Multitargeted Effects of Statin-Enhanced Thrombolytic Therapy for Stroke With Recombinant Human Tissue-Type Plasminogen Activator in the Rat

Li Zhang, MD; Zheng Gang Zhang, MD, PhD; Guang Liang Ding, PhD; Quan Jiang, PhD; Xianshuang Liu, MD, PhD; He Meng, MD, PhD; Ann Hozeska, BS; Chunling Zhang, BS; Lian Li, PhD; Dan Morris, MD; Rui Lan Zhang, MD; Mei Lu, PhD; Michael Chopp, PhD

Background—Microvascular dysfunction posttreatment of stroke with recombinant human tissue-type plasminogen activator (rht-PA) constrains the therapeutic window to 3 hours. Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) promote vascular thrombolysis and reduce the inflammation response. We therefore investigated the neuroprotective effects of a combination of atorvastatin and delayed rht-PA treatment in a rat model of embolic stroke.

Methods and Results—Rats subjected to embolic middle cerebral artery occlusion were treated with atorvastatin in combination with rht-PA 4 hours after stroke. Magnetic resonance imaging measurements revealed that combination treatment with atorvastatin and rht-PA blocked the expansion of the ischemic lesion, which improved neurological function compared with saline-treated rats. Real-time reverse transcription–polymerase chain reaction analysis of single endothelial cells isolated by laser-capture microdissection from brain tissue and immunostaining showed that combination treatment downregulated expression of tissue factor, von Willebrand factor, protease-activated receptor-1, intercellular adhesion molecule-1, and matrix metalloproteinase-9, which concomitantly reduced cerebral microvascular thrombosis and enhanced microvascular integrity. Combination treatment did not increase cerebrovascular endothelial nitric oxide synthase (eNOS) levels or eNOS activity, and inhibition of NOS activity with N-nitro-L-arginine methyl ester did not block the beneficial effects of combination treatment on stroke. Furthermore, combination treatment compared with thrombolytic monotherapy increased cerebral blood flow and reduced infarct volume in eNOS-null mice.

Conclusions—These data demonstrate that combination treatment with atorvastatin and rht-PA exerts a neuroprotective effect when administered 4 hours after stroke and that the therapeutic benefits are likely attributed to its multitargeted effects on cerebrovascular patency and integrity. (Circulation. 2005;112:3486-3494.)

Key Words: cerebral ischemia ■ plasminogen activators ■ thrombosis ■ microcirculation

Vascular inflammation, disruption of the blood-brain barrier (BBB), and secondary thrombosis all contribute to limiting the therapeutic window for thrombolysis of stroke with tissue-type plasminogen activator (tPA) to 3 hours.1,2 There have been multiple efforts at extending the therapeutic window for recombinant human tPA (rht-PA) beyond 3 hours.3-5 Agents have been used as adjunctive therapy to rht-PA that reduce vascular inflammation, platelet activity, and thrombin and that are neuroprotective.3-5 Moving adjunctive rht-PA therapy into the clinic requires agents with a good safety profile, that will not increase the incidence or severity of bleeding, are relatively inexpensive, and are effective in enhancing the thrombolytic efficacy of rht-PA.

Clinical Perspective p 3494

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are a class of compounds that have beneficial effects well beyond their ostensibly targeted purpose of reducing serum cholesterol.6 Pluripotent actions of statins include reducing hemostasis by reducing platelet activation and the procoagulation cascade and increasing fibrinolysis and the anticoagulation cascade.7 Statins have a broad safety profile, are widely used in humans, and are relatively inexpensive.7 Thus, statins appear to be ideal compounds to test as adjunctive agents for rht-PA. In this study, we selected a widely used statin, atorvastatin (Lipitor [Pfizer]), as the test agent to be used with rht-PA. Atorvastatin is contained under
normal conditions within the vasculature of the brain, and there are substantial data that suggest it is an antithrombotic agent and reduces vascular inflammation. Our study demonstrates that combination treatment with atorvastatin and rht-PA at 4 hours after stroke onset is neuroprotective in the rat.

Methods
All experimental procedures were approved by the institutional animal care and use committee of Henry Ford Hospital.

