Decreased brain edema after collagenase-induced intracerebral hemorrhage in mice lacking the inducible nitric oxide synthase gene

Laboratory investigation

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Object. Hematoma size and brain edema after intracerebral hemorrhage (ICH) are important prognostic factors. Inducible nitric oxide synthase (iNOS) is induced after cerebral ischemia and is known to be involved in secondary neuronal injury, but its significance in ICH is unknown. The authors tested whether iNOS would influence hematoma size and brain edema after ICH.

Methods. The authors used C57BL/6 and iNOS knockout mice for all the experiments. Experimental ICH was induced by the intrastriatal stereotactic administration of bacterial collagenase. Brain tissue was obtained at 72 hours after ICH. The volume of hematoma was quantified by spectrophotometric assay, and the brain water content was measured. The investigators also measured blood-brain barrier permeability using Evans blue dye.

Results. There was no significant difference in hematoma size between the 2 groups. The brain water content of the lesional hemisphere was higher in C57BL/6 mice than in iNOS knockout mice. More Evans blue leakage in the brain was observed in C57BL/6 control mice than in iNOS knockout mice. Immunohistochemistry showed iNOS immunoreactivity in the perihematoma areas of C57BL/6 mice but not in the iNOS knockout mice.

Conclusions. When hematoma size was similar, iNOS knockout mice had significantly less brain edema than their littermates. These results suggest that iNOS modulation might become an antiedematous therapy for ICH.

(DOI: 10.3171/2009.3.JNS081285)

Key Words • intracerebral hemorrhage • brain edema • inducible nitric oxide synthase • knockout mice • collagenase

Intracerebral hemorrhage accounts for 8 to 14% of all strokes and approximately 37,000–52,400 people in the US suffer ICH each year.2,6,26 This rate is expected to double during the next 50 years as a result of the increasing age of the population and changes in racial demographics.29 The prognosis of ICH is worse than that of ischemic stroke, and the 30-day mortality rate of ICH has been reported to be 45%, much higher than that of ischemic stroke.1 In contrast to ischemic stroke, in which medical complications are the major causes of death, brain death by hematoma itself or cerebral edema is more important in ICH.38

Brain edema is known to be a strong predictor of functional outcome in patients with hyperacute supratentorial spontaneous ICH.8,9 Inflammation occurs after ICH and may have an important role in the pathogenesis of brain damage.10,13,34 Recently, inflammatory cell–produced reactive oxygen species was demonstrated in the perihematoma areas.34 These reactive oxygen species may be responsible for brain edema and cell death following ICH. Nitric oxide produced by NOS is one of the reactive oxygen species by which postischemic inflammation contributes to cerebral ischemic damage.17 Nitric oxide synthase comprises a group of enzymes that catalyze the conversion of L-arginine to L-citrulline and NO. Three isofoms have been identified: NOS-1, -2, and -3. Both NOS-1 (neuronal NOS) and NOS-3 (endothelial NOS) are constitutively expressed and produce relatively small amount of NO; NOS-2 (iNOS) can produce relatively large amount of NO. In the brain, iNOS is
not normally present but appears in several pathological states including tumors, Alzheimer disease, and cerebral ischemia. Delayed administration of the relatively selective iNOS inhibitor aminoguanidine reduces the size of the infarct produced by middle cerebral artery occlusion. In iNOS-null mice, smaller infarcts and a better neurological outcome have been observed after focal ischemic injury. Furthermore, compared with the control group, BBB permeability was significantly reduced after inhibiting iNOS. These observations suggest that large amounts of NO produced by iNOS are toxic to the injured brain and contribute to brain edema and the late stages of cerebral ischemia. However, the role of NO produced by iNOS has not been defined in the mechanisms of brain damage after ICH.

In this study, we used iNOS knockout mice to test the hypothesis that iNOS would influence hematoma size or brain edema after collagenase-induced ICH.

**Methods**

**Animals and ICH Model**

Our institute’s animal care and ethics committee approved all experiments.

