Aggravation of Focal Cerebral Ischemia by Tissue Plasminogen Activator Is Reversed by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor but Does Not Depend on Endothelial NO Synthase

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Background and Purpose—It has repeatedly been reported that the thrombolytic tissue plasminogen activator (tPA) may aggravate ischemic injury after stroke. The underlying mechanisms, however, remain unknown. We hypothesized that tPA induces an inhibition of endothelial NO synthase (eNOS) after focal ischemia that is responsible for ischemic damage and may be restored by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors.

Methods—We examined the effects of tPA, administered either alone or in combination with rosuvastatin, on ischemic injury, on eNOS expression, and cell signaling after 90 minutes of intraluminal middle cerebral artery occlusion.

Results—in wild-type mice, tPA delivered immediately after ischemia significantly increased infarct volume 24 hours after reperfusion. Coadministration of rosuvastatin completely reversed the tPA-induced brain damage. Western blots of ischemic brain lysates showed that tPA markedly diminished eNOS levels, increased extracellular regulated kinase (ERK)-2, and decreased MAP kinase/p38 activity. Cotreatment with rosuvastatin prevented the decrease in eNOS, reduced ERK-1/-2 and normalized p38 levels. To elucidate the role of eNOS in tPA-induced ischemic injury, we also evaluated tPA effects in eNOS knockout mice. In eNOS knockout animals, tPA again significantly increased infarct size after transient focal ischemia.

Conclusions—in a mouse model of focal cerebral ischemia, tPA induces eNOS inhibition, ERK-2 activation, and p38 inhibition, possibly as part of a more complex signaling response exacerbating brain injury. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition reverses the effects of tPA by a mechanism independent of eNOS. (Stroke. 2005;36:332-336.)

Key Words: hydroxymethylglutaryl-CoA reductase inhibitors ■ ischemia ■ stroke, acute ■ thrombolysis

Intravenous thrombolysis with recombinant tissue plasminogen activator (tPA) is the only efficacious treatment of acute ischemic stroke in humans to date.1 Animal studies using intraluminal middle cerebral artery (MCA) occlusions have shown that tPA may also have detrimental effects after stroke,2,3 possibly through secondary disturbances of regional cerebral blood flow in ischemic brain areas.3 The molecular mechanisms underlying the aggravation of injury remain unknown.

We hypothesized that the adverse effects of tPA are induced by disturbances of endothelial NO synthase (eNOS) signaling. In focal cerebral ischemia, eNOS closely reflects endothelium-dependent vasorelaxation ability and, in turn, cerebral blood responses to ischemia.4 Because 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) upregulate eNOS activity and thereby ameliorate brain perfusion after stroke,5,6 we were interested whether tPA-induced brain damage would be reversed by a statin. We therefore exposed wild-type and eNOS knockout mice to 90 minutes intraluminal MCA occlusions and tested the effects of tPA, administered either alone or in combination with rosuvastatin, on ischemic injury, on eNOS expression, and on the phosphorylation (ie, activation) state of the mitogen activated protein (MAP) kinases extracellular regulated kinase (ERK)-1/2 and p38. ERK-1/2 and p38 were chosen because they are known to mediate signaling actions of serine proteases in the endothelium.7,8

Materials and Methods

Animal Experiments

Studies were carried out according to Swiss Veterinarian guidelines for animal experimentation and approved by local authorities. Male
C57BL/6 mice or eNOS knockout mice (B6.129P2-Nos3<tm1Unc>/J obtained from Jackson Laboratories, Bar Harbor, Me) weighing 20 to 25 g were anesthetized with 1% halothane (30% O2, remainder N2O). Rectal temperature was maintained at 37.0°C using a feedback-controlled heating system. Focal ischemia was induced using an intraluminal filament technique as described. Briefly, the left common and external carotid arteries were isolated and ligated. A silicon-coated 8 to 0 nylon monofilament was introduced into the common carotid artery and advanced 9 mm distal to the carotid bifurcation, thus interrupting MCA blood flow. Ninety minutes later, reperfusion was initiated by thread withdrawal.

Immediately after reperfusion, either tPA (10 mg/kg; in 0.2 mL carrier solution) or 0.2 mL normal saline was administered over a femoral venous catheter by continuous infusion during 30 minutes. At the same time, 30 μL of normal saline or normal saline containing rosuvastatin (20 mg/kg) was injected intraperitoneally (n = 5 to 7 animals per group). Because this dose induces ~90% inhibition of HMG-CoA reductase activity in rodents, 20 mg/kg was chosen. During the MCA occlusions, until 30 minutes after reperfusion onset, laser Doppler flow (LDF; Perimed AB) was monitored above the core of the MCA territory. Then anesthesia was discontinued and animals were placed back into their cages.

