An Activated Protein C Analog With Reduced Anticoagulant Activity Extends the Therapeutic Window of Tissue Plasminogen Activator for Ischemic Stroke in Rodents

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Background and Purpose—Tissue plasminogen activator (tPA) is the only approved therapy for acute ischemic stroke. However, tPA has a brief therapeutic window. Its side effects include intracerebral bleeding and neurotoxicity. Therefore, a combination therapy with tPA and agents that can extend the therapeutic window of tPA and/or counteract its side effects are warranted. Here, we studied whether 3K3A-APC, a neuroprotective analog of activated protein C with reduced anticoagulant activity, can enhance the therapeutic effects of tPA in models of ischemic stroke in rodents.

Methods—Human recombinant tPA (10 mg/kg), alone or in combination with human recombinant 3K3A-APC (2 mg/kg), was administered intravenously 4 hours after proximal or distal transient middle cerebral artery occlusion in mice and embolic stroke in rats. The 3K3A-APC was additionally administered for 3 to 4 consecutive days after stroke. The neuropathological and neurological analyses were performed at 1 to 7 days after stroke.

Results—In all models, tPA alone had no effects on the infarct volume or behavior (ie, neurological score, foot-fault, forelimb asymmetry, adhesive removal) compared with vehicle. The tPA and 3K3A-APC combination therapy reduced the infarct volume 24 hours and 7 days after proximal or distal transient middle cerebral artery occlusion in mice and 7 days after embolic stroke in rats by 65%, 63%, and 52%, respectively, significantly (P<0.05) improved behavior and eliminated tPA-induced intracerebral microhemorrhages.

Conclusions—The 3K3A-APC extends the therapeutic window of tPA for ischemic stroke in rodents. Therefore, this combination therapy also should be considered for treating stroke in humans. (Stroke. 2012;43:2444-2449.)

Key Words: ischemic stroke ▪ neuroprotection ▪ proteases ▪ thrombolytic therapy

The majority of strokes are ischemic and thrombotic. The reperfusion therapy with tissue plasminogen activator (tPA) is the only approved therapy for acute ischemic stroke. Still, the rate of recombinant tPA use is unfortunately <4% of stroke patients. Problems with tPA include blood–brain barrier (BBB) disruption causing intracerebral bleeding, a brief therapeutic window, and postischemic neuronal toxicity as shown by several studies using transient middle cerebral artery occlusion model in rodents. Therefore, a combination therapy with tPA and agents that can increase the therapeutic window of tPA and/or protect against tPA-induced intracerebral hemorrhage and neurotoxicity are warranted.

Activated protein C (APC) is a protease with systemic anticoagulant activity that is independent of its cell signaling effects, resulting in a blockade of various pathological pathways in brain endothelium, neurons, and microglia during an acute or chronic central nervous system insult. The cell-signaling actions of APC require activation of protease-activated receptor 1 by proteolytic cleavage of its extracellular N-terminal tail that generates an intramolecular tethered ligand, which subsequently triggers intracellular signaling. Endothelial protein C receptor, sphingosine-1-phosphate receptor-1, and protease-activated receptor-3 also are required for the actions of APC on brain endothelium and neurons, respectively. Mutations of APC residues that are not part of the immediate APC enzymatic active site result in diminished anticoagulant activity of APC without altering the cell signaling activity of APC because, for example, replacement of 3 lysine residues 191 to 193 by 3 alanine residues...
produces the 3K3A-APC variant, causing loss of >90% of the anticoagulant activity of APC.\textsuperscript{10,11} The significance of such APC-engineered mutations is that they provide APC variants for therapeutic purposes in which the risk of serious bleeding caused by the systemic anticoagulant activity of APC is diminished, whereas the cytoprotective activities of the direct effects of APC on cells and its pharmacological benefits are preserved.

APC and its cell-signaling recombinant analogs with reduced anticoagulant activity, including 3K3A-APC and 5A-APC, exert direct vascular-protective effects in the brain endothelium\textsuperscript{12–16} and can enhance integrity of the endothelial barrier.\textsuperscript{17,18} Moreover, APC, 3K3A-APC, and 5A-APC cross the BBB via endothelial protein C receptor–dependent transcytosis\textsuperscript{19–21} and have direct neuronal protective actions.\textsuperscript{20,22,23} By inhibiting transporting of leukocytes across the BBB\textsuperscript{24} and by suppressing microglia activation,\textsuperscript{20} APC therapy exerts a significant anti-inflammatory activity.

