Plasminogen Activators Potentiate Thrombin-Induced Brain Injury

Bryan E. Figueroa, BS; Richard F. Keep, PhD; A. Lorris Betz, MD, PhD; Julian T. Hoff, MD

Background and Purpose—Evidence suggests that cerebral edema following intracerebral hemorrhage (ICH) results from a mass effect in combination with neurotoxic injury from clot-derived substrates such as thrombin. Thrombolytics can compete for thrombin inhibitors endogenous to the brain. This study examines the effect of intracerebral infusion of thrombolytics, tissue plasminogen activator (tPA), and urokinase (uPA), individually and in combination with thrombin.

Methods—Various 100 µL solutions were stereotactically infused into the right basal ganglia of adult male rats. Animals were euthanized 24 hours later, and brain sections were taken for measurement of water, sodium, and potassium content.

Results—Regardless of dose, when infused independently tPA (2 µg) and uPA (2000 and 5000 Plough units) failed to produce any significant tissue edema compared with vehicle control tissues. However, when either thrombolytic was infused concomitantly with thrombin (1 or 5 U), brain water, sodium, and potassium content all demonstrated a potentiation of thrombin-induced brain injury (P<0.05). In addition, animal deaths were significantly greater than expected in animals receiving a combination of tPA (2 µg) and thrombin (5 U) compared with either drug alone (P<0.001).

Conclusions—This study indicates that brain edema caused by thrombin can be greatly amplified by the presence of plasminogen activators, perhaps because the latter compete for naturally occurring thrombin inhibitors. In the context of ICH, our results suggest that the use of tPA or uPA to lyse clotted blood in brain parenchyma may promote edema formation in surrounding tissue. (Stroke. 1998;29:1202-1208.)

Key Words: brain edema • intracerebral hemorrhage • thrombin • thrombolysis • plasminogen activator, tissue-type

Intracerebral hemorrhage has an average annual incidence of 14 to 15 per 100 000, with a mortality rate approaching 60%. If the patient survives the initial ictus, ICH invariably results in the progressive accumulation of edema fluid around the hematoma. Increasing evidence suggests that intracerebral hematomas have a toxic effect as well as a mass effect, which result in the development of brain edema.1,2 Thrombin is one component of the hematoma that can be toxic to brain tissue, although the mechanisms of toxicity involved have yet to be elucidated. Low concentrations of thrombin have been shown to alter neuronal3 and astrocyte4 morphology in vitro, and high concentrations are cytotoxic.5,6 Intracerebral thrombin infusions cause cerebral edema and seizures in vivo,7,8 while intracerebral injections of thrombin inhibitors have been found to reduce edema formation in a rat model of ICH.9,10

The potential toxic effect of a hematoma argues for its removal. In certain neurosurgical protocols, hematoma evacuation is achieved by stereotactic injection of a fibrinolytic substance such as tPA or uPA, followed by drainage of the lysed coagulum.11,12 However, the probable contribution of thrombin to the toxic effect of a hematoma complicates the rationale for this form of removal. Thrombin is inhibited by two serine protease inhibitors in the brain, protease nexin-I (PN-1) and PAI-1, both of which inhibit tPA and uPA.13–18 Therefore, we hypothesized that the addition of either tPA or uPA to the clot could blunt the inhibition of thrombin by competing for these endogenous inhibitors and lead to progressive edema beyond that normally expected. This hypothesis was examined by investigating whether tPA and uPA potentiate thrombin-induced injury in the brain.

Materials and Methods

Animal Preparation

All animal procedures were conducted in accordance with institutional guidelines. A total of 105 adult male Sprague-Dawley rats weighing 250 to 350 g were anesthetized with 5% isoflurane and intubated with a 16-gauge catheter. The rats were ventilated with a mixture of oxygen (21%), nitrogen (76% to 77%), and isoflurane (2% to 3%). A polyethylene catheter was placed in the right femoral artery to monitor arterial blood pressure, which was maintained at a mean pressure of 100 mm Hg by titrating the isoflurane. Body temperature was maintained at 37.5°C with a rectal thermometer and a feedback-controlled heating pad. Ventilator rate and tidal volume were adjusted to maintain normal pH, Po2, and PCO2 as determined by blood gas analysis of samples drawn from the arterial line.
Selected Abbreviations and Acronyms

BSA = bovine serum albumin
ICH = intracerebral hemorrhage
PN-1 = protease nexin-1
PAI-1 = plasminogen activator inhibitor-1
tPA = tissue plasminogen activator
uPA = urokinase

