Thrombin Mediates Severe Neurovascular Injury During Ischemia

Bo Chen, BS; Qun Cheng, MD; Kai Yang, BS; Patrick D. Lyden, MD

**Background and Purpose**—Cerebral ischemia initiates cascades of pathological events such as edema, blood–brain barrier breakdown, and tissue degeneration. Thrombin activation is a key step in coagulation, and thrombin has recently been shown to mediate endothelial permeability and cellular toxicity in vitro. We examined the effect of thrombin on vasculature during ischemia in vivo.

**Methods**—Focal ischemia was induced in adult Sprague-Dawley rats by occlusion of the middle cerebral artery for 4 hours followed by a short period of reperfusion. High-molecular-weight fluorescein isothiocyanate–dextran was injected before surgery to label the severe vascular disruption. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to identify dying cells, which were quantified with manual counts. Intra-arterial thrombin or intravenous thrombin inhibitors were infused during ischemia and reperfusion.

**Results**—Infusion of thrombin (3 U/kg) intra-arterially during ischemia greatly enlarged the volume of severe vascular disruption, as visualized by fluorescein isothiocyanate–dextran extravasation \((P < 0.05)\). Thrombin also promoted blood–brain barrier leakage of IgG during ischemia. Vascular disruption was blocked by intravenous infusion of the direct thrombin inhibitor argatroban (1.69 mg/kg, \(P < 0.05\)). Greater numbers of dying cells were found in regions of severe vascular disruption, and interventions that reduced vascular leakage also reduced the numbers of dying cells.

**Conclusions**—Thrombin mediates severe vascular disruption during ischemia and thrombin inhibitors may partially ameliorate vascular disruption. Further work is needed to establish whether thrombin, entering parenchyma due to increased vascular permeability, augments neurotoxicity during ischemia. (Stroke. 2010;41:2348-2352.)

**Key Words:** blood–brain barrier, cerebral ischemia, thrombin, vascular disruption

Cerebral ischemia triggers several interrelated events, including cytotoxic and vasogenic edema, microvascular thrombosis, and tissue degeneration.1–4 Although the molecular mechanisms of cellular death in ischemia have not been fully understood, blood–brain barrier leakage of serum constituents such as thrombin might exert a direct toxic effect on brain parenchyma5–7 in addition to well-known opening effects on the blood–brain barrier and thrombotic effects.

Thrombin activation plays a key role during ischemic injury. Thrombin is a major regulator in the coagulation pathway.8,9 During endothelial damage or brain trauma, thrombin is activated from the cleavage of prothrombin by Factor X. Thrombin then will convert soluble fibrinogen to fibrin and activate platelets, forming the meshwork of a blood clot.10–12 The continuous formation of microthrombus is likely to exacerbate focal ischemia by occluding the vasculature beyond the initial occlusion sites.

Thrombin has also been shown to cause neuronal cell death both in vitro and in hemorrhage models in vivo.13,14 The direct cytotoxic effects of thrombin appear to be mediated by protease-activated receptors (PARs),15–17 most significantly the PAR-1 receptor.18,19 Activation of PAR-1 leads to neuronal cell death, astrocytic proliferation, microglial activation, and toxin release.20,21 In hemorrhagic stroke models, continuous release of activated thrombin from existing clots would exert severe damage to the brain tissue,22 whereas infusion of argatroban, a direct inhibitor of thrombin, showed a reduced level of perihematomal infarction.23–25 In addition, previous studies have related thrombin to blood–brain barrier damage and edema formation.26,27 These preliminary data suggest that thrombin is a likely candidate to mediate vascular and tissue injury after stroke.

We sought to determine whether thrombin mediated endothelial barrier dysfunction using an in vivo ischemia model.

**Materials and Methods**

All protocols were approved by the Animal Research Committee of the Veterans Affairs Medical Center, San Diego, and by the Institutional Animal Care and Use Committee of the University of California–San Diego, following all national guidelines for the care of experimental animals.