Twenty-four hours later, mice were reanesthetized and decapitated. Brains were removed, frozen on dry ice, and cut into 18-μm coronal sections on a cryostat (Leica Microsystems AG). Brain sections were collected from a total of 5 equidistant brain levels 2 mm apart, which were stained with cresyl violet. From the rostrocaudal level of the bregma onwards up to 2 mm further caudally, tissue samples were also retrieved involving both ipsilateral and contralateral brain tissue for Western blots. This procedure of tissue sampling was chosen to minimize influences of the infarct size on histochemical results, which may otherwise seriously compromise data interpretation. Cresyl violet-stained brain sections were digitized, brain infarcts were outlined, and infarct volume and brain swelling were measured.

Western blots

Tissue samples were dissected, complemented with lysis buffer, homogenized, and centrifuged. Supernatants were used for SDS-PAGE. Equal amounts of protein were diluted in 6X sample buffer, boiled, loaded on polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. Membranes were dried, incubated in blocking solution, and immersed with monoclonal mouse anti-eNOS (1:10000; BD Biosciences, Basle, Switzerland), antiphospho–ERK-1/2 (M8159; Sigma, St Louis, Mo), or antiphospho–p38/MAP kinase (M8177; Sigma, St Louis, Mo) antibodies, each diluted 1:500 in 0.1% Tween 20 per 0.1 mol/L Tris buffered saline. Membranes were rinsed, incubated with peroxidase-coupled secondary antibodies, washed, immersed in enhanced chemiluminescence (ECL) solution and exposed to ECL-Hyperfilm (Amersham). Blots were carried out with protein samples pooled from all animals belonging to the same group. Protein loading was controlled using a monoclonal mouse antibody against β-actin (A5316; Sigma). Protein levels were analyzed densitometrically, corrected with values determined on β-actin blots, and finally expressed as relative values compared with normal saline-treated animals.

Statistics

Differences between groups were calculated by 1-way ANOVA, followed by least significant difference tests (SPSS for Windows 10.1). Values were given as means±SD, n values indicating the number of different samples analyzed. P<0.05 were considered significant.

Results

Laser Doppler Flow During Experiments

To ensure reproducibility of ischemias, LDF recordings were performed. In all groups, both in wild-type and eNOS-deficient mice, thread occlusion resulted in a similar decrease of LDF to 15% to 20% of preischemic values. Thread retraction was followed by a rapid restoration of blood flow to baseline levels. LDF values during and immediately after ischemia did not differ between groups.

HMG-CoA Reductase Inhibitor Reverses Detrimental Effects of tPA on Infarct Size in Wild-Type Mice

In wild-type mice, tPA significantly increased the infarct volume after transient focal ischemia (Figure 1). Rosuvastatin, on the other hand, significantly reduced infarct size, both when administered alone and in combination with tPA (Figure 1). Brain swelling was not different between groups.

tPA Inhibits eNOS and Modifies ERK-2 and p38 Signaling: Reversal by HMG-CoA Reductase Inhibition

To characterize changes of eNOS expression and cell signaling, Western blots were performed with tissue samples obtained from wild-type mice. Interestingly, tPA treatment markedly diminished eNOS levels in homogenized brain tissue (Figure 2). Furthermore, tPA increased ERK-2 activity...
and decreased p38 activity (Figure 2). In contrast, cotreatment with rosuvastatin prevented the decrease of eNOS, reduced ERK-1/-2 and normalized p38 levels (Figure 2).

**tPA-induced Brain Injury Is Not Abolished in eNOS Deficient Mice**

To identify whether eNOS inhibition was responsible for the tPA-induced aggravation of injury, mice deficient for eNOS were subjected to transient focal ischemia and effects of tPA were then tested in these mice. Contrary to our assumption, tPA significantly increased infarct volume in eNOS knockout mice (Figure 3). Our data indicate that changes of eNOS levels are not responsible for the tPA-induced brain damage. Apparently, other mechanisms must also be involved.

**Discussion**

Intravenous thrombolysis with tPA is an efficacious treatment of ischemic stroke in humans when applied within 3 hours after the onset of stroke symptoms.1 In recent years, however, animal experiments have pointed out that tPA may also have adverse effects. Indeed, we and others have shown that tPA aggravates brain injury after intraluminal MCA occlusions in mice,2,3 possibly because of secondary hemodynamic disturbances.3 To find out whether these effects are mediated through disturbances of endothelial NO signaling, we here exposed mice to transient focal ischemias and tested the effects of tPA, administered either alone or in combination with rosuvastatin, on ischemic injury, on eNOS levels, and on the phosphorylation (ie, activation) state of ERK-1/-2 and p38.

**Role of eNOS, ERK-2, and p38 in tPA-Induced Brain Injury**

Indeed, tPA induced a marked reduction of eNOS levels in our study, which closely accompanied the augmentation of brain damage, which we have already demonstrated previously3 and which we again confirmed here. Interestingly, both the increase in infarct size and the decrease in eNOS levels were completely abolished by add-on treatment with rosuvastatin. tPA is a serine protease, which, besides cleaving plasminogen, catalyzes various other enzymatic reactions.2,3 It has already been shown in vitro in human aortic endothelial cells that thrombin, another serine protease, markedly decreases eNOS levels,7 whereas cotreatment with simvastatin reversed these changes, implicating a protective role of statins on endothelial integrity.8 In human aortic endothelial cells, administration of thrombin was followed by an activation of ERK-1/-2 and p38 pathways.7,8 It is noteworthy that we observed an increase in ERK-2 activity, but a reduction of p38 activity after tPA treatment. The different observations in this study may be related to specific actions of tPA, which may differ from thrombin, or to differences between in vitro and in vivo conditions. It remains unclear whether the inhibition of eNOS by tPA takes place on a transcriptional or posttranscriptional level. Further studies may clarify this issue.