Animal Model
Male Wistar rats weighing 350 to 450 g or male C57BL/6J endothelial nitric oxide–null (eNOS−/−) mice weighing 28 to 36 g were subjected to embolic middle cerebral artery occlusion (MCAO).10

Experimental Protocols
Atorvastatin (Lipitor [Pfizer]) was given at a dose of 20 mg/kg SC 4 hours after embolic MCAO and was followed by a second dose of 20 mg/kg 24 hours after the first dose. rht-PA (Genentech) was infused at a dose of 10 mg/kg IV 4 hours after ischemia. Four hours after the MCAO, animals were randomly assigned to monotherapy with atorvastatin (n=14), rht-PA (n=18), or saline (n=26) or combination therapy with atorvastatin and rht-PA (n=26). Rats were humanely killed at 1 or 7 days after MCAO. eNOS−/− mice were treated with either tenecteplase–tissue plasminogen activator (TNK-PA, 1.5 mg/kg IV, n=4; Genentech) or TNK-PA (1.5 mg/kg IV) and atorvastatin (20 mg/kg IP, n=6) 4 hours after stroke.

Measurement of Infarct Volume and Hemorrhage
Seven days after MCAO, infarct volume was measured on 7-hematoxylin and eosin–stained coronal sections, as described previously. Gross hemorrhagic rate is presented as the percentage of animals per group with visible gross hemorrhage on any coronal section.10

Measurement of Microvascular Patency
To examine the patency of cerebral microvessels, fluorescein isothiocyanate (FITC)–dextran (2×10⁶ molecular weight, Sigma; 1 mL of 50 mg/mL) was administered intravenously to the rats 24 hours after stroke, as previously reported.11 Three coronal sections (100 μm) from each rat were analyzed with a MicroComputer imaging device system (MCID, Imaging Research Inc). Ten fields of view (4.4 mm²) from each coronal section were acquired in the territory supplied by the right MCA. Data are presented as the numbers of FITC pixels divided by the total number of pixels within the field of view and expressed as a percentage.

Immunohistochemistry
Immunohistochemistry was performed as previously described. The following primary antibodies were used in the present study: a rat monoclonal antibody against rat thrombocytes (Inter-Cell Technologies Inc, 1:200), a monoclonal antibody (mAb) against von Willebrand factor (vWF; Dako, 1:200), a polyclonal anti-human myeloperoxidase antibody (Dako, 1:200), an anti-rat intercellular adhesion molecule-1 (ICAM-1) mAb (Seikagaku Corp, 1:500), an anti-rat type IV collagen mAb (M3F7; Developmental Studies Hybridoma Bank, University of Iowa, 1:200), a goat anti-fibrinogen/fibrin antibody (Accurate Chemical & Scientific, 1:1000), an anti-rat matrix metalloproteinase-9 (MMP9) mAb (Chemicon International, 1:200), an anti-human protease-activated receptor (PAR)-1 mAb (Santa Cruz Biotechnology, 1:1000), and a polyclonal anti-human eNOS antibody (Cell Signaling Technology, 1:500). Immunohistochemical measurements were performed 1 day after stroke except for myeloperoxidase immunoreactivity, which was measured 7 days after stroke.

Western Blot Analysis
Rats were decapitated and the brains removed 24 hours after ischemia. Cytoplasmic proteins were extracted from the ischemic and contralateral homologous tissues. Western blotting was performed to detect eNOS protein with an antibody against eNOS (1:1000). For control of protein loads, a β-actin antibody (1:5000, Sigma) was used. Signal bands were visualized by the ECL system (Amersham). The relative density between the blot lanes was analyzed with the MCID system (Imaging Research Inc).

Measurements of eNOS Activity
eNOS activity was measured by monitoring the conversion of [¹⁴C]L-arginine to [¹⁴C]citrulline with the NOS assay kit (Cayman Chemicals, n=3/group) according to the manufacturer’s instructions.

LCM and Real-Time RT-PCR
Frozen brain coronal sections were incubated with an antibody against vWF (1:50 dilution) for 3 minutes and exposed to a CY3-conjugated F(ab’), secondary antibody (Dako, 1:50 dilution). With use of a PixCell IIe laser-capture microdissection (LCM) instrument (Arcturus Engineering), vWF-immunoreactive cerebral endothelial cells (~250 cells per rat) were isolated. Total RNA was isolated with use of the RNeasy micro kit (Qiagen Inc). Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed on an ABI 7000 PCR instrument (Applied Biosystems) with 3-stage program parameters provided by the manufacturer. Each sample was assayed in triplicate, and the TaqMan PCR Ct values obtained from 3 independent experiments were used for analysis of relative gene expression according to the 2⁻ΔΔCt method.13,14 The TaqMan primer/hybridization probes for genes of tissue factor, eNOS, vWF, and glyceraldehyde 3-phosphate dehydrogenase were obtained from ABI.

