Differential Neuroprotection and Risk for Bleeding From Activated Protein C With Varying Degrees of Anticoagulant Activity

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Background and Purpose—Activated protein C (APC), a protease with anticoagulant and cytoprotective activities, protects neurons and endothelium from ischemic injury. Drotrecogin-alfa activated, a hyperanticoagulant form of human recombinant APC, is currently being studied in patients with ischemic stroke. How changes in APC anticoagulant activity influence APC’s neuroprotection and risk for bleeding is not clear.

Methods—We used neuronal and brain endothelial cell injury models and middle cerebral artery occlusion in mice to compare efficacy and safety of drotrecogin-alfa activated and human 3K3A-APC, an APC nonanticoagulant mutant.

Results—Drotrecogin-alfa activated and 3K3A-APC exhibited 148% and 10% of plasma-derived APC’s anticoagulant activity and differ in the carbohydrate content. 3K3A-APC protected mouse neurons from \textit{N}-methyl-D-aspartate-induced apoptosis and human brain endothelial cell from oxygen-glucose deprivation with 1.8- and 3.1-fold greater efficacy than drotrecogin-alfa activated. Given 5 minutes before transient middle cerebral artery occlusion, 3K3A-APC and drotrecogin-alfa activated (0.5 and 2 mg/kg intravenously) reduced comparably and dose-dependently the infarction lesion up to 85%. 3K3A-APC, but not drotrecogin-alfa activated, improved neurological score dose-dependently (\textit{P}<0.05). 3K3A-APC did not cause bleeding. In contrast, drotrecogin-alfa activated dose-dependently increased hemoglobin content in postischemic brain. After permanent middle cerebral artery occlusion, 3K3A-APC multidose therapy (1 mg/kg intravenously at 12 hours and 1, 3, 5, and 7 days) improved functional recovery and reduced infarction by 60% with no risk for bleeding, whereas drotrecogin-alfa activated induced increased hemoglobin deposition in the postischemic brain and showed relatively modest neuroprotection.

Conclusions—Nonanticoagulant 3K3A-APC exhibits greater neuroprotective efficacy with no risk for bleeding compared with drotrecogin-alfa activated, a hyperanticoagulant form of APC. (\textit{Stroke}. 2009;40:1864-1869.)

Key Words: anticoagulant activity ■ brain ischemia ■ intracerebral microhemorrhage ■ neuroprotection ■ proteases

A ctivated protein C (APC) is a serine protease with systemic anticoagulant, anti-inflammatory, and antiapoptotic activities.\textsuperscript{1} Its anticoagulant activity is independent of its cellular effects. APC is generated from its zymogen protein C that is activated by thrombin on the surface of the endothelial cells, which requires 2 membrane receptors, thrombomodulin and endothelial protein C receptor. APC’s anticoagulant activity is mediated by irreversible proteolytic degradation of factors Va and VIIIa with contributions by various cofactors, whereas its cytoprotective activities are mediated by proteolytic activation of protease activated receptor 1.\textsuperscript{1} APC’s cellular signaling results in cytoprotective alterations of gene expression profiles, anti-inflammatory activity, and antiapoptotic activity.\textsuperscript{2–6} APC protects neurons and brain endothelial cells from ischemic injury and divergent inducers of apoptosis by inhibiting the mitochondria-mediated and death receptor-mediated apoptotic pathways.\textsuperscript{4,7–9} Early posts ischemic administration of APC within 4 hours of an ischemic insult is neuroprotective in rodent models of transient ischemia\textsuperscript{4,10} and embolic stroke.\textsuperscript{11} APC also protects against diab etic endothelial and glomerular injury\textsuperscript{12} and in animal models of sepsis, ischemia–reperfusion injury of kidney and lung, and thrombosis models.\textsuperscript{13} Regarding central nervous system disorders, APC is protective in rodent models of spinal cord injury,\textsuperscript{14} amyotrophic lateral sclerosis,\textsuperscript{15} and multiple sclerosis.\textsuperscript{16} A form of human recombinant APC with increased anticoagulant activity, ie, xigris (drotrecogin-alfa acti-
vated), has been approved by the US Food and Drug Administration for use in patients with severe sepsis.17 Drotrecogin-alfa activated is currently being studied in patients with ischemic stroke (The Activated Protein C in Acute Stroke Trial [APCAST]; http://clinicaltrials.gov/ct2/show/NCT00533546?term=apc&rank=25). The Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) trial for APC infusion in patients with severe sepsis suggested low but significant risk for bleeding, including increased risk for intracerebral hemorrhage.17 Moreover, at higher pharmacological doses, APC is toxic to nonhuman primates and produces disseminated bleeding (see www.emea.europa.eu/humandocs/PDFS/EPAR/xigris/247102en6.pdf).