We used C57BL/6 and iNOS knockout mice for all the experiments. Male C57BL/6 mice were purchased from Koatech. Dr. Oh kindly provided iNOS knockout mice (iNOS−/−), which have been described previously. The mice were 12–13 weeks of age and weighed 22–25 g at the beginning of this study. They were housed, 4 per cage, in a room maintained at 22 ± 3°C with an alternating 12-hour light-dark cycle. All mice had free access to food and water. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health. Experimental ICH was induced by the stereotactic intrastriatal administration of bacterial collagenase Type VII (Sigma) according to previously described methods. After an intraperitoneal injection with 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg), the mice were placed on a stereotactic frame. A 30-gauge Hamilton syringe needle was inserted through a bur hole into the left striatum (located 2.0 mm left-lateral to the midline, 0.1 mm posterior to the bregma, 4.0 mm deep below the skull). An ICH was induced by the administration of collagenase Type VII (0.5 µl saline containing 0.075 U collagenase) over 1 minute. After being in place for another 4 minutes, the needle was gently removed, and the wound was sutured closed. Rectal temperature was maintained at 37 ± 0.5°C with the use of a thermostat-controlled heating blanket. The mice were allowed to recover from surgery in a cage with free access to food and water. Animals were kept in air-ventilated cages at 24 ± 0.5°C for the duration of the experiment.

**Spectrophotometric Assay of ICH**

The volume of the hematoma was quantified with a spectrophotometric assay as described previously. Brain tissue was obtained in 12 C57BL/6 mice or 10 iNOS knockout mice at 72 hours after ICH. The mice were subjected to complete transcardial perfusion to remove intravascular blood. Distilled water was added to each brain sample to reach a total volume of 500 µl, followed by homogenization for 30 seconds, sonication on ice for 1 minute, and centrifugation at 14,000 rpm for 30 minutes. After the hemoglobin-containing supernatant was collected, Drabkin reagent (160 µl, Sigma) was added to 40-µl aliquots and allowed to stand for 15 minutes at room temperature. This reaction converts hemoglobin to cyanmethemoglobin, which has an absorbance peak at 540 nm. Optical density was measured and recorded at 540 nm with a spectrophotometer. As an additional measure, blood was obtained in control mice by cardiac puncture after induction of anesthesia. Incremental aliquots of this blood were then used to generate a standard absorbance curve.

**Measuring Brain Water Contents**

The mice were anesthetized and killed by decapitation 72 hours postoperatively (15 C57BL/6 mice, 9 iNOS knock out). The brains were harvested immediately and divided into 2 hemispheres along the midline, and the cerebellum was removed. The brain samples were immediately weighed on an electronic analytical balance to determine the wet weight and were then dried in an oven at 100°C for 24 hours to obtain the dry weight. Water content was expressed as percentage of wet weight; the formula for calculation was as follows: (wet weight − dry weight)/wet weight × 100, as described previously.

**Blood-Brain Barrier Permeability Determined by Evans Blue Extravasations**

Vascular permeability was quantified using the fluorescent detection of extravasated Evans blue dye. Two percent Evans blue in PBS was infused intravenously (4 mg/kg) into mice via tail vein over 3 minutes as a BBB permeability tracer at 48 hours after ICH. The mice (8 C57/BL6 mice, 9 iNOS knock out mice) were anesthetized with 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg), the mice were placed on a stereotactic frame. A 30-gauge Hamilton syringe needle was inserted through a bur hole into the left striatum (located 2.0 mm left-lateral to the midline, 0.1 mm posterior to the bregma, 4.0 mm deep below the skull). An ICH was induced by the administration of collagenase Type VII (0.5 µl saline containing 0.075 U collagenase) over 1 minute. After being in place for another 4 minutes, the needle was gently removed, and the wound was sutured closed. Rectal temperature was maintained at 37 ± 0.5°C with the use of a thermostat-controlled heating blanket. The mice were allowed to recover from surgery in a cage with free access to food and water. Animals were kept in air-ventilated cages at 24 ± 0.5°C for the duration of the experiment.

**Immunohistochemistry**

Animals were anesthetized at 24 hours after ICH and then perfused through the heart with 50 ml cold saline and 50 ml of 4% paraformaldehyde in 0.1 mol/l PBS (2 each, respectively). After 24 hours of fixation in 4% paraformaldehyde, the brains were cryoprotected with 30% sucrose for 24 hours. Adjacent serial coronal sections
Inducible NOS and brain edema after ICH

through the needle entry site, which was identifiable on the brain surface, were cut at 30 µm on a freezing microtome, and sections were stored in PBS (pH 7.4), as previously described. Tissue sections were then rinsed 3 times for 10 minutes each in PBS, and sections were incubated in PBS containing 10% normal goat serum and 0.3% Triton X-100 for 3 hours. Sections were then incubated with a mouse rabbit polyclonal anti-iNOS (1:300) at 4°C for 24 hours. The next day, after washing all tissue sections 3 times in PBS, the sections were incubated in biotinylated secondary antibody (Envision™/HRP, rabbit/mouse; Dako) for 30 minutes at room temperature. After three 10-minute washes, sections were reacted for peroxidase enzyme activity by using 3,3′-diaminobenzidine tetrahydrochloride (Dako). The reaction was terminated by transferring the sections to PBS. Sections were mounted on gelatinized slides, dehydrated, and placed on cover slips.

