24 h after the infusion of 30 μ l autologous arterial blood (Fig. 6A, $p < 0.01$) and at 48 h and 72 h after the infusion of 50 μ l autologous arterial blood (Fig. 6B, $p < 0.01$).

Discussion

The nNOS is constitutive NOS (Zhou and Zhu, 2009). In the current study, we mainly focused on changes of its activities after ICH. We assessed the activity of NADPH-d using NADPH-d histochemical staining. A selective nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) was also used (Furfin et al., 1994). Although there are a few enzymes that are known to be responsible for NADPH-d activation, only NOS is insensitive to formaldehyde fixation, which was used in our method (Morris et al., 1997). It has been reported that NADPH-d staining accounts for the function of NOS in neuronal tissue (Morris et al., 1997, Hoque et al., 2010). SMTC had its relative specificity for nNOS inhibition both *in vitro* (Human nNOS/eNOS = 9, nNOS/iNOS = 33) and *in vivo* (17-fold selective for rat nNOS in neuronal tissue compared with rat eNOS in vascular endothelial tissue) (Furfin et al., 1994). The systemic administration of SMTC attenuated the concentration of NO in brain tissue for several hours and had neuroprotective effects (Colado et al., 2001, Darvesh et al.,

2005). In the current study, we demonstrated that ICH increased NADPH-d activity in the striatum 24 h after ICH. A dose of 3 mg/kg SMTC has been used, which appeared to be unable to inhibit eNOS in the *in vivo* study (Wakefield et al., 2003). Administering SMTC 3 h after ICH reduced NADPH-d activity. Previous studies indicated that ICH increased the level of glutamate in the perihematomal area starting from 30 min, with a peak at 6 h to 12 h (Qureshi et al., 2003a, Nilsson et al., 2006). There was a 25-fold increase in the CSF glutamate level in ICH patients (Chiang et al., 2006). Glutamate induces the activation of the NMDA receptor (NMDAR) and leads to the formation of a complex of NMDAR-PSD95-nNOS. A Ca^{2+} influx through NMDAR can efficiently activate nNOS (Kiedrowski et al., 1992, Boehning and Snyder, 2003). Memantine, which is an uncompetitive NMDA receptor antagonist, reduced ICH brain injuries at day 3 and improved functional recovery in a collagenase-induced ICH rat model (Lee et al., 2006a). Previous studies also indicated that iron and bilirubin lead to neurotoxicity partially through activating the NMDA-nNOS signaling cascade (Gulturk et al., 2008, Brito et al., 2010). Following ICH, non-heme iron and unconjugated bilirubin increased (Wu et al., 2003, Clark et al., 2008), which could contribute to nNOS activation. These results support our speculation that nNOS was activated in the ICH brain in early stage of ICH.

ICH induced the upregulation of MMP-2, -3, -7, -9 and -12 and the activation of MMP-2 and -9 (Wang and Tsirka, 2005, Florczak-Rzepka et al., 2012). Activated MMPs caused the loss of collagen IV-stained microvessels, damage to endothelial barrier integrity, extravasation of plasma proteins and brain edema (Wang and Tsirka, 2005, Wasserman and Schlichter, 2007, Katsu et al., 2010). In the current study, the administration of SMTC 3 h after ICH reduced gelatinolytic activity. Because *in situ* gelatin zymography is highly sensitive to MMP-2 and -9 activation (Lu et al., 2008), the decrease in gelatinolytic activity with nNOS inhibition following ICH could indicate that nNOS contributed to MMP-2 and/or MMP-9 activation and could be treated by the administration of SMTC. We also demonstrated that treatment with SMTC 3 h after ICH protected against ICH-induced basal lamina and tight junction injuries. Previous studies indicated that NO that was produced by nNOS activated MMP-9. After cerebral ischemia, activated MMP-9 co-localized with the nNOS and was abrogated in nNOS knockout mice or in wild-type animals that were treated with the nNOS inhibitor. Neuroprotection has been demonstrated under each of these conditions of NOS inhibition (Huang et al., 1994, Gu et al., 2002). nNOS has also been connected with BBB interruption and brain edema formation in hyperthermic brain injury (Alm et al., 2000), focal brain and spinal cord trauma (Sharma et al., 2005, Sharma et al., 2006), transient cerebral ischemia and brain hypoxic-ischemia (Hara et al., 1996, Ishida et al., 2001). These results are consistent with our findings.

In the current study, we also demonstrated that that administration of SMTC attenuated ICH-induced neuronal death. Experimental models and human brain specimens with ICH show progressive cell death in hemorrhagic brain (Qureshi et al., 2003b, Wang and Tsirka, 2005). The level of elevated glutamate in ICH brain could lead to excitotoxicity (Chiang et al., 2006, Nilsson et al., 2006). MMP-3, 9 activation and iron toxicity played a deleterious role in acute neuron degeneration after ICH (Wang and Tsirka, 2005, Caliaperumal et al., 2012). Increased unconjugated bilirubin in ICH brain after ICH caused neuronal death

(Clark et al., 2008, Almaas et al., 2012). Treatment with a nNOS inhibitor or knocking out nNOS can protect neurons against glutamate and ischemic injury, NMDA toxicity and improve functional recovery (Huang et al., 1994, Dawson et al., 1996, Coert et al., 2003). A nNOS inhibitor or nNOS knockout abrogated increased MMP activity in the ischemic cortex (Gu et al., 2002). A nNOS blocker also decreased iron-induced brain toxicity (Gulturk et al., 2008). NOS inhibitor attenuated bilirubin-induced neuron death (Brito et al., 2010) and nNOS inhibitor reduced bilirubin neurotoxicity in newborn piglets (Park et al., 2002). Thus, the nNOS inhibitor reduced ICH-induced neuronal death possibly *via* decreased MMP activation and the toxicities of glutamate, iron and bilirubin.