To find out whether eNOS inhibition was responsible in a causative manner for the tPA-induced aggravation of injury, we tested effects of tPA in eNOS knockout mice, evaluating whether and how tPA influenced infarct size in these animals. Contrary to our assumption, eNOS knockout mice again exhibited an exacerbation of their infarct size on tPA treatment. Our data indicate that tPA-induced brain damage does not depend on the inhibition of eNOS. This finding basically allows 2 different interpretations. First, the eNOS inhibition may represent an epiphenomenon of other injury cascades which, albeit clearly detectable, is functionally irrelevant. The important role of eNOS in regulating vascular reactivity of ischemic blood vessels may argue against that view.4–6

**Figure 2.** Western blots for eNOS, diphospho–ERK-1/-2, and diphospho–MAP kinase/p38 of ischemic brain tissue from wild-type mice treated with normal saline or tPA. Note that tPA treatment significantly diminishes eNOS levels, increases ERK-2 activity and decreases p38 activity. In contrast, add-on treatment with rosuvastatin reverses the inhibition of eNOS, reduces ERK-1/-2, and increases p38 activity without affecting Bcl-XL and caspase-3. *P<0.05 compared with saline-treated animals; †P<0.05 compared with tPA-treated animals (n=3 different samples per group). Data were normalized with corresponding blots for β-actin.
rosuvastatin. This may be because of the late application of the drug, providing insufficient time to upregulate eNOS.

Statins as Add-On Treatment to Thrombolytics?
In our study, HMG-CoA reductase inhibition completely abolished the effects of tPA on infarct size. This raises the question whether statins may be suitable as add-on treatment in thrombolysis. A number of reasons support the use of statins in stroke patients: statins are (a) considered safe in humans,\textsuperscript{15,16} (b) widely prescribed in patients with vascular disease,\textsuperscript{17,18} and (c) according to recent secondary stroke prevention studies, able to decrease cardio- and cerebrovascular events in high-risk patients irrespective of plasma cholesterol levels.\textsuperscript{18} In the context of our data presented here, it is important to note that we applied rather high rosuvastatin doses, which were considerably above those usually recommended in human patients. Our reason for these high doses was that the pharmacodynamics of rosuvastatin differs considerably between rodents and humans.\textsuperscript{11} In rodents, doses of 20 mg/kg have been shown to be required for a \textsuperscript{90}\% inhibition of HMG-CoA reductase activity,\textsuperscript{11} which is appropriate in animal studies. Hence our data should clearly be interpreted as experimental results. Regarding tPA, further in vivo research will be necessary in the future to elucidate how this serine protease exacerbates the injury development after stroke. Such research might help to establish strategies maximizing the therapeutic potential, while minimizing adverse side effects of tPA.

Acknowledgments
This research was supported by the Swiss National Center of Competence in Research “Neural plasticity and repair,” the Swiss Heart Foundation, and a grant from Astra Zeneca.

References

Effects of HMG-CoA Reductase Inhibitors in Acute Ischemic Stroke
Postischemic delivery of rosuvastatin significantly reduced infarct size in our study, both when administered alone and in combination with tPA. It has repeatedly been shown in the past that statins reduce ischemic injury when given before the onset of a stroke, most likely through restoration of endothelial NO signaling.\textsuperscript{5,6} On the other hand, only 1 study until now has assessed effects of postischemic statins in focal cerebral ischemia using MCA electrocoagulations in rats, demonstrating that simvastatin protects against ischemic damage.\textsuperscript{14} Rosuvastatin is a very potent HMG-CoA reductase inhibitor\textsuperscript{15} with a long elimination half-life (\textasciitilde 18 to 21 hours) that, in contrast to most other statins, does not need to be metabolized in the liver to be active.\textsuperscript{14} Because there is no time delay until rosuvastatin can exert its function, rosuvastatin appeared well suited for poststroke delivery. Indeed, we were able to show tissue protection; although, in contrast to earlier studies,\textsuperscript{5} we did not see an increase of eNOS levels after treatment with

Figure 3. Infarct volume in eNOS-deficient mice subjected to 90 minutes intraluminal MCA occlusion followed by 24 hours of reperfusion. Animals were treated with (a) normal saline or (b) tPA (see also representative brain sections). Note that tPA exacerbates brain injury in eNOS knockout mice, indicating that tPA-induced brain injury does not depend on the eNOS pathway. Scale bar, 2 mm. *P<0.05 compared with saline-treated animals (n=6 animals per group).


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*Stroke*. 2005;36:332-336; originally published online December 29, 2004; doi: 10.1161/01.STR.0000152273.24063.f7

*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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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