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Increase in brain thrombin activity after experimental intracerebral hemorrhage

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Summary

Thrombin has been shown to play a major role in brain injury after intracerebral hemorrhage (ICH). In this study, we measured thrombin activity in the perihematomal zone and examined the role of thrombin in ICH-induced brain tissue loss.

There were 2 experiments in this study. In the first part, adult male Sprague-Dawley rats received 100 μL of either autologous whole blood or saline. The rats were killed at 1 h or 24 h later for thrombin activity measurement. Thrombin activity was measured using the thrombin-specific chromogenic substrate, S2238. In the second part, rats received a 50-μL intracaudate injection of either thrombin or saline, and the rats were killed at days 1, 3, or 28 for determination of neuronal death and brain tissue loss.

We found that brain thrombin activity was elevated in ipsilateral basal ganglia 1 h after ICH. Intracerebral injection of thrombin rather than saline caused significant neuronal death at days 1 and 3, and resulted in significant brain tissue loss at day 28. These results suggest that thrombin inhibition in the acute phase may reduce ICH-induced brain damage.

Keywords: Cerebral hemorrhage; thrombin activity; neuronal death.

Introduction

Intracerebral hemorrhage (ICH) is a subtype of stroke with high morbidity and mortality, accounting for approximately 15% of all deaths from stroke [8]. Many factors affect outcomes in ICH patients. Experimental investigations have indicated that thrombin formation and iron toxicity play a major role in ICH-induced injury [24].

Thrombin is a serine protease and an essential component in the coagulation cascade. It is produced in the brain immediately after an ICH to stop the hemorrhage. Thrombin at low concentrations is neuroprotective [22]. However, direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration and brain edema formation [22, 24]. We have demonstrated that thrombin is responsible for early brain edema formation following ICH and that such edema results partly from a direct opening of the blood–brain barrier [13]. Because thrombin can be harmful at high concentrations and protective at low concentrations, it is important to know what concentrations of thrombin may occur in the brain after ICH.

In this study, we measured thrombin activity around the hematoma. We also examined whether or not thrombin causes neuronal death and brain tissue loss.

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Materials and methods

Animal preparation and intracerebral injection

Our animal protocol was approved by the University of Michigan Committee on the Use and Care of Animals. Male Sprague-Dawley rats weighing 300–400 g were used in this study. The animals were anesthetized with pentobarbital (40 mg/kg, i.p.). Aseptic precautions were taken for all surgical procedures. The right femoral artery was catheterized for continuous blood pressure monitoring and for blood sampling during surgery. Arterial blood was obtained for analysis of pH, PaO_2, $PaCO_2$, hematocrit, and blood glucose. Core body temperature was maintained at 37.5 °C using a feedback-controlled heating pad. The rats were positioned in a stereotactic frame and a 1-mm cranial burr hole was drilled in the right coronal suture 4.0 mm lateral to the midline. Autologous whole blood, thrombin, or saline were infused into the right caudate nucleus through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma) at a rate of 10 μL per minute using a microinfusion pump. The needle was removed and the skin incision closed with suture.

Experimental groups

There were 2 parts to this study. In the first part, rats received a 100-μL intracaudate injection of either autologous whole blood or saline. The

rats were killed 1 h or 24 h later for thrombin activity measurement. In the second part, rats received a 50-μL injection of either thrombin (5 U) or saline into right caudate and the rats were killed at day 1, 3, or 28 for determination of neuronal death and brain tissue loss.

Thrombin activity measurement

For thrombin activity measurements, rat brains were perfused transcardially with saline. Brain samples were homogenized and thrombin activities were measured using the thrombin-specific chromogenic substrate, S2238 (Chromogenix, Milano, Italy) [4]. The final concentration of S2238 was 0.3 mmol/L in phosphate-buffered saline, and absorption at 405 nm of supernatant was measured 1 h later.