MRI Measurements
To measure dynamic changes in the ischemic lesion, magnetic resonance imaging (MRI) measurements were performed with a 7-T, 20-cm-bore superconducting magnet (Magnex Scientific) interfaced to a SMIS console. Perfusion-weighted and T2-weighted images (T2WI) measurements were performed 2, 24, and 48 hours after onset of embolization.15

Image Analysis
Areas of ischemic damage were calculated from MRI parameters of T2WIs with threshold values of 2 SDs for T2WIs of the contralateral nonischemic hemisphere. (Areas associated with cerebrospinal fluid and its elevated T2WI values were distinct from parenchymal tissue and were excluded from analysis). Areas of abnormal cerebral blood flow (CBF) were calculated with a threshold value of 50 mL · 100 g⁻¹ · min⁻¹. Data were normalized to 100% at the 2-hour time point and presented as percentage changes relative to the 2-hour time point.

Functional Outcomes
Neurological severity scores were measured at 1 and 7 days after ischemia. The neurological severity score is a composite of motor, sensory, reflex, and balance tests.16

Body Weight Loss
Animals were weighed before and 7 days after stroke. Body weight loss is presented as a percentage of preischemic body weight.

Statistics
All values are presented as mean±SD. Data were evaluated for normality. Ranked data were used in the analysis when they were not normally distributed (eg, neurological severity score). Two-way ANOVA was used to test the combination treatment effects on lesion reduction at 7 days after stroke. Analysis began testing for treatment interactions at the critical value of 0.05. An additive effect would be observed if there was no interaction detected at the 0.05 level. Otherwise, the effect of rht-PA and atorvastatin was tested.
further for a superadditive effect (i.e., the combined rht-PA and atorvastatin effect was superior for recovery from stroke to that of the combined effects of each treatment alone) or a subadditive effect (vice versa). We estimated the coefficient of the super/subadditive effect and its 95% confidence intervals (CIs). CIs excluding zero indicate a super/subadditive effect of the combined rht-PA and atorvastatin treatment. The same analytic approach was used to study treatment effects on functional recovery and weight loss. With a similar approach, we tested for treatment effects on the incidence of hemorrhage at 7 days by logistic regression. Pairwise comparisons were conducted for measurements at day 1 after adjustment for multiple comparisons according to Hochberg’s approach.

Table 1. Infarct Volume, Incidence of Hemorrhage, and Functional Outcome

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lesion Volume, % of Hemisphere</th>
<th>1 Day</th>
<th>7 Days</th>
<th>Body Weight, % of Loss</th>
<th>Incidence of Hemorrhage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=8)</td>
<td>37.5±9.4</td>
<td>10.5±0.8</td>
<td>8.3±0.7</td>
<td>28.6±8.9</td>
<td>13</td>
</tr>
<tr>
<td>rht-PA (n=8)</td>
<td>39.0±5.8</td>
<td>11.3±0.7</td>
<td>8.3±0.7</td>
<td>32.9±5.1</td>
<td>25</td>
</tr>
<tr>
<td>Atorvastatin (n=8)</td>
<td>33.6±5.4</td>
<td>11.3±1.5</td>
<td>8.5±1.6</td>
<td>24.9±6.7</td>
<td>25</td>
</tr>
<tr>
<td>Atorvastatin + rht-PA (n=8)</td>
<td>23.1±9.6*</td>
<td>10.9±1.1</td>
<td>6.6±1.2</td>
<td>19.4±5.9*</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are mean±SD.  
*P<0.05 vs saline-treated rats.

Results

Effects of Combination Treatment on Infarct Volume and Functional Outcomes

We previously demonstrated that intravenous administration of rht-PA 4 hours after stroke did not reduce infarct volume.18 To examine whether short-term, high-dose atorvastatin might be effective for enhancing thrombolysis, atorvastatin (20 mg/kg) was administered 4 and 24 hours after stroke, and rht-PA (10 mg/kg IV) was administered 4 hours after stroke in rats subjected to embolic MCAO. Rats in control groups were treated with atorvastatin alone (20 mg/kg at 4 and 24 hours).

