Background and Purpose—Argatroban, a specific thrombin inhibitor, has been shown to reduce ischemic lesion size after focal cerebral ischemia in rats. In addition, recombinant tissue plasminogen activator (rtPA) has been shown to reduce ischemic lesion size in both rats and humans if given within 3 hours of symptom onset. We tested the hypothesis that the administration of argatroban with rtPA could extend the treatment window of stroke to 4 hours without increasing gross cerebral hemorrhage rates or reducing efficacy.

Methods—Male Wistar rats were subjected to middle cerebral artery (MCA) occlusion by a single fibrin-rich clot. After embolization, rats were administered argatroban at the following dose levels: 2.08, 6.25, and 18.75 μg·kg⁻¹·min⁻¹. In a second experiment, rats received argatroban (6.25 μg·kg⁻¹·min⁻¹) or argatroban in combination with rtPA 4 hours after MCA occlusion. Tissue sections were then analyzed for lesion volume, gross hemorrhage and fibrin deposition.

Results—The 6.25 μg·kg⁻¹·min⁻¹ dose demonstrated a significant reduction (P<0.05) in lesion volume after 48 hours (27.2±6.3%) compared with controls (35.3±3.7%). A significant reduction (P<0.05) in lesion volume was observed in the argatroban-plus-rtPA group (17.1±10.4%) compared with controls (35.3±3.7%). No increase in hemorrhagic transformation was observed. Fibrin deposition in the ipsilateral cortical microvasculature was significantly decreased in the 4-hour combination argatroban-plus-rtPA group compared with the controls (P<0.05).

Conclusions—This study demonstrates that the combination of argatroban and rtPA extends the window of opportunity for treatment of stroke to 4 hours without increasing hemorrhagic transformation.

Key Words: cerebral ischemia ■ stroke ■ thrombin ■ tissue plasminogen activator ■ rats
intravenous injection. Anticoagulation is rapidly reversed after discontinuation of argatroban infusion by removal from the systemic circulation via hepatic metabolic clearance. Argatroban shows no antigenicity and directly inhibits thrombin without cofactors. In experimental rat models of stroke, argatroban reduced the formation of microthrombi and the size of the ischemic lesion up to 6 hours after MCA occlusion. A placebo-controlled double-blinded clinical study of argatroban in stroke showed significant improvements in neurological symptoms in humans when treatment was initiated within 48 hours of onset of symptoms. The clinical use of argatroban in Japan is approved for the treatment of chronic peripheral arterial obstructive disease and acute ischemic stroke. Furthermore, argatroban was approved in the United States in June 2000 for the treatment or prophylaxis of thrombosis in patients with heparin-induced thrombocytopenia.

The only FDA-approved drug for treatment of stroke in the United States is rtPA. The use of rtPA in stroke is limited to a 3-hour time window of symptom onset because of cerebral hemorrhagic complications and reduced efficacy. Administration of rtPA after 3 hours of stroke onset increases hemorrhagic transformation up to 20% in both human and animal models of stroke. In addition, rtPA upregulates the inflammatory response in the microcirculation with increased expression of intracellular adhesion molecule-1 (ICAM-1) and P- and E-selectins, which may promote BBB disruption. The increased rate of hemorrhagic transformation that is observed with treatment of rtPA beyond 3 hours may be related to the increased inflammatory response that promotes platelet activation, adhesion, and aggregation in the microvessels. These activated platelets are a source of thrombin, which deposits fibrin in the inflamed microvessel. Therefore, by inhibiting thrombin, the vicious cycle of platelet adhesion and aggregation can be halted.

Platelet adhesion and aggregation in the microvessels may promote BBB leakage and disruption. Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes that are released from activated platelets and endothelial cells. MMPs degrade components of the extracellular matrix in the microvascular bed leading to hemorrhagic transformation.

Platelet accumulation coincides with increases in MMP9 and parenchymal fibrin deposition in a rat model of embolic stroke. A major component of the cerebral microvascular basement membrane, collagen IV, was also observed to disappear over 4 hours of ischemia. Taken together, these data support the hypothesis that activated platelets and fibrin form aggregates that promote cerebral microvascular perfusion deficits and that platelet aggregation contributes to the loss of integrity of cerebral microvessels. Therefore, inhibiting platelet aggregation may preserve microvascular integrity. Based on the hypothesis that thrombin is an initiator and mediator of platelet activation and aggregation and that treatment of stroke with rtPA promotes platelet activation via the inflammatory response, we tested the hypothesis that the thrombin inhibitor argatroban extends the therapeutic window for treatment of stroke with rtPA.