Intracerebral Infusions

Rats were placed in a stereotaxic frame, and the scalp was incised along the sagittal midline. With the use of an operating microscope, a 1-mm burr hole was drilled on the right coronal suture 2.5 mm lateral to the bregma. A 26-gauge needle was inserted into the right caudate with stereotactic guidance (coordinates: 0.1 mm anterior, 6.0 mm ventral, and 2.5 mm lateral to bregma) and was fixed with cyanoacrylate glue. Solutions were infused into the brain at a rate of 10 μL/min with use of an infusion pump (Harvard). The needle was then withdrawn and the scalp incision closed with suture. The arterial pole was cut. This section was divided along the midline, and the groin incision was closed. Animals were then extubated and allowed to recover.

Experimental Groups

This study involved 3 parts. First, we examined the effect of tPA on brain edema formation both individually and in combination with increasing doses of thrombin. In the second part, the effects of low-dose and high-dose infusions of uPA were examined in the presence and absence of thrombin. Third, we calculated a dose-response curve for thrombin to estimate doses of thrombin that would produce equivalent injury to tPA/thrombin and uPA/thrombin combinations.

Part 1. Two sets of animals, each containing 4 groups, were examined. Each rat received a 100-μL infusion. In the first set 6 animals received a control infusion of the vehicle for the tPA and thrombin solutions (120 mmol/L NaCl, 30 mmol/L NaHCO₃, 20 ng/mL BSA, and 820 mmol/L mannitol), 6 received 2 μg tPA, 5 animals received 1 U thrombin, and 6 received a combination of 1 U thrombin and 2 μg tPA. In the second set 6 animals received the vehicle control, 7 received 2 μg tPA, 5 animals received 5 U thrombin, and 16 received a combination of 5U thrombin and 2 μg tPA.

Part 2. In the second part of the experiment, 6 groups of animals were studied, with each receiving a 100-μL intracerebral infusion. Five rats received a control infusion of the vehicle for uPA and thrombin (normal saline with 26 mg/mL BSA, 40 mmol/L mannitol, 50 mmol/L sodium citrate, and 150 mmol/L sodium chloride added). Seven received 2000 Plough units uPA, 5 received 5000 Plough units uPA, 6 received 5 U thrombin, 7 received a combination of 2000 Plough units uPA and 5 U thrombin, and 7 received a combination of 5000 Plough units uPA and thrombin.

Part 3. In the third part of the experiment, 2 additional doses of thrombin were injected into 2 groups of animals. Five rats received a thrombin dose of 8 U and 6 received a dose of 10 U, both in a 100-μL volume.

Brain Water, Sodium, and Potassium Contents

Rats were euthanized by decapitation 24 hours after intracerebral infusion. The brains were removed and a 2-mm-thick coronal slice containing the needle tract (approximately 4 mm from the frontal pole) was cut. This section was divided along the midline, and the cortex was separated from the basal ganglia bilaterally. In addition, the cerebellum was separated from the brain stem. The five tissue samples were immediately weighed on an electronic analytical balance (Mettler AF100) to the nearest 0.1 mg to obtain the wet weight (WW). The tissue was then dried in a gravity oven at 100°C for 48 hours and weighed again to obtain the dry weight (DW). The formula (WW–DW)/DW was used to calculate the water content.

The dehydrated samples were digested in 1 mL 1 mol/L nitric acid for 5 days to release the ions into the solution. The sodium and potassium contents were measured by flame photometry. Ion content was expressed in micromoles per gram of dehydrated brain (μmol/g dry weight).

Materials

Rat thrombin, human tPA, and human uPA were obtained from Sigma.

Statistical Analysis

Differences in brain water and ion content between groups of rats were evaluated using ANOVA and Scheffe’s F test of significance. A χ² test was used to evaluate mortality in parts 1 and 2 of the study. The relationship between thrombin dose and brain water content was assessed by linear regression analysis. A probability value of less than 0.05 was used to indicate a significant difference.

Results

Physiological Parameters

Table 1 shows the mean values for blood gases and blood pH for rats used in each of the three parts of the study. Normal blood gas, blood pH, and blood pressure values were recorded in all groups during the anesthetic period, and these parameters were not significantly affected by the infusion of the plasminogen activators, thrombin, or their combination.