Figure 1. MRI measurements of CBF and T2WIs. A, CBF and T2W MRI images at 2, 24, and 48 hours after MCAO of a representative rat treated with saline (n=10) or rht-PA and atorvastatin (n=6) 4 hours after stroke. B and C, Quantitative data of CBF and T2WI values, respectively. Solid and dashed lines represent saline and 4-hour rht-PA and atorvastatin groups, respectively. Data for CBF were normalized to 100% at the 2-hour time point and are presented as percentages changes relative to the 2-hour time point.
hours), rht-PA alone (10 mg/kg at 4 hours), or saline. Rats were humanely killed 7 days after stroke. Treatment with atorvastatin or rht-PA alone did not reduce infarct volume compared with the saline group (Table 1). In contrast, an interaction of rht-PA and atorvastatin on lesion volume at 7 days was detected \((P=0.02)\), with a superadditive effect with a coefficient of \(-12.0\) and a 95% CI of \(-22.1\) to \(-1.89\). Rats in the combination treatment group had a mean lesion volume reduction of 38% \((P<0.0001)\), and these rats did not show an increase in the incidence of hemorrhage compared with those in the control group (Table 1). Combination treatment also substantially improved neurological outcome (Table 1).

Using MRI, we dynamically measured the effects of combination treatment of atorvastatin and rht-PA on stroke. Oclusion of the right MCA resulted in a significant reduction of CBF in the territory supplied by the MCA 2 hours after stroke (Figure 1). There was no significant difference in CBF between saline-treated (Figure 1A) and combination-treated (Figure 1A) groups before treatment, suggesting that the initial severity of stroke was the same in both groups. A decline in CBF in the ipsilateral hemisphere persisted for the 48-hour duration of MCAO in the saline-treated group (Figure 1A). In contrast, combination treatment resulted in significantly enhanced CBF in the ipsilateral hemisphere during the same period (Figure 1A). Interestingly, combination treatment did not elevate contralateral hemisphere CBF levels (Figure 1A). In line with perfusion-weighted MRI, T2WI measurements revealed expansion of the ischemic lesion in the saline-treated rats during the 48-hour period (Figure 1A), whereas combination treatment blocked expansion of the ischemic lesion during the same period (Figure 1A). Quantitative analysis showed that combination treatment significantly increased CBF (Figure 1B) and reduced expansion of the ischemic lesion (Figure 1C).

Effects of Combination Treatment on Microvascular Patency
To examine whether the increased ipsilateral CBF observed on MRI resulted from increases in microvascular patency, FITC-dextran was administered before the animal was killed. FITC-dextran perfuses all cerebral microvessels in the normal brain.\(^{11}\) Combination treatment significantly increased microvascular areas perfused with FITC-dextran in the ipsilateral hemisphere (Figure 2C and 2E) compared with the same areas in rats treated with saline (Figure 2A and 2E), rht-PA (Figure 2E), or atorvastatin (Figure 2E) alone. In addition, combination treatment resulted in a significant reduction of intravascular fibrin and neutrophil deposition and a trend toward decreased platelet deposition (Table 2), which are all involved in thrombosis.\(^2\) These data are consistent with the MRI findings and demonstrate that combination treatment enhanced microvascular patency by decreasing microvascular thrombosis.

Effects of Combination Treatment on Procoagulant and Anticoagulant Systems
Statins regulate some key steps of coagulation and fibrinolytic processes, including tissue factor, tPA, vWF, and thrombin.\(^7\) To assess the effects of combination treatment on these processes, using TaqMan probes in combination with LCM techniques, we performed real-time RT-PCR analysis on cerebral endothelial cells obtained from brain coronal sections 24 hours after treatment. Analysis of mRNA isolated from vWF-immunoreactive vessels (Figure 3A through 3D) revealed that combination treatment significantly reduced the expression of vWF and tissue factor compared with rht-PA alone (Figure 3E and 3F). Immunostaining with antibodies against PAR-1, a receptor for thrombin, showed that combination treatment significantly \((P<0.05)\) reduced PAR-1 immunoreactivity compared with rht-PA–treated rats (Figure 3G through 3I).