Materials and Methods
All procedures were approved by the Care of Experimental Animals Committee of Henry Ford Health Sciences Center.

Animal Model
Male Wistar rats (n = 60) weighing 320 to 380 g were subjected to focal cerebral ischemia. The MCA was occluded by placement of an embolectomy at the origin of the MCA as previously described. Briefly, a single intact, fibrin-rich, 24-hour-old, homologous clot was positioned at the origin of the MCA via a 15-mm length of modified PE-50 catheter. Rats were anesthetized with 3.5% halothane and maintained with 1.0% to 2.0% halothane in 70% N2O and 30% O2 using a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure using a feedback-regulated water heating system. The right femoral artery and vein were cannulated for measuring physiological parameters, blood pressure, and drug administration, respectively.

Experimental Protocols
To test whether the dose-response inhibition of thrombin at the time of onset of cerebral ischemia reduces lesion size, animals were randomly divided into 4 groups: control group (n = 10) and 3 argatroban treatment groups: 2.08 (n = 10), 6.25 (n = 10), and 18.75 (n = 6) μg·kg⁻¹·min⁻¹. Saline vehicle was administered as control, and argatroban was administered immediately after MCA occlusion as a continuous infusion over 48 hours. Total dose of argatroban administered over 48 hours in the 2.08, 6.25, and 18.75 μg·kg⁻¹·min⁻¹ is 6, 18, and 27 mg/kg, respectively. An optimal dose that has effects on lesion size reduction will be used to study the effect of combination of argatroban and rtPA; as a result, the 6.25 μg·kg⁻¹·min⁻¹ group was chosen.

To test the hypothesis that rtPA administration beyond the 3-hour treatment window in the presence of a thrombin inhibitor would reduce lesion size without increasing hemorrhagic transformation, animals were randomly divided into 4 groups: control (n = 10), human recombinant rtPA (Genentech, Inc) (10 mg/kg) (n = 6), argatroban (6.25 μg·kg⁻¹·min⁻¹) treatment group (n = 6), and combination argatroban (6.25 μg·kg⁻¹·min⁻¹) and rtPA (10 mg/kg) treatment group (n = 6). Single and combination therapy was administered 4 hours after MCA occlusion; rtPA as a 10% bolus followed by an infusion over 30 minutes and argatroban as a continuous infusion over 44 hours. An additional combination therapy group was tested 6 hours after MCA occlusion using identical doses in the 4-hour combination group.

Animals were weighed before MCA occlusion and daily after MCA occlusion. Two days after MCA occlusion, all animals were anesthetized with ketamine (44 mg/kg) and xylazine (13 mg/kg) and killed. Each rat was transcardially perfused with heparinized saline followed by 10% formalin. The brain was removed from the skull and cut into 7 coronal blocks, each with 2-mm thickness. The brain tissue was processed and embedded, and 6-μm-thick paraffin sections from each block were cut and stained with H&E for evaluation of ischemic cell damage. A 6-μm-thick coronal section was used for immunohistochemical staining.

Measurement of Ischemic Lesion Volume
Lesion volume was measured using a Global Laboratory Image analysis software program (Data Translation). Each H&E-stained coronal section was evaluated at ×2.5 magnification. The area of both hemispheres and the area containing the ischemic neuronal damage (mm³) were calculated by tracing the area on the computer screen, and lesion volume (mm³) was determined by multiplying the appropriate area by the section interval thickness. To reduce errors associated with processing of tissue for histological analysis, the ischemic volume is presented as the percentage of infarction volume of the contralateral hemisphere (indirect volume calculation). The incidence of gross cerebral hemorrhage was determined by visualization of the brain slices under a ×10 microscope.
Measurement of Fibrin Deposition
A goat anti-mouse fibrinogen/fibrin antibody was used at a titer of 1:1000 to assess the deposition of fibrin and fibrinogen-related antigen within vessels and in the parenchyma (Accurate Chemical & Scientific). The specificity of this antibody to fibrin has been demonstrated.1 Coronal sections were incubated with the anti-fibrinogen antibody for 3 days at 4°C, and sections were then incubated with the secondary antibody conjugated to FITC. Control experiments consisted of staining brain coronal sections as outlined but with the primary antibodies omitted. Each anti-fibrinogen antibody was digested under a ×20 or ×40 objective (Axioplan 2; Zeiss) using a 3-CCD video camera (C4742-95; Hamamatsu) interfaced with MCID image analysis system (Imaging Research). The total pixels of staining present in the coronal section were divided by the total tissue pixels to determine the percentage of fluorescently marked tissue. Fibrin deposition in the parenchyma was determined by counting the number of microvessels with fibrin deposition outside the vessel.