APC is neuroprotective in multiple models of stroke in mice and rats, including transient brain ischemia,\textsuperscript{2,8,14,24,25} permanent distal middle cerebral artery occlusion (MCAo),\textsuperscript{23,26} embolic stroke,\textsuperscript{27} and neonatal hypoxic/ischemic brain injury.\textsuperscript{28} After proximal transient and distal permanent MCAo in mice, the 3K3A-APC variant with reduced anticoagulant activity had some advantages over the recombinant wild-type APC, including reduced risk for bleeding that was particularly noticeable when treatments were administered at later time points after MCAo.\textsuperscript{23,26} In the present study, we asked whether 3K3A-APC can enhance tPA therapy for focal ischemic stroke in mice and rats.

Materials and Methods

Reagents

Human recombinant tPA (alteplase) was purchased from Genentech (South San Francisco, CA). Human recombinant 3K3A-APC was prepared in Chinese hamster ovary cells and manufactured by the Laureate Biopharmaceutical (Princeton, NJ, contracted by ZZ Bio-tech). Methods for the generation, purification, and characterization of recombinant 3K3A-APC have been described (Williams, Zlokovic, Griffin, Pryor, and Davis, unpublished data, 2012). Based on analysis of 3K3A-APC using nonreduced and reduced SDS polyacrylamide gels, the protein showed the stained bands typical for disulfide-linked heavy chain and light chain, characteristic of APC. SDS gel analysis and size exclusion chromatography showed that the 3K3A-APC used for these studies was >96% pure (data not shown).

Animals

The protocol in mice was approved by the Animal Care Committee at the University of Rochester in compliance with the National Institutes of Health guidelines. Male C57Bl6 mice (22–26 g; Jackson Laboratory, Bar Harbor, ME) were anesthetized intraperitoneally with 100 mg ketamine/10 mg xylazine per kg body weight. Rectal temperature was maintained between 36.5°C and 37.0°C using a feedback-controlled heating system. The protocol in rats was approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital in compliance with the National Institutes of Health guidelines. Adult male Wistar rats (Jackson Laboratory, Bar Harbor, ME) 8 to 12 weeks of age and weighing 300 to 350 g were anesthetized with 3% isoflurance, and the anesthesia was maintained with 1.0% to 1.5% isoflurance. Rectal temperature was maintained at 37.0°C ± 0.5°C using a feedback-regulated water heating system.

Proximal Transient MCAo in Mice

The MCA was occluded for 45 minutes using a silicon-coated nylon monofilament (DOCCOL CO) as described.\textsuperscript{2,8,14} The tPA alone (10 mg/kg, 10% as a bolus and 90% as a 30-minute infusion), tPA (10 mg/kg, infused as noted) and 3K3A-APC (2 mg/kg, 50% bolus/50% 30-minute infusion), 3K3A-APC alone (2 mg/kg, infused as noted), or vehicle were administrated intravenously 4 hours after stroke. For combination therapy, tPA was administered via the femoral vein and 3K3A-APC was administered via the tail vein. Cerebral blood flow was monitored by laser Doppler flowmetry (Transonic Systems). Motor neurological examination was determined after 24 hours,\textsuperscript{2,8,14} no neurological deficit, 0; failure to extend left forepaw fully, 1; turning to left, 2; circling to left, 3; unable to walk spontaneously, 4; and stroke-related death, 5. Mice were euthanized 24 hours after the MCAo. It is of note that the dose of tPA of 10 mg/kg used in this and other studies described is equivalent to a therapeutic dose of tPA of 1 mg/kg in humans.\textsuperscript{29–31} whereas the dose of human recombinant 3K3A-APC of 2 mg/kg was shown to provide maximal neuroprotection in different stroke models in mice.\textsuperscript{26,32}