Effects of Combination Treatment on ICAM-1 Expression
To examine whether the antiinflammatory effect of atorvastatin affected ICAM-1 expression, immunostaining for ICAM-1 was performed. Treatment with rht-PA alone significantly increased the numbers of ICAM-1–immunoreactive vessels in the ipsilateral hemisphere (Figure 4A and 4C). However, combination treatment significantly reduced the rht-PA–augmented ICAM-1 expression (Figure 4B and 4C).

**Table 2.** Vascular Immunoreactivity in the Ischemic Hemisphere

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fibrin Deposition, % of Total Area</th>
<th>Neutrophils, No. of Cells</th>
<th>Platelets, No. of Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.4 ± 0.9</td>
<td>39.5 ± 9.0</td>
<td>52.7 ± 14.0</td>
</tr>
<tr>
<td>rht-PA</td>
<td>2.5 ± 0.4</td>
<td>50.0 ± 27.0</td>
<td>76.0 ± 13.6</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>2.7 ± 0.6</td>
<td>13.8 ± 17.7</td>
<td>59 ± 4.5</td>
</tr>
<tr>
<td>Atorvastatin + rht-PA</td>
<td>1.4 ± 0.4*</td>
<td>14.7 ± 15.7*</td>
<td>34.2 ± 13.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*\(P<0.05\) vs saline-treated rats.
Western blot analysis (Figure 4D and 4E) revealed that atorvastatin significantly diminished rht-PA–induced ICAM-1 expression.

Effects of Combination Treatment on Microvascular Integrity

To examine the effects of combination treatment on cerebral microvascular integrity, immunostaining for collagen type IV, one of the major basal lamina components of cerebral microvessels, and fibrin was performed. Stroke reduced collagen type IV–immunoreactive vessels (Figure 5B) compared with collagen IV–positive vessels in the homologous area of the contralateral hemisphere (Figure 5A). A reduction in collagen type IV was associated with significant increases in the numbers of vessels with parenchymal fibrin deposition (Figure 5C), an indication of BBB leakage. Treatment with rht-PA alone significantly increased the numbers of MMP9–immunoreactive vessels (Figure 5D and 5F), which degrades extracellular matrix components, including collagen type IV. However, combined treatment with atorvastatin and rht-PA

Figure 3. LCM of single endothelial cells and expression of tissue factor, vWF, and PAR-1. A–D, vWF-immunoreactive vessels at the ischemic boundary before (A and C) and after (B and D) laser capture. C and D, Merged image of fluorescence and bright-field images. Loss of fluorescence signals (B and D) indicated that cells have been captured. Analysis of mRNA isolated from single cerebral endothelial cells revealed the effects of combination treatment on the expression of tissue factor (E) and vWF (F). Immunostaining shows PAR-1– (G–I) immunoreactive vessels at the ischemic boundary area in representative rats treated with rht-PA (G) and a combination of atorvastatin and rht-PA (H). I, Quantitative data of PAR-1–immunoreactive vessels (saline, n=6; rht-PA, n=3; atorvastatin, n=3; rht-PA+atorvastatin, n=6). Bar=40 μm for A-D and 20 μm for G and H. *P<0.05 vs saline and **P<0.05 vs rht-PA.

Figure 4. Expression of ICAM-1. A and B, ICAM-1–positive vessels and C, quantitative data (saline, n=6; rht-PA, n=3; atorvastatin, n=3; rht-PA+atorvastatin, n=6). *P<0.05 vs saline and +P<0.05 vs rht-PA. D, Western blots of ICAM-1 from 4 representative rats; line 1 is the ICAM-1 protein used as a positive control. E, Quantitative data (n=4/group). Bar=40 μm for A and B.
resulted in a significant reduction in the numbers of MMP9-immunoreactive vessels (Figure 5F) and a significantly smaller reduction in the number of collagen IV–immunoreactive vessels than monotherapy with saline or rht-PA alone (Figure 5E). Atorvastatin alone did not significantly reduce the loss of collagen IV– and MMP9–positive vessels (Figure 5E and 5F). Furthermore, combination treatment significantly ($P<0.05$) reduced the numbers of vessels with parenchymal fibrin deposition (12±4.9, n=6) compared with the numbers in the rht-PA–alone group (39±8.9, n=3).