Statistical Analysis
The study was conducted into 2 parts: a dose-finding study of argatroban and an efficacy/safety study of the combination of tPA and argatroban. We considered the lesion size reduction as primary interest for treatment efficacy. We also observed incidence of gross cerebral hemorrhage for safety. One-way ANOVA was used to test the dose effect on lesion size compared with the controls if data were normal. Nonparametric Kruskal-Wallis test or data transformation would be considered if data were otherwise. Two-way ANOVA was conducted to test the effect of the combination of tPA and argatroban on lesion size, including 2 factors of tPA and argatroban. We began testing the interactions between argatroban and tPA at the 0.05 level, followed by pairwise comparisons at the significant level of 0.05 using 2-sample t test. If the interaction was detected. Otherwise, pairwise tests were considered as exploratory. We used the mixed regression model to test the treatment effect with adjustment for multiple comparisons.

Results
The arterial blood gas and mean arterial blood pressure values were measured before MCA occlusion, 10 and 20 minutes after treatment with single or combination therapy and before the animals were killed. All values for each animal were within the normal physiological range, and no significant differences between groups were observed (data not shown). Indirect lesion volumes (mm3) are shown in Table 1. The percentage lesion volume in rats administered argatroban at the time of MCA occlusion at the infusion rates of 2.08, 6.25, and 18.75 μg · kg−1 · min−1 for 48 hours were 28.9±10.3% (n=10), 27.2±6.3% (n=10), and 32.4±6.4% (n=6), respectively. Rats administered saline exhibited a lesion volume of 35.3±3.7% (n=10). Only the 6.25 μg · kg−1 · min−1 group showed a statistically significant reduction (P=0.049) in lesion volume compared with controls (Figure 1).

Because the 6.25 μg · kg−1 · min−1 group showed a significant reduction in lesion volume, this dose was chosen to be tested in the combination therapy group. Rats treated with rtPA at 4 hour post MCA occlusion showed a lesion volume of 43.4±8.4% (n=6) while rats treated with argatroban (6.25 μg · kg−1 · min−1) alone at 4 hour post MCA occlusion showed a lesion volume of 36.5±5.7% (n=6). No significant differences were observed between control and 4-hour argatroban and 4-hour rtPA groups. However, combination therapy of both rtPA and argatroban at 4 hours after MCA occlusion exhibited a lesion volume of 17.1±10.4% (n=6), demonstrating a significant decrease in lesion volume in comparison with single therapy or control (P<0.01) (Figure 2). Because of the significant result in the 4-hour combination therapy group, an additional combination treatment group was tested at 6 hours. The lesion volume in the 6-hour group was 35±5.7%, showing no differences in lesion size compared with the control group.

Gross cerebral hemorrhage was detected in the ipsilateral hemisphere in 2 of 10 rats in the control group. In this stroke model, hemorrhages typically occur in the striatum and preoptic regions.27 For rats treated immediately with argatroban alone after the onset of MCA occlusion, the 2.08 and 6.25 μg · kg−1 · min−1 groups showed a gross cerebral hemorrhage in 2 of 10 rats, whereas the 18.75 μg · kg−1 · min−1 group had 1 of 6 rats with a gross cerebral hemorrhage. The 4-hour argatroban and combination groups (4 and 6 hours) both exhibited 1 of 6 rats with a gross cerebral hemorrhage, whereas the tPA-only group had 2 of 6 rats showing a gross cerebral hemorrhage (Table 2). No significant differences were detected between groups.

<table>
<thead>
<tr>
<th>TABLE 1. Indirect Lesion Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect Lesion Volume, mm3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>215.3</td>
</tr>
<tr>
<td>SEM</td>
</tr>
<tr>
<td>25.4</td>
</tr>
</tbody>
</table>

*P<0.05 compared with controls.
†Doseage was 6.25 μg · kg−1 · min−1.