Brain Water Content

In all parts of this study, cerebellar water content did not vary significantly with any drug or drug combination compared with vehicle controls. In the tPA study (Figure 1), contralateral brain water content was also not affected by drug infusions. In addition, water content in the ipsilateral cortex and basal ganglia was not significantly decreased on infusion of tPA or thrombin (1 or 5 U) alone, although there was a tendency for the ipsilateral basal ganglia water content to increase with the infusion of 5 U thrombin. In contrast to these findings, the combination of tPA and thrombin caused significant edema in the ipsilateral basal ganglia (tPA with 1 or 5 U thrombin) and cortex (tPA with 5 U thrombin only). These increases in water content were significant in comparison to the vehicle control group.

In the urokinase experiments, neither the 2000 nor the 5000 Plough units of uPA caused significant edema in the ipsilateral cortex or basal ganglia (Figure 2). Five units of thrombin alone did cause significant edema in the ipsilateral basal ganglia but not in the cortex. Combining the thrombin with 2000 Plough units uPA resulted in significant edema in both the ipsilateral cortex and basal ganglia. Furthermore, the combination of thrombin with 5000 Plough units uPA resulted in significantly greater edema formation than thrombin alone in both the ipsilateral cortex and basal ganglia, as well as the contralateral basal ganglia.

Brain Ion Content

The effects of the plasminogen activators with or without thrombin on sodium content of the brain followed brain water content. Neither tPA nor uPA at any dose affected sodium content of the brain in either the contralateral or ipsilateral tissues. Sodium content was also unaffected by thrombin at any dose except in the ipsilateral basal ganglia,
when the 5-U (but not the 1-U) dose caused a significant increase. In contrast, a significant increase in sodium accumulation was seen in the ipsilateral cortex in the groups that received a combination of thrombin and tPA or uPA (data not shown). There was also a greater sodium content in the ipsilateral basal ganglia in the combination experiments than found with either thrombin or the plasminogen activator alone (Figure 3).

### Table 1. Physiological Parameters in Rats Undergoing Intracerebral Infusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle Control</th>
<th>tPA (Dose 1)</th>
<th>Thrombin (1 U)</th>
<th>THR (1 U)+tPA</th>
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<tr>
<td>pH</td>
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<td>7.45±0.02</td>
<td>7.42±0.02</td>
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<td>Pco2, mm Hg</td>
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<td>Po2, mm Hg</td>
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<td>85.8±3.0</td>
<td>87.9±3.8</td>
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</table>

**Experiment 2**

<table>
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<th>Parameter</th>
<th>Vehicle Control</th>
<th>tPA (Dose 1)</th>
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<td>Po2, mm Hg</td>
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<table>
<thead>
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<th>Vehicle Control</th>
<th>tPA (Dose 1)</th>
<th>Thrombin (5 U)</th>
<th>THR (5 U)+tPA</th>
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</thead>
<tbody>
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<td>7.42±0.01</td>
<td>7.41±0.03</td>
</tr>
<tr>
<td>Pco2, mm Hg</td>
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<td>38.8±1.5</td>
<td>39.5±1.7</td>
</tr>
<tr>
<td>Po2, mm Hg</td>
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<td>90.3±2.6</td>
<td>87.4±4.6</td>
<td>88.4±2.7</td>
</tr>
</tbody>
</table>

**THR indicates thrombin; dose 1, dose used in experiment 1; and dose 2, dose used in experiment 2. Values are mean±SEM (n=3-5 animals in each group).**

Figure 1. Water content in the cortex (A, C) and basal ganglia (B, D) contralateral and ipsilateral to the site of intracerebral injection of differing combinations of tPA (t-PA) and thrombin (THR). The values are mean±SEM for 5 to 6 animals per group. *P<0.05, †P<0.01, and ‡P<0.001 vs control group; §P<0.01 vs thrombin group, by the Scheffé F test.

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Plasminogen Activators Potentiate Brain Injury
Infusion of the plasminogen activators alone did not affect the potassium content of any of the regions sampled. Thrombin did cause a decrease in the potassium content of the ipsilateral basal ganglia. However, rats receiving a combination of 5 U thrombin with either tPA or 5000 Plough units uPA had a significantly greater loss of potassium from that region than found with thrombin alone (Figure 4). They also had a significant decrease in the potassium content of the ipsilateral cortex, a change not found with thrombin or plasminogen activators alone (data not shown).