**Effects of Atorvastatin on eNOS Expression**

To examine whether the benefits of combination treatment with atorvastatin and rht-PA were associated with the upregulation of eNOS, eNOS mRNA and protein levels were measured. Quantitative real-time RT-PCR analysis of total RNA extracted from the ischemic boundary and the contralateral hemisphere revealed that combination treatment with rht-PA and atorvastatin did not significantly increase eNOS mRNA levels compared with those in saline-treated rats (Figure 6A). To further confirm the effects of atorvastatin on cerebrovascular eNOS, total RNA was extracted from cerebral endothelial cells isolated from brain tissue by LCM. Combination treatment did not significantly increase vascular eNOS mRNA levels (Figure 6B). Consistent with mRNA analysis, combination treatment did not significantly increase the numbers of eNOS-immunoreactive vessels (n=4, 0.6±0.1/mm²) compared with the numbers in saline– (n=4, 0.7±0.4/mm²), rht-PA– (n=4, 0.7±0.1/mm²), and atorvastatin– (n=4, 0.6±0.2/mm²) treated groups. The same results were obtained from Western blot analysis (Figure 6C). These results demonstrate that atorvastatin does not change expression of cerebral eNOS.

**Figure 5.** MMP9 and collagen IV immunoreactivity in cerebral microvessels. Immunoreactivity of collagen IV in the contralateral (A) and ipsilateral (B) hemispheres, fibrin (C), and MMP9 (D). E and F, Quantitative data of collagen IV (* vs saline and ** vs rht-PA) and MMP9 (* vs saline and ** vs rht-PA), respectively, in the ipsilateral hemisphere (saline, n=6; rht-PA, n=3; atorvastatin, n=3; rht-PA+atorvastatin, n=6). Bar=10 μm.

**Figure 6.** Effects of atorvastatin on eNOS expression. Quantitative real-time PCR shows eNOS mRNA levels from brain extracts (A) and cerebral endothelial cells (B). β-Actin was used as an internal control. C, eNOS protein levels as assayed by Western blotting. Similar results were obtained in 3 independent experiments. Contra and ipsi represent the contralateral and ipsilateral hemispheres, respectively.
Effects of L-NAME on Combination Treatment

To examine whether blocking NOS activity affects the beneficial effects of combination treatment, rats were treated with atorvastatin, rht-PA, and L-NAME. Administration of L-NAME significantly reduced brain NOS activity (Figure 7). These rats (n = 3) had a mean infarct volume of 19.5 ± 8.1% in the contralateral hemisphere, which was significantly smaller than in rats treated with saline (37.5 ± 9.4%, n = 8) or rht-PA (39 ± 5.8%, n = 8) alone, but infarct volume was not significantly different from rats treated with atorvastatin in combination with rht-PA (23.1 ± 9.6%, n = 8).

Effects of Combination Treatment on eNOS−/− Mice

L-NAME inhibits both eNOS and neuronal NOS activity. To further examine whether eNOS was involved in the beneficial effects of atorvastatin on stroke, eNOS−/− mice were treated with TNK-tPA or TNK-tPA with atorvastatin 4 hours after stroke. Embolic stroke in the eNOS−/− mice resulted in an ∼70% reduction of CBF 4 hours after stroke but before treatment. Twenty-four hours after stroke, administration of TNK-tPA alone (n = 4) increased CBF by 32% (31 ± 6% at 4 hours to 45 ± 2% at 24 hours), whereas treatment with a combination of TNK-tPA and atorvastatin (n = 6) increased CBF by 46% (32 ± 3% at 4 hours to 58 ± 3% at 24 hours), and the difference between the 2 groups was significant (P < 0.05). Furthermore, eNOS−/− mice treated with TNK-tPA alone had a mean infarct volume of 32 ± 4% of the contralateral hemisphere, whereas eNOS−/− mice treated with TNK-tPA in combination with atorvastatin had a mean infarct volume of 11 ± 1% (P < 0.05).