**Figure 1.** Effects of argatroban on lesion volume. Rats received argatroban at time of MCA occlusion and were killed at 48 hours after MCA occlusion. *P<0.05.
Table 2. Incidence of Cerebral Hemorrhage

<table>
<thead>
<tr>
<th>Cerebral Hemorrhage</th>
<th>(4 and 6 h), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 (20)</td>
</tr>
<tr>
<td>Argatroban (4 h)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>rtPA (4 h)</td>
<td>6 (33)</td>
</tr>
<tr>
<td>Argatroban plus rtPA (4 h)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>Argatroban plus rtPA (6 h)</td>
<td>6 (17)</td>
</tr>
</tbody>
</table>

No significant differences were observed. Argatroban dosage was 6.25 μg · kg⁻¹ · min⁻¹.

Table 3. Weight Change at 48 Hours After MCA Occlusion

<table>
<thead>
<tr>
<th>Weight Change</th>
<th>n</th>
<th>Mean Reduction, %</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>22.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Argatroban</td>
<td>6</td>
<td>18.6</td>
<td>5.7</td>
</tr>
<tr>
<td>2.08 μg · kg⁻¹ · min⁻¹</td>
<td>10</td>
<td>16.5</td>
<td>4.7</td>
</tr>
<tr>
<td>6.25 μg · kg⁻¹ · min⁻¹</td>
<td>6</td>
<td>20.2</td>
<td>3.7</td>
</tr>
<tr>
<td>rtPA (4 h)</td>
<td>6</td>
<td>19.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Argatroban (4 h)</td>
<td>6</td>
<td>23.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Argatroban plus rtPA (4 h)</td>
<td>6</td>
<td>13.1*</td>
<td>4.0</td>
</tr>
<tr>
<td>Argatroban plus rtPA (6 h)</td>
<td>6</td>
<td>18.8</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*P<0.01 compared with controls.

Discussion

The present study demonstrates that combination therapy of the thrombin inhibitor argatroban and rtPA, administered 4 hours after the onset of MCA occlusion, reduces infarct volume in an embolic stroke rat model. The combination therapy of the thrombin inhibitor argatroban and rtPA, administered 4 hours after the onset of MCA occlusion, does not increase the risk of hemorrhagic transformation. However, we did not have sufficient statistical power to detect the difference due to the study design. To detect the reduction of 16.5% based on 33% apparent hemorrhage incidences (OR 0.40) with 0.05 2-sided significant level and power of 80%, we need 107 rats per group, which would be unrealistic for a preclinical study. These data support the hypothesis that the window of opportunity for treatment of stroke with rtPA can be extended to 4 hours in rats, supporting the hypothesis that inhibition of continuous endogenous generation of thrombin from partially lysed thrombus during thrombolysis can reduce combination groups (P<0.05). No other groups showed significant leakage of fibrin compared with controls.

Table 4. Effects of Argatroban and rtPA on Fibrin Deposition

<table>
<thead>
<tr>
<th>Fibrin Deposition/Tissue Volume</th>
<th>n</th>
<th>Mean, %</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
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<tr>
<td>4-h MCA occlusion—no treatment</td>
<td>6</td>
<td>2.80</td>
<td>1.54</td>
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<tr>
<td>Argatroban (t=0)</td>
<td>6</td>
<td>1.78</td>
<td>1.19</td>
</tr>
<tr>
<td>rtPA (4 h)</td>
<td>5</td>
<td>2.90</td>
<td>1.70</td>
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<tr>
<td>Argatroban (4 h)</td>
<td>4</td>
<td>2.10</td>
<td>1.69</td>
</tr>
<tr>
<td>Argatroban plus rtPA (4 h)</td>
<td>6</td>
<td>0.95*</td>
<td>1.12</td>
</tr>
<tr>
<td>Subcortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-h MCA occlusion—no treatment</td>
<td>6</td>
<td>3.04</td>
<td>2.20</td>
</tr>
<tr>
<td>Argatroban (t=0)</td>
<td>6</td>
<td>2.15</td>
<td>1.87</td>
</tr>
<tr>
<td>rtPA (4 h)</td>
<td>5</td>
<td>3.35</td>
<td>2.03</td>
</tr>
<tr>
<td>Argatroban (4 h)</td>
<td>4</td>
<td>2.25</td>
<td>1.65</td>
</tr>
<tr>
<td>Argatroban plus rtPA (4 h)</td>
<td>6</td>
<td>2.46</td>
<td>1.51</td>
</tr>
</tbody>
</table>

*P<0.05.

