Adjuvant Treatment With Neuroserpin Increases the Therapeutic Window for Tissue-Type Plasminogen Activator Administration in a Rat Model of Embolic Stroke

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Background—After stroke, the thrombolytic effect of tissue-type plasminogen activator (tPA) in the intravascular space is beneficial, whereas its extravascular effect on ischemic neurons is deleterious. We tested the hypothesis that neuroserpin, a natural inhibitor of tPA, reduces tPA-induced neuronal toxicity and increases its therapeutic window for treatment of embolic stroke.

Methods and Results—Rats were subjected to embolic middle cerebral artery occlusion (MCAO). Ischemic brains were treated with neuroserpin in combination with recombinant human tPA (n=7), tPA alone (n=7), or saline (n=9). Neuroserpin (20 μL of 16 μmol/L active neuroserpin) was intracisternally injected 3 hours after ischemia. MRI measurements were performed to study blood brain barrier (BBB) leakage and ischemic lesion volume. Administration of tPA alone 4 hours after ischemia significantly (P<0.05) increased BBB leakage in the ischemic core measured by Gd-DTPA–enhanced MRI compared with rats treated with saline. However, treatment with neuroserpin in combination with tPA significantly (P<0.05) reduced BBB leakage, brain edema, and ischemic lesion volume compared with rats treated with tPA alone, although ischemic lesion volumes were the same in both groups before the treatment. Immunostaining revealed that MCAO resulted in reduction of neuroserpin immunoreactivity in the ipsilateral hemisphere after 2 to 6 hours of ischemia. Zymographic assay showed increased plasminogen activity in areas with BBB leakage in rats treated with tPA.

Conclusions—Administration of neuroserpin after stroke is neuroprotective, seemingly because it blocks the extravascular effect of tPA, leading to subsequent decrease in stroke volume and widening of the therapeutic window for the thrombolytic effect of tPA. (Circulation. 2002;106:740-745.)

Key Words: stroke ▪ plasminogen activators ▪ magnetic resonance imaging ▪ thrombolysis

The serine proteinase tissue plasminogen activator (tPA), the predominant plasminogen activator in the blood, plays a thrombolytic role by converting plasminogen to plasmin.1 Treatment with recombinant human tPA significantly improves long-term neurological function when intravenous tPA is administered to ischemic stroke patients within 3 hours of the onset of stroke.2 Recent studies in experimental stroke indicate that tPA, in addition to its thrombolytic role, may be deleterious to neurons in the brain.3,4 Using an intraluminal filament to occlude the middle cerebral artery (MCA), Wang et al3 demonstrated that tPA-deficient mice exhibit significantly smaller ischemic lesion volumes than wild-type mice after MCA occlusion (MCAO) and that administration of tPA increases ischemic lesion volume in wild-type mice. Reduction of ischemic lesion volume in tPA-deficient mice is additionally demonstrated after ligation of the MCA.4 In contrast, Tabrizi et al5 showed that tPA-deficient mice have a significant increase in cerebrovascular fibrin deposition and in the ischemic lesion volume compared with wild-type mice with similar genetic backgrounds. Furthermore, Klein et al6 demonstrated that administration of recombinant tPA fails to exacerbate ischemic injury after global and focal cerebral ischemia in the rat.

Neuroserpin, a natural inhibitor of tPA in the central nervous system, is primarily expressed in neurons.7 We recently demonstrated that intracortical injection of neuroserpin significantly reduces the infarct volume in rats subjected to MCAO,8 suggesting that tPA, independently of its thrombolytic activity, has detrimental effects on ischemic cell survival. Moreover, using an embolic model of MCAO, we demonstrated that administration of tPA 1 hour after the onset of ischemia significantly reduces infarct volume and im-
proves functional outcome, whereas administration of tPA 4 hours after the onset of ischemia increases blood brain barrier (BBB) leakage and exacerbates ischemic cell damage.\textsuperscript{8,9} In light of these data, we hypothesize that after stroke the thrombolytic effect of tPA in the intravascular space is beneficial, whereas its extravascular effect on ischemic neurons is deleterious, and that neuroserpin reduces the adverse effects of tPA on parenchymal tissue. To test this hypothesis, we administered neuroserpin in combination with tPA to a rat model of focal cerebral embolic ischemia. We demonstrate that administration of neuroserpin at 3 hours and tPA at 4 hours after the onset of stroke significantly reduces infarct volume and brain edema. Moreover, adjuvant treatment with neuroserpin widens the therapeutic window for tPA treatment of embolic stroke.