Effects of Atorvastatin on Blood Pressure, Serum Cholesterol Levels, and Regional CBF

We examined the effects of short-term, high-dose treatment of stroke with atorvastatin on blood pressure, cholesterol levels, and CBF. Treatment with atorvastatin did not significantly reduce mean arterial blood pressure at 5 (n = 3, 94 ± 10.3 mm Hg) and 30 (98 ± 10.8 mm Hg) hours after stroke compared with rats treated with saline (n = 3, 98 ± 10.4 mm Hg at 5 hours and 99 ± 9.3 mm Hg at 30 hours). Serum total cholesterol levels were not significantly different between atorvastatin- (n = 4, 68 ± 9 mg/dL) and saline- (n = 4, 56 ± 1 mg/dL) treated groups. Treatment with atorvastatin also did not significantly increase regional CBF as measured by laser Doppler flowmetry (n = 3, 434 ± 84 perfusion units before treatment versus 451 ± 57 perfusion units during a 5-hour period after treatment).

Discussion

We demonstrate for the first time that a widely used statin compound with an excellent safety profile exerts neuroprotective effects when combined with thrombolytic treatment with rht-PA at 4 hours after stroke onset. The therapeutic benefits observed with combination therapy can likely be attributed to atorvastatin’s multitargeted effects on cerebral microvascular thrombosis and microvascular integrity.

Statins have been used prophylactically or within 3 hours of onset to effectively treat ischemic stroke in the rodent model. Chronic statin administration upregulates eNOS expression resulting in augmentation of CBF, which leads to a reduction in infarct volume, whereas the prophylactic effects of statins on stroke are absent in eNOS−/− mice subjected to MCAO. These data indicate that the major therapeutic mechanism is statin-induced upregulation of eNOS expression. However, studies in postnatal rats subjected to hypoxia-ischemia showed that prophylactic administration of simvastatin reduced brain injury and improved functional recovery and that this prophylactic effect was associated with a reduction in cytokine expression but did not affect eNOS expression. In parallel, our data demonstrate that administration of atorvastatin after stroke extended the therapeutic window of rht-PA by enhancement of cerebral microvascular patency and integrity, primarily by decreasing thrombosis. Combination treatment did not increase eNOS levels, and blocking NOS activity with the NOS inhibitor L-NAME did not abolish the beneficial effects of combination treatment on stroke. Furthermore, treatment with TNK-tPA and atorvastatin significantly increased restoration of CBF and reduced infarct volume compared with TNK-tPA treatment alone in eNOS−/− mice. Thus, the mechanism underlying the neuroprotection after combination treatment likely derived from the statin’s eNOS-independent ability to mitigate rht-PA–induced microvascular thrombosis, inflammatory responses, and vasculature disruption. Consistent with our results, a recent study showed that rosuvastatin reduced rht-PA–induced brain damage by a mechanism independent of eNOS.

In this model of embolic stroke, we have demonstrated that secondary thrombosis develops in the cortex during the first 6 hours of stroke, which contributes to impairment of cerebral microvascular patency. Consistent with previous findings, the present study has shown that combination treatment with atorvastatin and rht-PA significantly improved restoration of CBF and enhanced vascular patency in the ipsilateral cortex, indicating that combination therapy reduced secondary thrombus formation. Mechanisms for generation of thrombosis after cerebral ischemia include procoagulant transformation of brain microvascular endothelium and platelet activation. Stroke-induced tissue factor initiates the coagulation
cascade. Thrombin promotes expression of vWF on endothelial cells and facilitates thrombosis formation. PAR-1 is the prototype thrombin receptor, and plasmin, which is generated from rht-PA’s cleavage of plasminogen, activates PAR-1, which also promotes thrombosis formation. Our data have shown that combination treatment with atorvastatin and rht-PA blocked the rht-PA– and stroke-induced increases in PAR-1, vWF, and tissue factor expression, suggesting that the beneficial effects of atorvastatin on coagulation and fibrinolysis inhibit secondary thrombosis and improve cerebral microvascular patency, which reduce infarct volume.

Delayed fibrinolysis with rht-PA further increased ICAM-1 expression and neutrophil accumulation in ipsilateral cerebral vessels. In the present study, administration of atorvastatin in combination with rht-PA significantly reduced ICAM-1 expression and neutrophil accumulation in cerebral microvessels, suggesting that atorvastatin attenuates the adverse effects of inflammation after thrombolysis with rht-PA and thereby enhances the efficacy of thrombolytic therapy. Our data are comparable with previous studies that have demonstrated that treatment of diabetic retinopathy in rats with simvastatin reduced ICAM-1 expression and inhibited leukocyte accumulation, whereas treatment did not change eNOS expression.