Morphometric analyses

The rat brains were removed and kept in 4% paraformaldehyde for 4–6 h, then immersed in 25% sucrose for 3–4 days at 4 °C. Brains were embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) and 18-μm-thick sections were taken on a cryostat. Coronal sections from 1 mm posterior to the blood injection site were stained with hematoxylin and eosin. The caudate, cortex, and lateral ventricle were outlined on a computer and the outlined areas were measured using NIH Image software, version 1.62 (National Institutes of Health, Bethesda, MD). All measurements were repeated 3 times and the average value was recorded [6].

Fluoro-Jade staining

Brain sections were kept 15 min in 0.06% potassium permanganate (KMnO4) and rinsed in distilled water. Sections were stained by gently shaking for 30 min in working solution of Fluoro-Jade composed of 10 mL 0.01% Fluoro-Jade in distilled water and 90 mL 0.1% acetic acid, then rinsed in distilled water 3 times. After drying with a blower, slides were quickly dipped into xylol and covered for microscopic examination [16].

Statistical analysis

All data in this study are presented as mean ± SD. Data were analyzed using Student *t*-test. Statistical significance was set at $p < 0.05$.

Results

One hour after ICH, thrombin activity was elevated in ipsilateral basal ganglia (3.3 ± 1.4 vs. 0.1 ± 0.2 U/g in saline control, $p < 0.01$, Fig. 1). Twenty-four hours after ICH, the level of thrombin activity in ipsilateral basal ganglia was still higher (2.4 ± 1.0 vs. 0.3 ± 0.2 U/g in contralateral side, $p < 0.01$). Intracerebral saline injection did not increase thrombin activity in the brain. One hour after saline injection, thrombin activity in ipsilateral basal ganglia and contralateral basal ganglia were the same (0.1 ± 0.2 and 0.1 ± 0.1 U/g, respectively).

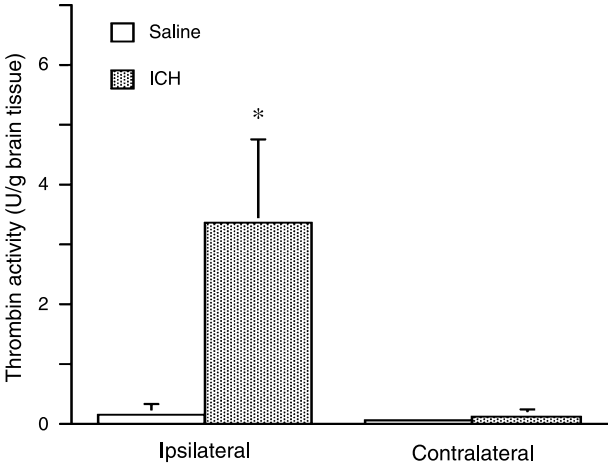

Fig. 1. Thrombin activity in ipsilateral and contralateral basal ganglia 1 h after ICH or saline injection into the ipsilateral basal ganglia. Values are mean ± SD; $n = 6$; $^{*}p < 0.05$ vs. saline

Fluoro-Jade can be effectively utilized to stain degenerating neuronal cells in the central nervous system of mammals. There were many Fluoro-Jade-positive cells in ipsilateral basal ganglia at day 1 and day 3 after 5-U thrombin injection. Only a few Fluoro-Jade-positive cells were detected in ipsilateral basal ganglia after saline injection (Fig. 2).

Thrombin caused a significant loss of brain tissue. At 28 days after thrombin injection, marked brain tissue loss occurred in ipsilateral basal ganglia (6.2 ± 1.4 mm^2 vs. 0.7 ± 0.4 mm^2 in saline control, $p < 0.01$). In addi-