**Methods**

The Care of Experimental Animals Committee of Henry Ford Hospital approved all experimental procedures.

**Animal Model**

Male Wistar rats (\(n=46\); Charles River Laboratories, Wilmington, Mass) weighing 300 to 350 g were anesthetized with halothane. The MCA was occluded by placement of a single intact fibrin-rich 24-hour-old homologous clot at the origin of the MCA.\textsuperscript{11} After MCAO, all rats exhibited neurological deficits, including turning to the left.\textsuperscript{11}

**Experimental Protocol**

Neuroserpin (20 \(\mu\)L of 16 \(\mu\)mol/L active neuroserpin) was injected 3 hours after ischemia via percutaneous injection into the cisterna magna. tPA (Genentech, Inc) was intravenously administered 4 hours after MCAO at a dose of 10 mg/kg as a 10% bolus and the remainder continuously infused over a 30-minute interval. Animals were randomly assigned to neuroserpin in combination with tPA (\(n=7\)), tPA alone (\(n=7\)), or saline (\(n=9\)) treatment.

**MRI Measurements**

To measure dynamic changes in the ischemic lesion, MRI measurements were performed using a 7-T, 20-cm bore-superconducting magnet (Magnex Scientific). Diffusion-weighted image, perfusion-weighted image, and \(T_2\)-weighted image measurements were performed before and during ischemia (before and after neuroserpin in combination with tPA, tPA alone, or saline treatment for a total of 6 hours of measurements) and 24 hours after onset of embolization.\textsuperscript{10}

Gd-DTPA contrast multislice \(T_2\)-weighted images were obtained using multislice (9 slices) spin-echo (TR=500 ms, TE=20 ms) sequences with a 1-mm slice thickness, 32 mm FOV, and 128\(\times\)128 matrix.\textsuperscript{15}

**Image Analysis**

Areas of ischemic damage were calculated from MRI parameters of \(T_2\) or ADC, using threshold values of 2 SDs for \(T_2\) or 1.5 SD for ADC, of contralateral nonischemic hemisphere. Areas of abnormal cerebral blood flow (CBF) were calculated using threshold of 50 mL/100 g-min.

Areas of contrast leakage were calculated from Gd-DTPA contrast MRI. Postcontrast images were subtracted from the precontrast images to create subtraction images. A threshold of 2 SDs of contralateral nonischemic hemisphere in the subtraction images was used to identify the areas exhibiting Gd-DTPA contrast leakage.

**Immunohistochemistry**

A rabbit polyclonal antibody against human neuroserpin was used to detect neuroserpin\textsuperscript{14} and an antibody against microtubule-associated protein 2 (MAP2; Serotec) was used as a marker for early ischemic neuronal damage.\textsuperscript{13} Double immunofluorescent staining was performed on frozen coronal sections, as previously described.\textsuperscript{13}

**In Situ Zymography**

In situ zymographies were performed on 8-\(\mu\)m cryostat brain coronal sections, as previously described.\textsuperscript{14}

**Measurements of the Ischemic Lesion Volume**

The ischemic volume is presented as the percentage of infarct volume in relation to the volume of the contralateral hemisphere (indirect volume calculation).\textsuperscript{11}

**Statistics**

Data were analyzed using one-way ANOVA followed by a Student-Newman-Keuls test. All values are presented as mean\(\pm\)SE. Statistical significance was set at \(P<0.05\).

**Results**

To examine the effects of tPA administration on BBB leakage 4 hours after MCAO, the MRI contrast agent Gd-DTPA was administered to rats with MCAO at the end of tPA (\(n=7\)) or saline infusion (\(n=9\)). The presence of Gd-DTPA within the tissue causes hyperintensity on \(T_2\)-weighted MRI and reflects a breakdown of the BBB.\textsuperscript{12} In the saline-treated ischemic rats, the hyperintense area localized to the ipsilateral MCA supplied subcortex (Figures 1A through 1C). Administration of tPA at 4 hours of ischemia resulted in increases of the hyperintense areas localized to the ipsilateral subcortex (Figures 1D through 1F). Quantitative data analysis revealed that the hyperintense areas in tPA-treated rats (58\(\pm\)4\% of hemisphere) were significantly (\(P<0.05\)) larger than the areas in the control animals (33\(\pm\)5\% of hemisphere).