Mortality and Behavior
Table 2 displays the overall mortality in the tPA and uPA parts of this study. The vehicle and tPA groups used in part 1 have been combined. In the tPA study, no mortality was found in rats injected with tPA or thrombin alone, but a 50% mortality rate was seen in rats receiving thrombin (5 U) combined with tPA. All animals in this latter group experienced recurrent convulsive behavior. Mortality at 24 hours was not significantly greater than expected in animals infused with a combination of uPA and thrombin. Nonetheless, animals receiving 5000 Plough units uPA combined with 5 U thrombin became unresponsive and immobile.

Correlation Between Thrombin Dose and Edema Formation and Mortality
A dose-response-curve was determined for the effect of thrombin on edema formation by plotting 4 doses (0, 1, 5, and 8 U) against the absolute water content in the ipsilateral basal ganglia (Figure 5). A fifth dose of 10 U caused death in 5 of 6 rats; that dose was not included in the analysis. Linear regression analysis allowed an estimation of the thrombin doses that would cause elevations in brain water content comparable to those seen in animals receiving combinations of thrombin and plasminogen activators. The combination of tPA and thrombin (1 U) or uPA (2000 Plough units) and thrombin (5 U) caused an average increase in brain water content equivalent to a 7-U dose of thrombin. The combination of 5 U tPA and 5 U thrombin would equate to approximately an 11-U dose of thrombin, and the combination of 5000 Plough units uPA and 5 U thrombin would be the approximate equivalent of a 13-U dose of thrombin.

Discussion
Although tPA and uPA do not cause significant brain edema when infused directly into the brain, they do markedly accentuate thrombin-induced brain edema formation. A com-

Figure 3. Sodium content in the basal ganglia ipsilateral to the site of intracerebral injection of differing combinations of tPA and thrombin (THR; A, B) and of uPA and thrombin (C). The values shown are mean±SEM for 5 to 6 animals per group. *P<0.05 and †P<0.001 vs control group; ‡P<0.01 and §P<0.001 vs thrombin group, by the Scheffe F test. PU indicates Plough units.

Figure 2. Water content in the cortex (A) and basal ganglia (B) contralateral and ipsilateral to the site of intracerebral injection of differing combinations of uPA (URO) and thrombin (THR). The values shown are mean±SEM for 5 to 6 animals per group. *P<0.05, †P<0.01, and ‡P<0.001 vs control group; §P<0.01 and ¶P<0.001 vs thrombin group, by the Scheffe F test. PU indicates Plough units.
parison of co-infusion of these plasminogen activators with thrombin against infusion of thrombin alone demonstrates an increased sodium accumulation within the brain, a major factor involved in edema formation; enhanced loss of brain potassium, a potential marker of brain cell injury; and, in the case of tPA with thrombin, increased mortality.

The doses of thrombin, tPA, and uPA used in this study are pathophysiologically relevant. The 5-U dose of thrombin used in this study induces a degree of edema (4.5 g/g dry weight) in the ipsilateral basal ganglia similar to that induced by a 50-μL intracerebral hematoma (4.7 g/g dry weight), edema that could be markedly reduced by thrombin inhibitors. The doses of tPA and uPA used in this study are in the range of those that have been used in animals to lyse intracerebral hematomas. They are also, in terms of units per gram of brain tissue weight, similar to the doses that have been used clinically.

Other work suggests that there might be an interaction between thrombin and plasminogen activators involved in brain injury. Mice lacking the gene for tPA are resistant to kainate-induced seizures. In addition, intracerebral infusions of high doses of thrombin can induce epileptiform activity in rats. Although not a specific aim of this study, it was noted that animals receiving 5 U thrombin with tPA underwent convulsions that often resulted in death.

The original hypothesis of this study was that co-injection of uPA or tPA with thrombin would result in increased brain injury because of competition of these compounds for the endogenous protease inhibitors PN-1 and PAI-1. PAI-1 is an inhibitor of uPA and tPA expressed in cerebrovascular tissue and in astrocytes and neurons. Although alone it is not a good inhibitor of thrombin, the inhibitory potential of PAI-1 is vastly increased by the presence of the glycoprotein vitronectin, expressed in cerebrovascular tissue and in astrocytes and neurons. PN-1 is a potent inhibitor of thrombin expressed in glial cells and neurons in the brain. PN-1 is analogous to thrombomodulin, a potent thrombin inhibitor found outside the central nervous system. PN-1 also inhibits uPA and tPA. Our study suggests that the combination of tPA or uPA with thrombin results in a depletion of shared proteolytic inhibitors that enhances the neurotoxic effects of thrombin. While the