Argatroban dosage was 6.25 μg · kg⁻¹ · min⁻¹.
Inhibition of thrombin may dampen the inflammatory response and thus the damage to the integrity of the vessel wall during thrombolysis. Moreover, the important observation that E-selectin, P-selectin, and ICAM-1 are upregulated with rtPA treatment initiated 4 hours after stroke supports the hypothesis that delayed thrombolysis increases the inflammatory response. Combination treatment with an anti-ICAM antibody or an anti-CD-18 antibody and rtPA significantly reduced ischemic lesion volume and neutrophil accumulation compared with rtPA treatment alone. At high concentrations, thrombin has been shown to induce apoptosis in cell cultures of neurons and astrocytes. Apoptosis is a common feature of cerebral ischemia, trauma, or chronic degenerative diseases such as Alzheimer’s. Thrombin is produced at sites of cerebrovascular trauma and most likely exists at high concentrations. Therefore, speculation exists that high concentrations of thrombin in central nervous system, injury promotes neuronal cell death by apoptosis. It is conceivable that in ischemic insults in which the BBB is compromised, that uncontrolled leakage of thrombin in the brain parenchyma could promote neuronal apoptosis and thus worsen functional outcome.

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Thrombin is a potent activator of platelets; it triggers the change in shape of platelets and promotes the release of the platelet activators ADP, serotonin, and thromboxane A2. The actions of thrombin on platelets are of particular importance in the mechanism of cerebral ischemia. In animal models of focal cerebral ischemia, platelets accumulate in regions of low blood flow during the postischemic period. Inhibition of platelet aggregation by GP IIb/IIIa antagonists improves cerebral blood flow in the ischemic lesion and reduces infarct volume. Platelet aggregation is associated with increases in MMPs, which have been implicated in the disruption of the BBB during focal cerebral ischemia. In addition, type IV collagen, a major component of the cerebral microvascular basement membrane, is degraded in the ischemic core 1 hour after MCA occlusion and completely disappears in the microvessels in the ischemic core after 4 hours of ischemia. This degradation of type IV collagen coincides with the platelet aggregation. Therefore, evidence exists that activated platelets during cerebral ischemia contributes to impairment of the microcirculation and disruption of the vascular integrity. By inhibiting thrombin with argatroban during thrombolysis with rtPA in cerebral ischemia, the adverse effects of activated platelets may be negated, thereby preserving the vascular integrity and reducing hemorrhagic transformation.

At the cellular level, thrombin promotes its effects through protease-activated receptors (PARs). PARs are G protein-coupled receptors that are activated when a protease like thrombin cleaves the N-terminal exodomain of the receptor, unmasking a new N terminus that functions as a tethered ligand, docking intramolecularly with the receptor site to effect transmembrane signaling. Changes in the cell cytoskeleton, granule secretion, upregulation of adhesion molecules and chemokinics, and calcium mobilization are mediated by this PAR mechanism. These actions of thrombin occur on endothelial cells, leukocytes, and platelets. Expression of adhesion molecules, P-selectin, VWF, CD40 ligand, and the GP IIb/IIIa integrin receptor promote the well-described inflammatory response during cerebral ischemia. Inhibition of thrombin may dampen the inflammatory response and thus the damage to the integrity of the vessel wall during thrombolysis. Moreover, the important observation that E-selectin, P-selectin, and ICAM-1 are upregulated with rtPA treatment initiated 4 hours after stroke supports the hypothesis that delayed thrombolysis increases the inflammatory response. Combination treatment with an anti-ICAM antibody or an anti-CD-18 antibody and rtPA significantly reduced ischemic lesion volume and neutrophil accumulation compared with rtPA treatment alone.

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leakage and vessel disruption. The absence of hemorrhagic transformation or microvessel disruption in the rtPA-plus-argatroban group suggests that the window for rtPA can be extended to 4 hours in this rat model. Moreover, significant reduction in lesion volume with reduced fibrin deposition supports the hypothesis that dampening the inflammatory response caused by thrombin activation reduces lesion size without the complications of BBB leakage and hemorrhagic disruption.

In summary, this study demonstrates that combination therapy of rtPA and a thrombin inhibitor, argatroban, administered at 4 hours from the onset of MCA occlusion reduces lesion size in an embolic stroke rat model without increasing hemorrhagic transformation. The mechanism of action may involve decreasing fibrin deposition and platelet activation in the microcirculation, thus protecting the vascular integrity and preventing hemorrhagic transformation.

Acknowledgment
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Extension of the Therapeutic Window for Recombinant Tissue Plasminogen Activator With Argatroban in a Rat Model of Embolic Stroke
Daniel C. Morris, Li Zhang, Zheng Gang Zhang, Mei Lu, Kurt L. Berens, Philip M. Brown and Michael Chopp

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