Increases in BBB leakage after treatment with tPA may permit circulating exogenous tPA to leak into the parenchyma. To examine plasminogen activity (PA) after treatment with tPA, in situ zymography was performed in rats treated with tPA 4 hours after MCAO. Increases in PA activity were observed in the ipsilateral subcortex in rats treated with tPA (Figure 2A), and areas with increased PA activity corresponded closely to the hyperintense areas on Gd-DTPA–
Figure 2. PA activity and BBB leakage. PA activity on in situ zymography of coronal sections revealed black zones of substrate lysis in the ipsilateral subcortex (A, arrows), and this area closely corresponded to the hyperintense area on Gd-DTPA–enhanced MRI (B) from a representative rat treated with tPA at 4 hours of the onset of ischemia. Morphological analysis on H&E-stained sections revealed many ghost (loss of hematoxylino-philia, C, arrowheads) and red (pyknosis/eosinophilia, C, arrows) neurons in areas with PA activity, whereas only red neurons (D, arrows) were detected in areas adjacent to PA activity. E, Morphologically intact neurons in the homologous area of the contralateral hemisphere. Panels C through E were obtained from the areas correspondingly labeled (c to e) in panel B. Bar in E=100 μm.

enhanced MRI (Figure 2B). Neurons in areas with PA activity exhibited pyknosis/eosinophilia and loss of hematoxylinophilia (Figure 2C) compared with adjacent areas without PA activity (Figure 2D).

To examine acute effects of MCAO on expression of neuroserpin, immunoreactivity of neuroserpin was measured 2 and 6 hours after ischemia. Neuroserpin immunoreactivity was present in the cortex and subcortex in the nonischemic rats (Figure 3A). Double immunostaining with neuroserpin and MAP2 showed colocalization of these 2 proteins, indicating that neuroserpin is primarily localized to neurons (Figures 3A through 3D). MCAO resulted in an acute decrease of neuroserpin immunoreactivity in the subcortex 2 hours after ischemia (Figures 3E through 3H), and areas with decreased neuroserpin immunoreactivity extended from the subcortex to the cortex during 2 to 6 hours of ischemia (Figures 3I through 3L). Some MAP2 immunoreactive cells (Figures 3F and 3J, arrows) in the ischemic lesion did not show neuroserpin immunoreactivity (Figures 3E and 3I).

Reduction in endogenous neuroserpin and leakage of tPA into parenchyma may exacerbate neuronal damage. To examine whether an increase of exogenous neuroserpin reduces ischemic cell damage after delayed tPA treatment, rats were treated with neuroserpin at 3 hours and tPA at 4 hours after ischemia. In preliminary experiments (n=4), neuroserpin was directly injected into the ipsilateral striatum 3 hours after ischemia, whereas tPA was intravenously administered 4 hours after ischemia. The injection site was observed on diffusion-weighted (Figure 4A, arrow) and Gd-DTPA–enhanced (Figure 4B, arrow) MRI. Neurons in the ipsilateral striatum surrounding the injection site exhibited less ischemic damage (Figure 4E) compared with neurons in the striatum distant from the injection site (Figure 4F) 24 hours after ischemia. However, excessive bleeding induced by the burr hole in the skull after treatment with tPA precluded using this route for administration of neuroserpin.

Dynamic comparison of the ischemic lesion volume assayed on diffusion-weighted MRI measurements between tPA alone (n=7) and tPA in combination with neuroserpin-treated rats (n=7) revealed that treatment with tPA in combination with neuroserpin reduced the ischemic lesion volume by 41% (P<0.05) at 24 hours of the onset of ischemia, from 73.3±3.2% of the ipsilateral hemisphere in the tPA-treated group to 43.3±12.3% in the combination-treatment group, although the ischemic lesion volume was not significantly different between 2 groups 2 hours after onset of ischemia (44.2±6% in the tPA versus 44.4±8% in the combination group, Figures 5A through 5H). Perfusion-weighted MRI showed 34±1% and 35±1% of the ipsilateral hemisphere with 50% reduction in CBF in rats treated with tPA alone and tPA in combination with neuroserpin, respectively, 2 hours after MCAO (Figures 5A through 5H). However, when the ischemic lesion volume was measured on H&E-stained coronal sections from these rats, a significant (P<0.05) reduction of the ischemic lesion volume was detected in rats treated with neuroserpin and tPA compared with rats treated with tPA alone (Figure 6). Furthermore, rats treated with neuroserpin and tPA had a significantly (P<0.05) smaller ischemic lesion volume than ischemic rats.