Distal Transient MCAo in Mice

Distal transient MCAo was performed using a modified technique as reported.\textsuperscript{33,34} The right common carotid arteries were transiently occluded for 20 minutes and the MCA was occluded for 60 minutes. The tPA only (10 mg/kg, 10% as a bolus and 90% as a 30-minute infusion), tPA (10 mg/kg, infused as noted) and 3K3A-APC (2 mg/kg, 50% bolus/50% 30-minute infusion), 3K3A-APC alone (2 mg/kg, infused as noted), and vehicle were administrated intravenously 4 hours after stroke. When tPA and 3K3A-APC were administered together, tPA was administrated via the femoral vein and 3K3A-APC was administered via the tail vein. 3K3A-APC (2 mg/kg, intraperitoneally) was additionally administrated at 1, 3, 5, and 7 days after stroke. Foot-fault test\textsuperscript{26,27} and forelimb asymmetry test\textsuperscript{26,27} were performed at 0, 1, 3, and 7 days after the MCAo. Mice were euthanized 7 days after the MCAo.

Focal Embolic Stroke in Rats

The MCA of male Wistar rats was occluded by placement of an embolus at the origin of the MCA, as described.\textsuperscript{35} Four hours after stroke, tPA (10 mg/kg) was infused as a 10% bolus through the tail vein, and the remainder was infused continuously over a 30-minute interval. The 3K3A-APC (2 mg/kg) was administered intravenously 4 hours after stroke as a single bolus (100 µL). The 3K3A-APC was additionally injected intravenously for 3 consecutive days. A modified neurological severity score, a composite of motor, sensory, reflex, and balance tests (no deficit, score 0; maximal deficit, score 18),\textsuperscript{36} adhesive removal test for sensorimotor activity,\textsuperscript{37} and foot-fault test for locomotor assessment\textsuperscript{37} were performed 1 and 7 days after stroke. Rats were euthanized 7 days after stroke.

Neuropathological Analysis

The injury volumes were measured on coronal sections using either cresyl violet staining (mice) or hematoxylin and eosin staining (rats), as described previously.\textsuperscript{38,39} The infract test for locomotor assessment\textsuperscript{26,27} were performed at 0, 1, 3, and 7 days after the MCAo. Mice were euthanized 7 days after stroke.

Hemoglobin Assay

Hemoglobin levels were determined by a spectrophotometric assay using Drabkin reagent (Sigma).\textsuperscript{2,40}

Microscopic Hemorrhage

Microscopic hemorrhage, defined as a cluster of red blood cells outside of the lumen of blood vessels, was measured under a ×40 objective with a Global Laboratory Image (Data Translation, Marlboro, MA) analysis; the area of hemorrhage (um²/section) was calculated as described.\textsuperscript{27,35}
human recombinant 3K3A-APC (2 mg/kg) alone was neuro-
protective, as shown by 57% reduction in the infract volume
and improved neurological score from 2.7 to 1.2 compared
with vehicle, respectively.

As shown in Figure 2A–C, in mice subjected to 60 minutes
distal transient MCAo, tPA (10 mg/kg) alone compared with
vehicle did not improve performance on foot-fault and
forelimb asymmetry tests at days 1, 3, and 7 after stroke,
neither reduced the infract size 7 days after stroke. In contrast,
tPA and 3K3A-APC combination compared with tPA alone
improved significantly performance on foot-fault and fore-
limb asymmetry tests at days 1, 3, and 7 after stroke, and
reduced the infract volume by 63% as well as edema volume
(not shown). The tPA alone increased hemoglobin levels in
the ischemic hemisphere by 2.1-fold (Figure 2D), which was
normalized by adding 3K3A-APC. The 3K3A-APC alone
reduced the infarct volume by 63% as well as edema volume
compared with vehicle, respectively.

As in other models, tPA (10 mg/kg) standalone therapy
compared with vehicle had no effects on modified neurolog-
ical severity score, foot-fault test, and adhesive removal test
at day 1 or day 7 after embolic stroke in rats (Figure 3A–C).
In contrast, tPA and 3K3A-APC combination therapy significantly \((P<0.05)\) improved performance on all studied behavioral tests both on days 1 and 7 after stroke. The 3K3A-APC alone modestly improved performance on behavioral tests compared with vehicle or tPA, but the differences were not significant except for the foot-fault test. The tPA alone did not have any effect on the infarct volume determined 7 days after stroke, whereas tPA and 3K3A-APC combination therapy compared with tPA alone reduced the infarct volume by 53% (Figure 4A). The 3K3A-APC reduced the infarct volume by \(\approx30\%\) compared with tPA alone. The tPA alone increased by 3-fold the microscopic hemorrhage compared with vehicle (Figure 4B), as reported,21 which was completely normalized by 3K3A-APC.