How changes in APC anticoagulant activity influence APC’s neuroprotection and risk for intracerebral bleeding is not clear. To address this question, we compared drotrecogin-alfa activated with an APC nonanticoagulant mutant containing 3 alanine substitutions for 3 protease domain positively charged lysines (KKK191–193AAA) designated as 3K3A-APC.18 This mutation alters factor Va binding exosite in APC resulting in greatly reduced anticoagulant activity, ie, >90%,18 but does not affect exosites that recognize endothelial protein C receptor or protease activated receptor 1, which are important for APC’s cellular activity, resulting in normal antiapoptotic activity in a model of staurosporine-induced apoptosis in human umbilical vein cells.19 Here, we show that nonanticoagulant 3K3A-APC mutant exerts enhanced neuroprotective activity and eliminates risk for intracerebral bleeding that has been seen with drotrecogin-alfa activated, a hyperanticoagulant form of APC.

Methods

Reagents

Human 3K3A-protein C (3K3A-PC) stable cell line was generated in Chinese hamster ovary (CHO) cells. The cells were grown in suspension in CD OptiCHO medium (Invitrogen) containing 2 mmol/L CaCl2, 10 μg/mL vitamin K, and 2 mmol/L GlutaMAX (Invitrogen) in a 2-L BioWave bioreactor for production. A 4-step purification procedure was used: capturing PC using FQF resin (GE Health), purification of PC using Uno Q column (BioRad), activating with recombinant human thrombin (Zymogenetics), and removal of thrombin using Uno Q column. The purity of 3K3A-APC was determined by reduced SDS-PAGE/silver staining, the enzymatic activity by amidolytic assay, and the concentration by enzyme-linked thrombin using Uno Q column. The purity of 3K3A-APC was 90%,18 but does not affect exosites that recognize endothelial protein C receptor or protease activated receptor 1, which are important for APC’s cellular activity, resulting in normal antiapoptotic activity in a model of staurosporine-induced apoptosis in human umbilical vein cells.19 Here, we show that nonanticoagulant 3K3A-APC mutant exerts enhanced neuroprotective activity and eliminates risk for intracerebral bleeding that has been seen with drotrecogin-alfa activated, a hyperanticoagulant form of APC.

Activated Partial Thromboplastin Time Assay

This assay was accomplished as previously described.19 Briefly, 50 μL of protein C-deficient plasma (American Diagnostica) was mixed with 50 μL Automated APTT Reagent (Biomerieux) and incubated at 37°C for 1 minute. Human plasma-derived APC, drotrecogin-alpha activated, and 3K3A-APC were diluted into buffer (30 mmol/L HEPES, 150 mmol/L NaCl, and 0.3% BSA) and added to the mixture at 30 and 50 nM in a volume of 25 μL and incubated for 3 minutes at 37°C. The clotting was then initiated with the addition of 50 μL of 50 mmol/L CaCl2. Clotting time was measured by the Start4 Coagulometer (Diagnostica Stago). All samples were tested in duplicate.

Carbohydrate Analysis

Sialic Acid Analysis

Sialic acids (glycoly and acetyl) were hydrolyzed from 3K3A-APC and drotrecogin-alpha activated, labeled with a fluorescent dye orthophenylenediamine, and analyzed using reversed-phase high-pressure liquid chromatography (high-performance liquid chromatography) coupled with fluorescence detection.

N-linked Glycan Profiling

To obtain oligosaccharide profiles for 3K3A-APC and drotrecogin-alfa activated, sugar moieties were removed from protein backbone with N-glycanase and labeled with 2-aminobenzamide and analyzed by high-performance liquid chromatography coupled with fluorescence detection.

Mouse Neuronal Cell Cultures

Primary neuronal cultures were established as described previously.20 In brief, cerebral cortex was dissected from fetal CS7BL6 mice (Charles River Laboratories, Inc; Wilmington, Mass) at 16 days of gestation, treated with trypsin for 10 minutes at 37°C, and dissociated by trituration. Dissociated cell suspensions were plated at 5 x 106 cells per well on 12-well tissue culture plates or at 4 x 106 cells per dish on 60-mm tissue culture dishes coated with poly-L-lysine, in serum-free Neurobasal medium plus B27 supplement (Gibco; Rockville, Mass). The medium suppresses glial growth to <2% of the total cell population. Cultures were maintained in a humidified 5% CO2 incubator at 37°C for 7 days before treatment. Medium was replaced every 3 days.