Figure 3. Double immunofluorescent staining of neuroserpin and MAP2 from the cortex of a representative nonischemic rat (A through D), the ipsilateral striatum of a rat subjected to 2 hours of ischemia (E through H), or the ipsilateral cortex at boundary of ischemic lesion of a rat 6 hours after ischemia (I through L). Neuroserpin (A, E, and I) and MAP2 (B, F, and J) immunoreactivity are presented as red and green colors, respectively. Nuclei were stained by DAPI as a blue color. The combined reactivities are shown in panels D, H, and L. Neuroserpin was colocalized to MAP2 immunoreactive cells in nonischemic cortex (D). Reduction of neuroserpin (E) and MAP2 (F) immunoreactivity was detected in the ipsilateral striatum. However, some MAP2 immunoreactive cells (F and H, arrows) did not show neuroserpin immunoreactivity (E and H, arrows). Neurons in the ischemic boundary of the cortex lost neuroserpin immunoreactivity (I and L, arrows). Bar in L=10 μm.
treated with saline (Figure 6). In situ zymography shows that treatment with tPA in combination with neuroserpin decreased PA activity in the ipsilateral hemisphere (Figure 6B, arrowheads) compared with PA activity in rats treated with tPA alone (Figure 6C, arrowheads).

To examine the effect of exogenous neuroserpin on brain edema, T2-weighted MRI measurements were performed. Treatment with tPA in combination with neuroserpin significantly ($P<0.05$) reduced areas of elevation of $T_2$ (45±10% of the ipsilateral hemisphere) compared with values in tPA treated rats (75±10%) 24 hours after ischemia. In addition, combination treatment significantly ($P<0.05$) decreased hypointense areas (36±9% of the ipsilateral hemisphere) in Gd-DTPA enhanced MRI compared with areas in the tPA group (58±4%).

Discussion

The present study demonstrates that administration of neuroserpin and tPA 3 and 4 hours, respectively, after onset of stroke significantly reduces the ischemic lesion volume and brain edema. Administration of neuroserpin after stroke appears to block the extravascular deleterious effect of tPA and thereby decreases stroke volume and widens the therapeutic window for tPA-induced thrombolysis.

Experimental and clinical studies have demonstrated that treatment with tPA is beneficial when treatment is initiated within 3 hours of the onset of ischemic stroke.2,9,15,16 Recent data obtained from tPA-deficient mice indicate that tPA may...
contribute to excitotoxic neuronal damage after stroke.\textsuperscript{3,4} In contrast, studies from other groups show that mice with tPA deficiency increase ischemic lesion volume and administration of tPA at clinically relevant doses fails to exacerbate ischemic cell damage after global and focal ischemia in the rat.\textsuperscript{5,6} In addition to differences in genetic background and the tPA regimen, these discrepancies could be the result of data obtained from nonembolic models of MCAO, which obscures the beneficial effects of tPA on brain thrombolysis.

In the present study, we used a well-characterized embolic model of MCAO in the rat, which mimics human stroke.\textsuperscript{10} Using this model, we previously demonstrated that administration of tPA 1 hour after ischemia significantly reduced infarct volume and improved neurological outcome, whereas administration of tPA 4 hours after ischemia exacerbated hemorrhagic transformation and infarct volume, which is comparable with the results obtained from treatment of human ischemic stroke with tPA.\textsuperscript{9,10} Detrimental effects of late treatment with tPA may be related to direct neurotoxic effects from tPA.

Neuroserpin is a natural inhibitor of tPA in the brain.\textsuperscript{7} Our observation of constitutive expression of neuroserpin in neurons of the nonischemic rat is consistent with previous studies.\textsuperscript{7,8} MCAO resulted in an acute decrease of neuroserpin in neurons within the ischemic core, which preceded a decrease of MAP-2 immunoreactivity, indicating that a reduction of neuroserpin immunoreactivity is a more sensitive marker of ischemic insult than a decrease in MAP-2 immunostaining. In addition, reduction of endogenous neuroserpin may accelerate neuronal damage, as we have previously demonstrated that administration of neuroserpin immediately after MCAO significantly reduces infarct volume.\textsuperscript{8}