**Discussion**

Thrombolytic therapy for acute ischemic stroke with tPA has clear benefits if administered within a narrow therapeutic window.1,3–5 However, side effects of tPA treatment such as intracerebral hemorrhage limit its use in humans.3,5 Studies in animal models have demonstrated serious side effects of tPA in the ischemic brain, including BBB breakdown,2,41,42 intracerebral bleeding if tPA is administered 3 to 4 hours after either transient MCAo or embolic stroke,2,30,43 and direct postischemic neuronal toxicity after transient MCAo.6–8 Previous reports have shown that tPA reduces neurological damage after cerebral embolism44,45 and does not exacerbate brain injury after global or focal ischemia.46 However, a recent study using a model of angiographically documented recanalization of the rabbit MCAo has shown that tPA produces bleeding at all doses in proportion to its thrombolytic potential.47 Taken together, these studies suggest that beneficial or detrimental effects of tPA therapy for stroke critically depend on the time of its administration after the MCAo embolism.48

Figure 3. Effects of combined tissue plasminogen activator (tPA) and 3K3A-APC treatment on behavior in rats after embolic stroke. The tPA (10 mg/kg) and human 3K3A-APC (2 mg/kg) were administered intravenously 4 hours after embolic stroke. The 3K3A-APC (2 mg/kg) was additionally injected intravenously for 3 consecutive days. Modified neurological severity score (mNSS) (A), foot-fault test (B), and adhesive removal test (C) were performed at days 1 and 7 after stroke. All values are mean\(\pm\)standard error of the mean (SEM), \(n=10\) rats per group.

Consistent with previous findings, the present study confirmed that tPA treatment administered 4 hours after either proximal or distal transient MCAo in mice or embolic stroke in rats is not neuroprotective, as indicated by no changes in performance on multiple behavioral tests at days 1 to 7 after stroke, no change in the infarction volume, and increased risk for intracerebral bleeding compared with vehicle. However, when tPA was administered in combination with 3K3A-APC, the combined therapy exerted a remarkable neuroprotection in all studied models.

Consistent with previous findings demonstrating that APC treatment in addition to being neuroprotective is also vasculo-protective4,12–16 and can stabilize the endothelial barriers,17,18 including the BBB,2,20 we showed that 3K3A-APC eliminates tPA-induced intracerebral microbleedings. The tPA induces postischemic brain hemorrhage by activating nuclear factor \(\kappa\)B–dependent matrix metalloproteinase-9 pathway at the BBB, which is inhibited by APC both in mice and rats, as reported.2 Reduced systemic anticoagulant activity of APC mutants such as 3K3A-APC or 5A-APC by 85% to 90% and >95% compared with wild-type APC, respectively, has been shown to contribute to reduced risk of bleeding observed with these APC mutants compared with wild-type APC in models of ischemic stroke,23 brain trauma,31 and sepsis.49 Interestingly, human recombinant tPA and 3K3A-APC alone had only moderate or no beneficial effects in the rat model of embolic stroke, whereas the combination of the 2 drugs exerted clearly significant synergistic effects on all studied parameters. In conclusion, the present data show that 3K3A-
APC extends the therapeutic window of tPA in ischemic stroke models in rodents, supporting further development of tPA and 3K3A-APC combination therapy for focal ischemic stroke in humans.

Sources of Funding
This work was supported by the National Institutes of Health grant HL63290 (B.V.Z.), HL052246 (J.H.G.), and ZZ Biotech LLC. ZZ Biotech LLC also provided human 3K3A-APC mutant.

Disclosures
Dr Zlokovic is the scientific founder of ZZ Biotech, a biotechnology company with a focus to develop APC and its functional mutants for stroke and other neurological disorders. Dr Davis and Dr Griffin are members of the Scientific Advisory Board of ZZ Biotech LLC.

References


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Stroke. 2012;43:2444-2449; originally published online July 17, 2012; doi: 10.1161/STROKEAHA.112.658997

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