N-methyl-d-aspartate-Induced Apoptosis in Neuronal Culture

For induction of neuronal apoptosis, cultures were exposed for 10 minutes to 300 μmol/L NMDA/5 μmol/L glycine in Mg2+-free Earle’s balanced salt solution.20 Control cultures were exposed to Earle’s balanced salt solution alone. After the exposure, cultures were rinsed with Earle’s balanced salt solution, returned to the original culture medium, and incubated with or without different concentrations of drotrecogin-alfa activated (1 to 50 nM) and human 3K3A-APC (1 to 50 nM). All experiments involving treatment with APC included hirudin (Sigma) to block any thrombin signaling.13,21

Human Brain Endothelial Cell Cultures

Primary brain endothelial cells (BECs) were isolated from specimens (<3 hours) from neurologically healthy, young individuals after surgery for epilepsy. BECs were characterized and cultured as we previously described.4

Oxygen–Glucose Deprivation in Human Brain Endothelial Cells

Cells were maintained in serum-free Dulbeco’s modified Eagle medium and exposed for 8 hours to oxygen–glucose deprivation (OGD; <2% oxygen, no glucose). Hypoxia was induced using an anaerobic chamber (Forma Scientific; Holbrook, NY).4 The levels of O2 were monitored by O2 Fyrite (Forma Scientific). Different concentrations of drotrecogin-alpha activated (3 to 100 nM) and human 3K3A-APC (3 to 100 nM) were added at the time of OGD treatment. Hirudin was included to block any thrombin signaling.13,21

Detection of Cell Survival and Cell Injury

Cell survival in mouse neuronal cultures was detected by water-soluble tetrazolium WST-8 assay (Dojindo Molecular Technologies; Gaithersburg, Md). Cell injury in BEC was detected by the release of lactic acid dehydrogenase (LDH assay, Sigma).
Transient Middle Cerebral Artery Occlusion
All procedures were done according to the National Institutes of Health guidelines for animal care and approved by the Institutional Animal Care and Use Committee at the University of Rochester. Male C57BL6 mice, 6 to 8 weeks old (Charles River Laboratories; Wilmington, Mass) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. We used a 1-hour transient middle cerebral artery occlusion (MCAO) suture model as previously reported \[4,10\] followed by 24-hour reperfusion. Physiological parameters (ie, blood gasses, pH, hematocrit, arterial blood pressure) were monitored as described. \[2,22\] Rectal temperature was kept within normal limits during the MCAO surgical procedure. Mice were randomly assigned to receive 5 minutes before ischemia either vehicle or human recombinant 3K3A-APC (0.4 or 2 mg/kg through the femoral vein) or drotrecogin-alfa activated (0.5 or 2 mg/kg intravenously). Motor neurological deficit was scored on a 5-point scale 24 hours after ischemia: no neurological deficit = 0; failure to extend left forelimb fully = 1; circling to the left = 2; falling to the left side = 3; unable to walk = 4; and stroke-related death = 5. After 24 hours, mice were euthanized and brains isolated and prepared for analysis. Coronal brain sections (1-mm thick) were cut and incubated with 2% 2,3,5-triphenyltetrazolium chloride solution in a phosphate-buffered saline at 37°C as reported. \[4,10\] The injury volume (cubed millimeters) was calculated by multiplying the surfaces of all injured areas in square millimeters by the thickness of brain sections. The infarction volume was obtained by subtracting the edema volume from the injury volume. Edema volume (tissue swelling) was calculated by subtracting the volume of the contralateral nonischemic hemisphere from the volume of the ipsilateral ischemic hemisphere as described. \[2\]

Permanent Distal Middle Cerebral Artery Occlusion
All procedures were approved by the Institutional Animal Care and Use Committee at the University of Rochester. Male C57BL6 mice, 6 to 8 weeks old, were anesthetized with ketamine (100 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally). Animals were allowed to breathe spontaneously. Rectal temperature was maintained at 37°C using a feedback-controlled heating system. The right femoral artery was cannulated for monitoring of blood pressure and blood analysis. A modified permanent distal MCAO technique was used. Under the surgical microscope, the left common carotid artery was isolated through a neck incision and ligated using a 5–0 silk. A skin incision was made between the right orbit and tragus. The zygomatic arch was removed and temporal muscle retracted. The dura was carefully opened and the M1 branch of the middle cerebral artery was visible through the temporal semitranslucent surface of the skull. Cranietomy was performed by drilling with a 0.9-mm round burr. The inner layer of the skull was removed with fine forceps. The dura was carefully opened and the M1 branch of the middle cerebral artery exposed and coagulated using a cauterizer, producing permanent distal MCAO. The wound was sutured, and rectal temperature was controlled until mice regained full consciousness.