The procedure for middle cerebral artery occlusion (MCAO) model was performed as described previously.28 The subjects were

Received March 21, 2010; final revision received June 3, 2010; accepted June 29, 2010.

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*Stroke* is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.110.584920

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Argatroban (Enzo Life Sciences, BML-PI146) was dissolved in saline at 0.56 mg/mL and was infused through jugular vein using a syringe pump. We started to infuse argatroban (1.69 mg/kg) immediately after the onset of ischemia and continued for the duration of ischemia. Dosage of argatroban was based on published results.24 In the thrombin group (n=6), the area of FITC–dextran in both the stratum and cortex was larger than in the vehicle group (n=5). C, Blood–brain barrier leakage, as visualized by IgG, was mostly confined to lateral stratum ipsilateral to the MCAO in the vehicle group, whereas in the thrombin group, IgG leakage involved cortex as well. D, After thrombin treatment, the area of IgG leakage increased modestly in stratum and very significantly in cortex. *P<0.05; **P<0.01.

Figure 1. Thrombin promoted vascular disruption during ischemia. A, Treatment of thrombin during 4-hour ischemia greatly enlarged the brain region of severe vascular disruption as visualized by 2 MDa FITC–dextran. B, The signals of FITC–dextran were quantified using the method previously described.30 In the thrombin group (n=6), the area of FITC–dextran in both the stratum and cortex was larger than in the vehicle group (n=5). C, Blood–brain barrier leakage, as visualized by IgG, was mostly confined to lateral stratum ipsilateral to the MCAO in the vehicle group, whereas in the thrombin group, IgG leakage involved cortex as well. D, After thrombin treatment, the area of IgG leakage increased modestly in stratum and very significantly in cortex. *P<0.05; **P<0.01.

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To characterize the extent of severe blood–brain barrier disruption in each animal, 9 sections spanning the middle cerebral artery territory were imaged under epifluorescence microscopy using a highly sensitive CCD camera (Apogee, KAF32MB). The signal of FITC–dextran was quantified using Image-Pro Plus (Cybermedia) as described previously.30 To characterize the extent of blood–brain barrier opening—which is different than severe vascular disruption—sections were processed for immunostaining of endogenous IgG. Briefly, sections were quenched in 3% hydrogen peroxide for 10 minutes, incubated in primary antibody for 1 day (Vector Laboratories, antuniversal IgG), and then incubated in biotinylated antirabbit secondary antibody for 1 day (Vector Laboratories, PK6100 and SK4100). The distribution of IgG was outlined and quantified using Image-Pro Plus using methods previously published.30

To detect the cellular death associated with ischemic vascular disruption, a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining protocol was used using the In Situ Cell Death Detection Kit (Roche, Cat. 12156792) with minor modification. Sections prepared above were pretreated with fresh 0.1% Triton X-100 and 0.1% sodium citrate buffer for 10 minutes on ice. Then the sections were rinsed twice with phosphate-buffered saline, and incubated with TUNEL reaction mixture prepared according to the manufacturer’s instruction for 3 hours at 37°C. Finally, the sections were rinsed in phosphate-buffered saline, mounted with ProLong Gold antifade reagent (Invitrogen), and imaged with a stereoscope (Olympus, MVX10 MacroView). Three images spanning the stratum on the ischemic side were recorded for each brain section, and the number of TUNEL-positive cells was counted.

Results

Thrombin Promoted Severe Vascular Disruption

Compared with the vehicle group, the thrombin group showed an increase in the volume of severe vascular disrup-
tion as labeled by the fluorescence of high-molecular-weight FITC-dextran (Figure 1A). The areas of vascular damage after thrombin treatment were greater in striatum and extended to most regions of the cortex, whereas after vehicle treatment, the severe vascular disruption was confined to the striatum (independent-samples t test, P<0.05; Figure 1B). The same brain slices were immunostained with IgG antibody, using diaminobenzidine as the reporter, to examine the blood–brain barrier leakage of smaller molecular weight plasma proteins (Figure 1C). The result demonstrated widespread regions of blood–brain barrier permeability across the brain section in the thrombin group compared with the vehicle group (independent-samples t test, P<0.05; Figure 1D).