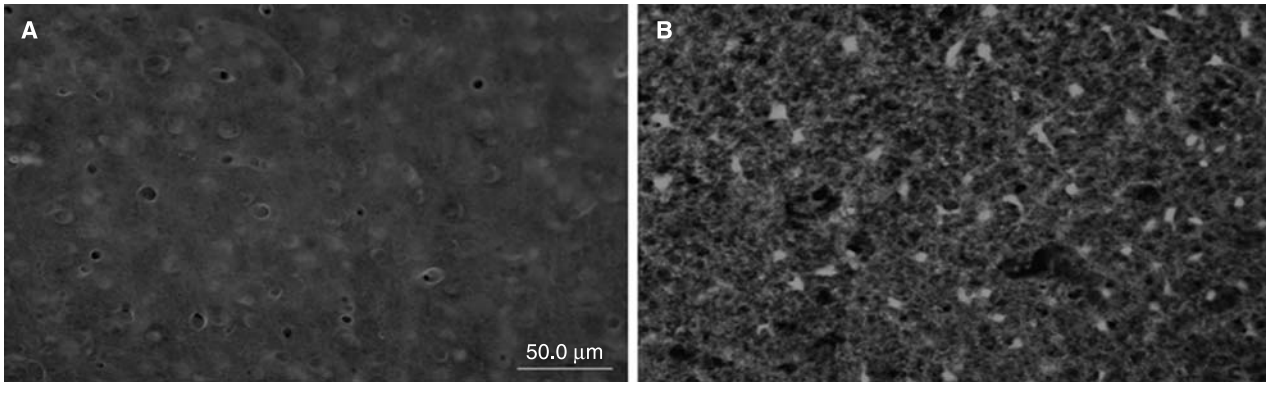

Fig. 2. Fluoro-Jade-positive cells in ipsilateral basal ganglia 24 h after injection of (A) saline or (B) 5 U thrombin. Scale bar = 50 μm

tion, lateral ventricle sizes were larger in thrombin-injected rats ($4.0 \pm 3.3\,mm^2$ vs. $0.3 \pm 0.2\,mm^2$ in saline control, $p < 0.05$).

Discussion

Our results show a significant increase in thrombin activity in the brain shortly after ICH. The concentration of prothrombin in plasma is high enough (1-5 μM) to produce a substantial amount of thrombin in brain parenchyma after hemorrhage. The brain as well as blood may be a site of thrombin production. In vitro studies have shown that prothrombin mRNA is expressed in the cells of the nervous system [1]. These results suggest that thrombin may be formed and cause brain injury, even if the blood–brain barrier is intact.

Thrombin is a serine protease and an essential component in the coagulation cascade. It is produced in the brain immediately after intracerebral hemorrhage, brain trauma, or blood–brain barrier breakdown following many kinds of brain injury [3]. Direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration, mesenchymal cell proliferation, scar formation, brain edema formation, and seizures [9–13, 15, 20, 21]. Thrombin in high concentrations also kills neurons and astrocytes in vitro [7, 18, 19]. Our previous studies have demonstrated that thrombin is responsible for early brain edema formation following ICH [11].

Clinical and experimental data have shown that brain atrophy occurs after ICH [2, 5, 17, 23]. Our recent study showed that iron has a role in ICH-induced brain atrophy [6]. In the present study, we found that thrombin causes neuronal death and also can result in brain tissue loss.

It is very important to determine thrombin activity in the brain after ICH, because high concentrations of thrombin are detrimental and low concentrations of thrombin are protective. In addition, thrombin also contributes to brain recovery following ICH [25]. Our findings in this study indicate that thrombin is harmful in acute phase, at least the first 24 h, after ICH. The concentration of thrombin in ipsilateral basal ganglia 1 h after ICH was about 3.3 U/g. Previously, we found that direct intracaudate injection of 5 U of thrombin causes marked brain damage, whereas 1 U is neuroprotective [22]. It should be noted, though, that the neurotoxicity of thrombin may be enhanced by the presence of other factors after an ICH, such as iron [14].

In summary, thrombin activity is increased in the brain after ICH, which results in neuronal death and brain tissue loss. The measured thrombin levels were 5-fold higher than we previously found in cerebral ischemia [4]. Limiting thrombin activation in the acute phase of ICH may reduce brain damage.

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