Thrombolysis with tPA exacerbates disruption of the BBB after stroke, which limits clinical use of tPA. Treatment with rht-PA upregulates MMP9 expression, which promotes the disruption of vascular integrity and intracerebral hemorrhage. We found that treatment with rht-PA significantly increased cerebral microvascular MMP9 immunoreactivity and parenchymal fibrin deposition, which is consistent with the hypothesis that thrombolysis with rht-PA promotes BBB disruption. MMPs degrade collagen IV. In hypercholesterolemic patients with coronary artery disease, treatment with simvastatin significantly reduced plasma levels of MMP activity. Consistent with previous studies, coadministration of atorvastatin and rht-PA significantly reduced cerebral microvascular MMP9 immunoreactivity, degradation of collagen IV immunoreactivity, and extravascular fibrin deposition compared with that in rht-PA–treated rats. Thus, our data suggest that atorvastatin reduces vascular MMP9 expression and thereby protects the integrity of cerebral vessels after thrombolytic rht-PA therapy for stroke.

Our study does not in any way exclude the possibility that other statins will behave similarly. Other statins, including the widely used simvastatin, have anticoagulant and thrombolytic effects similar to those of atorvastatin. Our goal in this study was to simply conduct a proof-of-principle study to demonstrate that a statin can be used safely and effectively to reduce cerebral infarct volume and enhance functional outcomes when applied with rht-PA 4 hours after stroke, a time point demonstrated to be beyond the therapeutic window for rht-PA treatment alone for embolic stroke in the rat. However, additional studies to more fully test and extend the utility of rht-PA adjunctive treatment with statins for stroke are warranted.

In preclinical rodent studies of stroke, both oral and subcutaneous delivery of the drug was performed. Plasma levels of atorvastatin after oral administration in the rat have been compared with human plasma levels. From an under-the-curve analysis, doses of 10 to 80 mg/kg in humans are equivalent to doses of 5 to 18 mg/kg in rats. However, because atorvastatin has extensive first-pass metabolism in the gut and liver in humans, plasma concentrations after subcutaneous administration could be several-folds greater than for oral administration. Pharmacodynamic studies are therefore warranted for subcutaneous administration of statins.

Acknowledgments
This work was supported by NINDS grants PO1 NS23393, RO1 NS38292, and RO1 HL64766. We thank Cynthia Roberts and Qing-e Lu for technical assistance.

References
Many efforts have attempted to extend the therapeutic window for the treatment of stroke with recombinant human tissue-type plasminogen activator (rht-PA) beyond 3 hours. Agents that reduce vascular inflammation, platelet activity, and thrombin and that are also neuroprotective have been used as adjunctive therapy to rht-PA. However, moving adjunctive rht-PA therapy into the clinic requires agents that are safe, do not increase the incidence or severity of bleeding, and are effective in enhancing the thrombolytic efficacy of rht-PA. Statins are a class of compounds that have beneficial effects well beyond their ostensibly targeted purpose of reducing serum cholesterol. Pluripotent actions of statins include reducing hemostasis by reducing platelet activation and the procoagulation cascade and increasing fibrinolysis and the anticoagulation cascade. In the present study, we have demonstrated that treatment of embolic stroke in the rat with atorvastatin (Lipitor [Pfizer]), in combination with rht-PA 4 hours after stroke, significantly reduced infarct volume without increasing the incidence of hemorrhagic transformation and substantially improved neurological function, which indicates that combination treatment with atorvastatin and rht-PA 4 hours after stroke onset extends the therapeutic window for rht-PA and is neuroprotective. The therapeutic benefits are attributed to its multitargeted effects on cerebrovascular patency and integrity. Statins are being used for the secondary prevention of ischemic stroke. The present data suggest that statins appear to be ideal compounds as adjunctive agents for rht-PA in treatment of acute stroke.
Multitargeted Effects of Statin-Enhanced Thrombolytic Therapy for Stroke With Recombinant Human Tissue-Type Plasminogen Activator in the Rat
Li Zhang, Zheng Gang Zhang, Guang Liang Ding, Quan Jiang, Xianshuang Liu, He Meng, Ann Hoseska, Chunling Zhang, Lian Li, Dan Morris, Rui Lan Zhang, Mei Lu and Michael Chopp

_Circulation_. 2005;112:3486-3494
doi: 10.1161/CIRCULATIONAHA.104.516757

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/22/3486

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/