Mice were randomly assigned to the vehicle-treated group, the 3K3A-APC-treated group and drotrecogin-alfa activated-treated group. 3K3A-APC and drotrecogin-alfa activated were administered by tail vein at 1 mg/kg at 12 hours and 1, 3, 5, and 7 days poststroke. Vehicle-treated group received saline. At days 1, 3, and 7 postischemia, the forelimb asymmetry test for sensorimotor activity \[23\] and foot-fault test for locomotor assessment \[24\] were studied at 25 and 50 nM. Values are mean ± SEM, n = 3 independent experiments. B, SA content in drotrecogin-alfa and 3K3A-APC preparations used in the present study. SA analysis was performed with high-performance liquid chromatography-fluorescence detection as described in “Methods.” N-acetyl-SA and N-glycolyl-SA are expressed in moles SA per mole APC. C, N-linked oligosaccharide profiles of drotrecogin-alfa and 3K3A-APC preparations used in the present study. The N-linked glycan maps were obtained using high-performance liquid chromatography–fluorescence detection as described in “Methods.” The overlay of the 2 chromatograms is shown. The relative peak quantification was determined by percent fluorescence (%F) for each APC preparation.

staining, the hemisections of the ischemic hemisphere were homogenized and treated with the Drabkin’s reagent (Sigma) to determine hemoglobin content. Bovine erythrocyte hemoglobin or mouse blood added to brain homogenates was used for standard curves.

Statistical Analysis
Data were presented as mean ± SEM. Student t-test and analysis of variance was used to determine statistically significant differences. P < 0.05 was considered statistically significant.

Results
Activated partial thromboplastin time values indicated that drotrecogin-alfa activated compared with plasma-derived
APC has by 48% increased anticoagulant activity, whereas 3K3A-APC has approximately 10% of plasma-derived APC anticoagulant activity (Figure 1A). The sialic acid (SA) analysis revealed that 3K3A-APC has 8.8 moles of SA per mole of APC, ie, 8.2 moles of acetyl forms and 0.6 moles of glycolyl forms, whereas drotrecogin-alfa activated had 8.1 moles of SA per mole of protein, or 7.6 moles of acetyl forms and 0.5 moles of glycolyl forms. The comparison of the released oligosaccharides showed that 3K3A-APC and drotrecogin-alfa activated have different N-linked glycan profiles and the content of N-linked glycans was significantly higher in 3K3A-APC (Figure 1B).

3K3A-APC was more potent than drotrecogin-alfa activated in protecting mouse cortical neurons from NMDA-mediated injury as indicated by a shift to the left of its respective dose–response curve (Figure 2A). The IC_{50} values, ie, the inhibitory concentrations corresponding to the half maximal protection effect, for drotrecogin-alfa activated and 3K3A-APC were 7.66 and 12.8 nM, respectively (Figure 2B), indicating 1.8-fold greater efficacy of 3K3A-APC. In the OGD model in human BEC, the 3K3A-APC protection curve similarly shifted to the left compared with the drotrecogin-alfa activated curve (Figure 3A). The respective IC_{50} values for drotrecogin-alfa activated and 3K3A-APC were 24.1 and 7.5 nM (Figure 3B), indicating 3.2-fold greater efficacy of 3K3A-APC.

During the MCAO surgical procedure, physiological variables (ie, PaO$_2$, Paco$_2$, pH, hematocrit, arterial blood pressure, rectal temperature) remained within normal limits compared with basal values (data not shown) as reported.4,10 Transient MCAO resulted in approximately 80% reduction in the cerebral blood flow compared with the preocclusion baseline values. During reperfusion, the cerebral blood flow recovered to 85% to 87% of the baseline values (not shown) as reported.10

Applied 5 minutes before 1-hour transient MCAO, 3K3A-APC and drotrecogin-alfa activated exhibited comparable dose–response reductions in the infarction volume at 24 hours (Figure 4A). At a higher dose, 3K3A-APC, but not drotrecogin-alfa activated, reduced significantly tissue swelling (edema; Figure 4B). Moreover, 3K3A-APC exhibited a dose-dependent reduction in motor neurological scores, whereas drotrecogin-alfa reduced neurological score only at a higher dose, but still significantly below 3K3A-APC (Figure 4C). The greatest difference between the 2 APC preparations was seen in hemoglobin accumulation within the ischemic hemisphere. Although 3K3A-APC did not increase hemoglobin levels at either concentration, there was a dose-dependent increase in hemoglobin content in the ischemic hemisphere with drotrecogin-alfa activated (Figure 4D), suggesting microbleeding. Microhemorrhages were also confirmed by counting clusters of red blood cells outside the vessels (data not shown) as reported.11