Previous studies also indicated that the degree of BBB breakdown, edema formation and brain tissue damage were correlated with the functional outcome of ICH patients (Lampl et al., 2005, Xi et al., 2006, MacLellan et al., 2008). In the current study, the administration of SMTC improved neurological deficits following ICH, which could be due to decreased neuronal death and injuries to the basal membrane and blood brain barrier. Because functional recovery is a goal in ICH treatment (Qureshi et al., 2009), the improvement of neurological deficits by administering SMTC is of clinical importance.

It has been reported that ICH also upregulated iNOS and produced NO. iNOS protein was not detectable until 24 h post ICH and reached the peak at 3 days after ICH (Zhao et al., 2007). iNOS knockout decreased brain edema at 3 day post ICH (Kim et al., 2009). Hemoglobin infusion into striatum increased eNOS levels and NO production (Yang et al., 2013). In an ICH rat model, EPO treatment activated eNOS and induced better functional recovery (Lee et al., 2006b). Since the peak of glutamate concentration increase was in the early phase of ICH (Qureshi et al., 2003a, Nilsson et al., 2006) and current study indicated that nNOS inhibitor decreased NAPH-d staining and ICH brain injuries at 24 h post ICH, nNOS activation is important in the early stage of ICH brain injuries and could be targeted for ICH treatment.

Conclusions

In this study, we reported that administering SMTC 3 h after ICH decreased the activity of NADH-d. The ICH-induced activation of gelatinolytic enzymes and the loss of laminin- and occludin-stained vessels in the perihematomal region of the striatum were reduced in SMTC-treated rats. The administration of SMTC after ICH also attenuated neuronal death and improved neurological deficits. These findings provide direct evidence that targeting nNOS effectively protected against ICH brain injury and improved neurological function at 24 h after ICH.

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Abbreviations

ICH	intracerebral hemorrhage
nNOS	neuronal nitric oxide synthase
SMTc	S-methyl-L-thiocitrulline
NADPH-d	nicotinamide adenine dinucleotide phosphate-diaphorase
MMP	matrix metalloproteinase
CSF	cerebral spinal fluid
NO	nitric oxide
MCID	microcomputer image device

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Highlights

- Administering SMTC decreased the activity of NADH-d in ICH brain.
- SMTC treatment reduced activation of gelatinolytic enzymes after ICH.
- SMTC decreased the loss of laminin- and occludin-stained vessels in ICH brain.
- SMTC treatment reduced neuronal death in the perihematoma region.
- The administration of SMTC improved neurological deficits after ICH.

Figure 1. NADPH-d histochemistry staining (representative figures, A–C) and quantification (D) in the striatum 24 h after an infusion of autologous arterial blood into the striatum of adult rats A sham operation (needle insertion without infusion) was performed on control animals. ICH: intracerebral hemorrhage. SMTTC: S-methyl-L-thiocitrulline (n = 4). ** $p < 0.01$ compared with the sham group. # $p < 0.05$ compared with the ICH group.

Figure 2. In situ gelatinolytic assays (representative figures, A–C) and quantification (D) in the perihematomal region 24 h after the infusion of autologous blood in adult rats
 ICH: intracerebral hemorrhage (n = 4). SMTC: S-methyl-L-thiocitrulline. ** $p < 0.01$ compared with the sham group. ## $p < 0.01$ compared with the ICH group.

Figure 3. Laminin immunostaining changes (representative figures, A–C) and quantification (D) in the striatum 24 h after the infusion of autologous blood in adult rats

ICH: intracerebral hemorrhage (n =4). SMTC: S-methyl-L-thiocitrulline. ** $p < 0.01$ compared with the sham group. ## $p < 0.01$ compared with the ICH group.

Figure 4. Occludin immunostaining changes (representative figures, A–C) and quantification (D) in the striatum 24 h after the infusion of autologous blood in adult rats

ICH: intracerebral hemorrhage. SMT: S-methyl-L-thiocitrulline (n =4). ** $p < 0.01$ compared with sham group. # $p < 0.05$ compared with the ICH group.

Figure 5. Fluoro-Jade staining (representative figures, A–C) and quantification (D) in the perihematomal region 24 h after the infusion of autologous blood in adult rats
 ICH: intracerebral hemorrhage. SMTC: S-methyl-L-thiocitrulline (n =4). ## $p < 0.01$ compared with the ICH group.

Figure 6. The change in the corner test score (%) after the infusion of 30 μ l autologous arterial blood within 24 h of observation (A, n = 4) and after the infusion of 50 μ l autologous arterial blood within 72 h of observation (B, n = 5) in adult rats

ICH: intracerebral hemorrhage. SMTC: S-methyl-L-thiocitrulline. * $p < 0.05$, ** $p < 0.01$ compared with the sham group. ## $p < 0.01$ compared with the ICH group.