In the blood, endogenous tPA circulates predominantly in a complex with plasminogen activator inhibitor 1 (PAI-1), whereas during thrombolytic therapy exogenous tPA saturates plasma PAI-1 and circulates predominantly as free tPA.\textsuperscript{17} In the brain, tPA activity is primarily regulated by neuroserpin.\textsuperscript{7} Therefore, it is possible that when the BBB breaks down, and once tPA has been administered as a thrombolytic therapy, the circulating free tPA leaks into the parenchyma. Our in situ zymography and Gd-DTPA–enhanced MRI data support this hypothesis by demonstrating increases of PA activity in BBB leakage areas in rats treated with tPA 4 hours after ischemia. Although PA activity in our in situ zymography may result from both tPA and uPA, increased PA activity in the present study is most likely attributable to exogenous tPA, because PA activity is lower in rats without treatment with tPA. Moreover, previous studies have demonstrated that the increase in uPA activity is a late event following MCA occlusion.\textsuperscript{3,14} In addition to exogenous tPA, circulating plasminogen, which has a high concentration in blood (2 \(\mu\)mol/L),\textsuperscript{19} may also leak into the parenchyma. In the absence of, or with lower levels of, endogenous neuroserpin, exogenous tPA in the ischemic parenchyma catalyzes plasminogen into plasmin, and high levels of plasmin may accelerate ischemic neuronal damage.\textsuperscript{18}

In the present study, we hypothesized that an increase in neuroserpin levels reduces ischemic lesion volume by counteracting the extravascular effects of tPA. We tested this hypothesis by administering neuroserpin and tPA 3 and 4 hours, respectively, after onset of ischemia and found that treatment with neuroserpin in combination with tPA significantly reduced ischemic lesion volume compared with tPA treatment alone 4 hours after ischemia. Our perfusion-weighted MRI data show that CBF values were not significantly different between the combination and tPA alone groups before treatment, suggesting that the initial severity of stroke is the same in both groups. This is important because minor differences in the extent of decline of CBF during the 2-hour period of MCAO may result in major differences in infarct volume.\textsuperscript{19} Consistent with CBF data, our dynamic diffusion-weighted MRI data demonstrated that the initial ischemic lesion volumes were relatively the same before treatment. However, evolution of the ischemic lesion was significantly reduced in animals treated with neuroserpin in combination with tPA compared with animals treated with tPA alone. In addition, animals treated with neuroserpin in combination with tPA exhibited less PA activity in the ipsilateral hemisphere. Furthermore, our preliminary data demonstrate that neurons near the neuroserpin injection site exhibited less ischemic damage than neurons at a distant area. Taken together, these data strongly suggest that exogenous neuroserpin significantly reduces ischemic lesion volume in rats treated with tPA 4 hours after stroke.

The present study was not designed to investigate the effects of neuroserpin on parenchymal cells and to tease out the cellular mechanism proceeding neuroprotection from tPA toxicity. Although the mechanisms by which exogenously administered neuroserpin in combination with tPA reduces ischemic lesion is not known, it seems that plasmin is involved because treatment with neuroserpin in combination with tPA significantly reduced BBB leakage and brain edema 24 hours after ischemia. Plasmin activates matrix-metalloproteinase (MMP) 2 and MMP-9\textsuperscript{20} and degrades laminin.\textsuperscript{20} Increased activity of MMP-2 and MMP-9 degrades collagen IV.\textsuperscript{20} Collagen IV and laminin are two major basal lamina constituents of cerebral microvessels.\textsuperscript{21} Degradation of these two proteins could break down the microvascular integrity and increase BBB permeability.\textsuperscript{22,23} Furthermore, plasmin cleaves protease-activated receptor 1, which interacts with thrombin. Thrombin might precipitate brain edema.\textsuperscript{18} Increases of neuroserpin could inhibit tPA activity, reduce plasmin, and consequently decrease BBB leakage and brain edema, which may account for the significantly decreased ischemic lesion. Consistent with our previous results, neuroserpin was morphologically intact in the ischemic region around the site of neuroserpin injected compared with distant regions (Figure 4), suggesting that neuroserpin may reduce degradation of matrix proteins.\textsuperscript{8} In addition, tPA may have direct excitotoxic effects on neurons independent of plasmin.\textsuperscript{4} In neuronal cultures, tPA exacerbates N-methyl-D-aspartate (NMDA) receptor–mediated neurotoxicity through cleavage of the NR1 subunit of NMDA receptor.\textsuperscript{24} Thus, it is possible to postulate that neuroserpin, by blocking the effects of tPA in the brain parenchyma, might prevent NMDA-mediated ischemic neuronal damage.

In summary, our data demonstrate that administration of neuroserpin (3 hours) in combination with tPA (4 hours)
significantly reduced BBB leakage and ischemic lesion volume, suggesting that thrombolytic therapy with tPA in combination with neuroserpin may provide a novel treatment of acute stroke not only by inhibiting the neurotoxic effects of tPA but also by widening the therapeutic window in embolic stroke.

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