### Argatroban Protected the Brain From Ischemic Vascular Injuries

To determine whether thrombin inhibition could ameliorate severe vascular injury during stroke, subjects were randomly assigned to receive intravenous argatroban or vehicle. In the group receiving argatroban treatment, the average clotting time increased by 30% (data not shown), confirming adequate delivery of the thrombin inhibitor. The brains exhibited a significant reduction in severe vascular disruption as labeled with high-molecular-weight FITC–dextran (Figure 2A). The areas of FITC–dextran distribution were significantly reduced in argatroban group (n=6) compared with the vehicle group (n=5). No cortical vascular damage was found in either group. **P<0.05.

![Figure 2.](image)

**Figure 2.** Intravenous infusion of argatroban reduced severe vascular damage during ischemia. A, Examples of brain slices in argatroban group (right) and vehicle group (left). Treatment with argatroban during ischemia protected the brain from ischemic vascular disruption labeled by FITC–dextran. B, The area of FITC–dextran distribution was significantly reduced in argatroban group (n=6) compared with the vehicle group (n=5). No cortical vascular damage was found in either group. *P<0.05.

### Argatroban Blocked Thrombin-Mediated Vascular Disruption

To confirm that the observed ischemic injury to vasculature was mediated in part through thrombin activity, we infused argatroban through the jugular vein simultaneously with the arterial infusion of thrombin through the internal carotid artery during ischemia and reperfusion. During arterial infusion of thrombin, 1.69 mg/kg argatroban infused intravenously alleviated the vascular disruption, but not to a significant extent. A higher dose of argatroban (3.4 mg/kg) significantly reduced severe vascular disruption during MCAO and arterial thrombin infusions (P<0.01, analysis of variance with Neuman-Keuls; Figure 3).

![Figure 3.](image)

**Figure 3.** Argatroban blocked thrombin-mediated vascular disruption. To test the specificity of thrombin toxicity during ischemia, thrombin was infused into the internal carotid artery in all animals during 4-hour MCAO with simultaneous infusion of vehicle or argatroban through the jugular vein. Treatment with argatroban ameliorated thrombin-induced vascular injury in a dose-dependent manner (analysis of variance, P<0.01). *P<0.05; **P<0.01.

### Thrombin Inhibition Reduced Neuronal Injury

In areas of reduced vascular injury, counts of TUNEL-stained cells were significantly reduced after argatroban treatment (Table). Intra-arterial thrombin significantly increased the extent of TUNEL staining during ischemia. When thrombin was infused into the middle cerebral artery at the time argatroban was given intravenously, the numbers of TUNEL-positive cells decreased.

![Table.](image)

**Table.** Thrombin Augmented Tissue Cell Death

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>TUNEL Staining (Mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (IA)</td>
<td>5</td>
<td>88±31</td>
</tr>
<tr>
<td>Thrombin (IA)*</td>
<td>6</td>
<td>151±42</td>
</tr>
<tr>
<td>Saline (IV)</td>
<td>6</td>
<td>118±47</td>
</tr>
<tr>
<td>Argatroban (IV, 1.69 mg/kg)</td>
<td>6</td>
<td>27±10</td>
</tr>
<tr>
<td>Thrombin (IA) + saline (IV)</td>
<td>10</td>
<td>147±35</td>
</tr>
<tr>
<td>Thrombin (IA) + argatroban (IV, 1.69 mg/kg)</td>
<td>7</td>
<td>121±46</td>
</tr>
<tr>
<td>Thrombin (IA) + argatroban (IV, 3.4 mg/kg)</td>
<td>3</td>
<td>53±24</td>
</tr>
</tbody>
</table>