### Table 2. Mortality at 24 hours Following Intracerebral Infusion in Parts 1 (Top) and 2 (Bottom) of the Study

<table>
<thead>
<tr>
<th>Part 1</th>
<th>Alive</th>
<th>Dead</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
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</tr>
<tr>
<td>tPA (2 μg)</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin (1 U)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin (5 U)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin (1 U)+tPA (2 μg)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin (5 U)+tPA (2 μg)</td>
<td>8</td>
<td>8*</td>
</tr>
<tr>
<td>Part 2</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5</td>
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<tr>
<td>uPA (2000 PU)</td>
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<tr>
<td>uPA (5000 PU)</td>
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</tr>
<tr>
<td>Thrombin (5 PU)</td>
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</tr>
<tr>
<td>Thrombin+uPA (2000 PU)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Thrombin+uPA (5000 PU)</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

*PU indicates Plough units.
P<0.001, x^2.*

![Figure 4](image-url) **Figure 4.** Potassium content in the basal ganglia ipsilateral to the site of intracerebral injection of differing combinations of tPA and thrombin (THR; A, B) and of uPA and thrombin (THR, C). The values shown are mean±SEM for 5 to 6 animals per group. *P<0.01 and †P<0.001 vs control group; ‡P<0.01 vs thrombin group, by the Scheffé F test. PU indicates Plough units.

![Figure 5](image-url) **Figure 5.** Scatterplot demonstrating the relationship between thrombin dose and water content. Open symbols represent mean±SEM for 5 to 10 animals per group. Data were examined via linear regression analysis (y=0.15x+3.8, r=0.77, P<0.001) Mortality (deaths at 24 h/total animals infused at given dose) is displayed in parentheses. Five of 6 animals injected with 10 U thrombin did not survive to 24 hours and were not included in this plot.
The converse relationship could be considered, that the introduction of thrombin enhances the effects of tPA or uPA, our study found no evidence of tissue injury from plasminogen activator infusion.

Other mechanisms may also be involved in the potentiation of thrombin-induced brain injury by plasminogen activators. In vitro studies have shown that brain endothelial cells exposed to tPA paradoxically diminish their levels of PAI-1 mRNA. Consequently, a reduction of thrombin inhibition may result. Additional consideration should be given to the secondary effects of plasmin, the proteolytic product of uPA and tPA. Plasmin may modulate the effects of thrombin because it has been shown to cleave the thrombin receptor of platelets.

A balance exists between fibrinogenic and fibrinolytic plasma zymogens that is maintained by a pool of serine protease inhibitors. Our study indicates that an excess of the primary fibrinogenic substance thrombin produces a degree of brain edema that can be amplified by the presence of plasminogen activators, possibly by depletion of the endogenous stores of serine protease inhibitors (PAI-1 and PN-1). These results suggest that further investigation into the use of tPA or uPA for stereotaxic hematoma evacuation should be undertaken, because the benefit achieved from relieving the mass effect of a large hematoma may be offset by an undertaken, because the benefit achieved from relieving the mass effect of a large hematoma may be offset by an}

**Acknowledgment**

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**References**

The extravasation of blood into the brain parenchyma is harmful in more ways than one. In addition to the loss of function that results from the destruction of brain tissues, the brain undergoes further damage from the resulting increase in the volume of the intracranial contents. The intracranial volume increases (and the perfusion pressure decreases) not only as a result of the extravasation of blood but also because of the development of brain edema. This fluid retention is particularly prominent in the tissues surrounding the hematoma, and its pathogenesis has been associated (at least in part) with the effects of blood products, thrombin in particular.

The authors of the accompanying article asked whether the “toxic” effects of thrombin on the brain would be potentiated by the simultaneous intracerebral injection of either tPA or uPA. The rationale for choosing these activators was based on the fact that in some treatment protocols for ICH, one of these activators may be topically applied following the surgical excision of an intracerebral hematoma, and also because both tPA and uPA may compete with the known inhibitors of thrombin.

The results of these experiments support the hypothesis explored by these investigators. Exposing the brain parenchyma to thrombin in combination with either tPA or uPA is worse (in terms of the toxic effects) than injecting any of these proteins alone. These observations, emanating from a highly productive research laboratory at the University of Michigan, may have significant implications in the development of protocols involving the surgical excision of intracerebral hemorrhages.

Julio H. Garcia, MD, Guest Editor
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References
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