Reverse Transcriptase Polymerase Chain Reaction for iNOS

Mice were killed at 24 hours after ICH, and their brains were removed and dissected on ice. Total RNA was extracted from the hemorrhage tissue by Trizol reagent, and 2 µg of total RNA was used to synthesize cDNA by a reverse transcription using RNA PCR kit (Promega); this was followed by PCR amplification using the following primers: 5′-ACAACGTGAAGAAAACCCCTTGTG-3′ (sense primer) and 5′-ACAGTTCCGGAGCTCAAAGAC-3′ (antisense primer) for iNOS, 5′-GGGTGATGCTGGTGCTGAGTATAGT-3′ (sense primer) and 5′-GGATGCCTTTAGTGGGCCCT-3′ (antisense primer) for glyceraldehyde-3-phosphate-dehydrogenase (560 bp), used as an internal control. The “hot start” method was used with the following cycle parameters: 94°C, 15 seconds; 65°C, 30 seconds; and 73°C, 25 seconds for 5 cycles, then 94°C, 15 seconds; 60°C, 30 seconds; and 73°C, 25 seconds for 35 cycles, and 73°C for 7 minutes for iNOS. The amplified sequences were resolved on 1.6% agarose gels, stained with ethidium bromide, and visualized with ultraviolet illumination.

Statistical Analysis

All data in this study are presented as mean ± SD. Data were analyzed using the unpaired Student t-test. A 2-tailed probability value of < 0.05 is considered significant.

Results

Difference in Hemorrhage Volume

There were no perioperative deaths. There was no significant difference in body weights between the groups (22.96 ± 5.20 vs 20.61 ± 2.41 g, p = 0.78). The volumes of hemorrhage, measured 72 hours after ICH induction, were 22.96 ± 5.20 µl in 12 C57BL/6 control mice and 20.61 ± 2.41 µl in 10 iNOS knockout mice, which were not statistically different (Fig. 1; p = 0.17).

Brain Water Contents

Brain water contents of the lesional (left) hemisphere were 79.71 ± 0.78% in 15 C57BL/6 control mice and 78.96 ± 0.69% (p = 0.03) in 8 iNOS knockout mice 3 days after ICH. Those of the nonlesional (right) hemisphere were 78.49 ± 0.64% in iNOS knockout mice (p = 0.75, Fig. 2). In iNOS knockout mice, we observed 0.48% ipsilateral relative brain edema compared with the contralateral hemisphere [(79.71 – 78.49)/(78.49)]. It was about 3-fold higher (1.55%) in C57BL/6 control mice [(78.96 – 78.58)/(78.59)].

Brain Evans Blue Dye

More Evans blue leakage from the whole brain was observed in C57BL/6 control mice (217.5 ± 6.84 ng) than in iNOS knockout mice (204.5 ± 3.35 ng, p = 0.03, Fig. 3A). Representative photographs show grossly evident Evans blue extravasation in a C57BL/6 control mouse, and less evident extravasation in iNOS knockout mouse with left basal ganglia hematoma. The brains of C57BL/6 control mice exhibited distinct Evans blue discoloration surrounding the hematoma, evident both on the brain surface and on coronal sections. These discolorations were less extensive in iNOS knockout mice (Fig. 3B).

Presence of iNOS mRNA and Protein Expression

Inducible NOS mRNA was detected by RT-PCR in C57BL/6 controls and iNOS knockout mice. The iNOS primer produced a 557-bp RT-PCR product. No iNOS signal was detected in iNOS knockout mice at 24 hours after ICH (Fig. 4A). Immunohistochemistry showed iNOS immunoreactivity in C57BL/6 controls but not in iNOS knockout mice (Fig. 4B–E). The iNOS immunoreactivity was preferentially localized in perihematoma areas, such as the basal ganglia and adjacent cerebral cortex, in C57BL/6 mice (Fig. 4B and D). In the nonlesional hemisphere, there was rare iNOS staining.
We demonstrated that mice lacking the iNOS gene, compared with C57BL/6 mice, had lower brain water content after ICH. Despite similar hematoma volumes between the groups, edema volumes were significantly different. To our knowledge, the present report provides the first evidence that iNOS may be considerably responsible for brain edema after ICH.