*Thrombin was used at the dose of 3 U/kg in all cases treated.
IA indicates intra-arterial infusion; IV, intravenous infusion.
Our data demonstrated that thrombin activation during an acute stage of ischemia resulted in severe neurovascular injury. Arterial infusions of thrombin augmented the extent of severe vascular injury, whereas intravenous treatment with the direct thrombin inhibitor argatroban inhibited the effect of thrombin on vascular disruption in a dose-related manner. Our data, obtained in a well-characterized MCAO model, confirm previous findings from in vitro and hemorrhagic models in vivo. Ischemic cellular injury, represented as counts of TUNEL-stained cells, increased with thrombin and decreased with argatroban treatments, suggesting that severe vascular injury and cellular damage are both influenced by thrombin activation.

The integrity of the blood–brain barrier is crucial for the homeostasis of the brain and it is often associated with perivascular tissue damage. The mechanisms of ischemic damage to the neurovascular unit caused by thrombin remain elusive. In general, thrombin might exert its toxic effect by 3 nonmutually exclusive pathways. One is to promote microthrombosis in the smaller distal vessels and exacerbate the ischemic condition downstream of the larger arterial occlusion; another is to induce vascular permeability by PAR-1/protein kinase C pathways, remodeling the endothelial junctional structure and causing edema; finally, thrombin might leak into parenchymal tissues and cause direct cytotoxic effects on glia and neurons. The pleiotropic effects of thrombin on neurovascular units are likely mediated by distinct signaling pathways dependent on the cell types. Further investigation into the thrombin toxicity mechanism can be directed to dissect the role of PAR-1 pathway at specific cell types in the context of ischemic stroke.

Thrombin could play multiple roles during cerebral ischemic insult. Despite the deleterious effects as discussed, however, some studies have suggested a beneficial role for thrombin. Low-dose thrombin was neuroprotective and was a potent mediator for neurogenesis and angiogenesis. Injection of low-dose thrombin could also serve as a method to induce brain tolerance to ischemic attack. It is likely that thrombin effects are dosage-dependent. In our experiment, we infused a small amount of thrombin (3 U/kg) over 4 hours, a dose that was previously used to precondition the brain against ischemia. Interestingly, in the present experiment, the infusion of low-dose thrombin resulted in severe damage to the brain vasculature. It is possible that ischemic tissues are more susceptible to thrombin insult, so the low-dose thrombin that is tolerated by the normal brain becomes extremely toxic in an ischemic setting. It is also possible that the infused thrombin potentiates the generation of additional thrombin and thus the effect of thrombin is further amplified by the coagulation cascade.

Further studies are essential to define the mechanisms by which thrombin augments ischemic damage. In particular, the role of PAR-1-mediated injury seems critical as well as the role of thrombin-mediated microthrombosis. Perhaps most importantly, it remains to be determined whether thrombin enters brain parenchyma to cause direct cytotoxic injury or simply exacerbates blood–brain barrier disruption leading indirectly to increased edema and tissue injury.

Acknowledgments
We thank Agnieszka Brzozowska-Prechtl at Burnham Institute Histology Core Facility for assisting with the microtomy of the brain tissue. We also thank Dr David Kleinfeld for use of epifluorescent microscopy and Dr Beth Friedman for discussions during the preparation of the manuscript. B.C. thanks the HHMI-NIBIB Interfaces Training Program at the University of California–San Diego.

Sources of Funding
This work was funded by the Veterans Affairs Medical Research Department (P.D.L.), National Institutes of Neurological Disorders and Stroke grants R01NS043300 (P.D.L.) and R21NS052565 (P.D.L.), and by American Heart Association Predoctoral Fellowship (B.C.). We also acknowledge the usage of the University of California–San Diego Neuroscience Microscopy Shared Facility (National Institutes of Health P30 NS047101).

Disclosures
None.

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Stroke. 2010;41:2348-2352; originally published online August 12, 2010;
doi: 10.1161/STROKEAHA.110.584920
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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