We used a collagenase-induced ICH model because this model mimics human ICH in the view of growing hematoma during first several hours. A clinical study showed that an increase in volume of > 33% was detectable on repeated CT scanning in 38% of patients initially scanned within 3 hours of onset; in two-thirds of cases with an increase in volume, this increase was evident within 1 hour. Recently, one group of authors compared the collagenase-induced ICH model with a blood infusion model. Despite similar initial hematoma volumes, collagenase-induced ICH resulted in growing hematoma during first 4 hours and in a greater BBB breakdown. There was faster hematoma resolution in the blood injection model.

Poor initial neurological status, large hematoma, old age, infratentorially located hematoma, and intraventricular hemorrhage have been considered major prognostic factors for death after ICH. Even though hematoma volume is the most important prognostic factor, perihematoma edema also leads to neurological deterioration. A recent study found increased relative edema volume within the first 3 hours of symptom onset to be the strongest independent predictor of improved 12-week functional outcome. The majority of perihematoma edema develops in the first 24–48 hours, and such edema growth may be associated with increased mass effect and neurological worsening. These findings suggest that post-ICH deficits are not simply attributed to the mechanical destruction by the hematoma, but that brain edema, which is substantially related to the inflammatory response, may also contribute to functional impairment. If controllable, brain edema after ICH might be a good target for treatment to improve neurological outcome.

Many factors influence the regulation of the permeability of the BBB, and NO has been reported to be one of them. Nitric oxide donors can increase BBB permeability, although the precise molecular mechanisms mediating NO-induced breakdown of the barrier are incompletely understood. Inflammatory conditions, iNOS appears to be the main producer of NO. In a septic shock model by cecal ligation and puncture, iNOS knockout mice exhibited less microvascular leakage in the cremaster muscle than wild-type septic mice. This suggests an important role of NO in modulating vascular permeability during sepsis.

It is well documented that relatively high concentrations of NO, such as those produced by iNOS, are neurotoxic. Previous studies have shown that NO was produced by iNOS from inflammatory cells in both ischemic infarct and traumatic brain injury models. Recently, one group of authors found that BBB permeability was
significantly reduced after inhibiting iNOS compared with the control group. Neutrophils and macrophage-infiltrating ICH tissue contribute to the expansion of brain edema. A number of mechanisms, such as hydrostatic pressure, activation of the coagulation cascade, thrombin production, and hemoglobin-induced neuronal toxicity, seem to be involved in edema formation after ICH. Recently, in response to ICH, iNOS mRNA was found to be maximally increased at 24 hours after ICH. In agreement with the iNOS mRNA upregulation time profile, iNOS protein was not detectable until 24 hours after ICH, and the maximal protein expression appeared at 3 days after ICH, which related to the peak time of brain edema. In a study of antioxidant treatment after ICH, behavioral performance was significantly improved following ICH in rats treated with dimethylthiourea or α-phenyl-N-tert-butyl nitrone. This suggests that free radicals play a role in the development of brain injury following ICH. The researchers, however, found that antioxidant treatment did not significantly affect edema, resolution of the hematoma, or neuronal injury in tissue adjacent to the hemorrhage. They measured white matter hyperintensity on MR imaging but did not measure brain water contents. They measured edema volume 2 days after ICH, although the peak time for brain edema after collagenase-induced ICH was known to be 3 days after ICH. In our study, iNOS knockout mice had 0.48% ipsilateral relative brain edema compared with contralateral hemisphere. Our results show that iNOS is responsible for about 69% decrement of edema 3 days after ICH, which might have an important clinical implication. Recently, we reported that decreased brain edema animals with cyclooxygenases inhibitor after ICH had a better neurological score than animals in the control group. These findings suggest that iNOS modulation might be antiedematous therapy for ICH.

Conclusions

Our study has several limitations. We could not perform behavioral tests after ICH in the present study. Therefore, the effects of edema control on long-term neurological status were not evaluated. Further studies are needed to elucidate whether iNOS affects delayed or early cell death following ICH. More importantly, it should be investigated if decreased brain edema due to iNOS modulation can improve neurological deficits after ICH. Despite these limitations, our results suggest that iNOS may have a clear role in brain edema formation after ICH. The applicability of our findings in this model to larger animals and ultimately humans remains to be determined.

Disclosure

This work was supported by a Korea Research Foundation grant (no. KRF-2006-003-E00217).

References

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