Finally, 2 APC preparations were administered as a multidose therapy (1 mg/kg intravenously) at 12 hours and 1, 3, 5, and 7 days after permanent distal MCAO. 3K3A-APC had superior effects both in terms of functional recovery, as shown by significantly better performance on the foot-fault...
test and forelimb asymmetry test (Figure 5A–B), as well as in significantly greater reduction in the infarction volume, ie, 60% compared with 25% of control (vehicle-treated) mice for 3K3A-APC and drotrecogin-alfa activated, respectively (Figure 5C). 3K3A-APC did not increase the risk for bleeding as shown by no increase in hemoglobin content in the ischemic hemisphere (Figure 5D) as well as by the absence of red blood cell extravasation (data not shown). In contrast, drotrecogin-alfa activated doubled hemoglobin accumulation in the ischemic brain (Figure 5D) suggesting significant postischemic microhemorrhages.

Discussion

The present study shows superior neuroprotective effects of human nonanticoagulant 3K3A-APC mutant compared with drotrecogin-alfa activated, a hyperanticoagulant form of human recombinant APC. 3K3A-APC was more efficacious in all models studied, including in vitro cultured mouse cortical neurons challenged by NMDA and human BEC subjected to OGD and in vivo models of transient MCAO and permanent distal MCAO in mice. 3K3A-APC’s neuroprotection was not associated with a risk of intracerebral bleeding in vivo. In contrast, treatment with drotrecogin-alfa activated resulted in a dose-dependent postischemic accumulation of hemoglobin in the lesioned hemisphere. Drotrecogin-alfa activated reduced the infarct lesion after transient MCAO to the degree comparable to that seen with 3K3A-APC, but its effect on motor neurological score was very modest and likely due to accumulations of hemoglobin-derived neurotoxic products in ischemic tissue that cause damage through oxidant stress as reported.

The anticoagulant action of APC mainly depends on its binding to factor Va, which is a prerequisite for APC-mediated inactivation of factor Va by proteolytic cleavage at Arg506. Factor Va binding to APC depends on positively charged residues in the surface loops on APC’s protease domains, including loop 37 (residues 190 to 193), the Ca²⁺-binding loop (residues 225 to 235), and the autolysis loop (residues 301 to 316). Substitution of positively charged lysine residues with 3 alanine residues in loop 37 (KKK191-193AAA) decreases APC’s anticoagulant activity by >90% and reduces the risk for bleeding through reductions in both APC’s anticoagulant and APC-dependent profibrinolytic activities. Reduced risk for bleeding has been confirmed in vivo in different models of sepsis and now in murine models of stroke. It is of note, the KKK191-193AAA mutation in 3K3A-APC leaves intact the N-terminal Gla domain of APC, which is important for its binding to endothelial protein C receptor on the endothelial cells, and does not alter the protease catalytic site that is responsible for activation of protease activated receptor 1.

The anticoagulant activity of APC is also influenced by its glycosylation profile, which primarily depends on the content of negatively charged sialic acid forms and distribution and branching of the N-linked glycan species. HEK 293-derived drotrecogin-alfa activated (human protein C; zigris) has intact factor Va and VIIIa binding sites, but compared with plasma-derived APC contains 50% less sialic acid forms and the N-linked oligosaccharides are less branched. It has been suggested that the reduced glycosylation pattern in HEK 293-derived drotrecogin-alfa activated may influence APC binding to factors Va and VIIIa and/or other cofactors resulting in its higher anticoagulant activity.

3K3A-APC was more potent than drotrecogin-alfa activated in protecting neurons and brain endothelial cells from injury in vitro, which must be attributed to structural differences between the 3K3A-APC mutant and drotrecogin-alfa activated, including the KKK191-193AAA mutation in loop 37, and significantly higher content of N-linked glycan species and sialic acid forms. Although the exact molecular mechanism for the enhanced efficacy of the mutant compared with drotrecogin-alfa activated remains to be explored, one might speculate that an enriched glycosylation profile in 3K3A-APC would either facilitate proteolytic activation of protease activated receptor 1 and/or 3K3A-APC binding to endothelial protein C receptor on endothelium, resulting in enhanced antiapoptotic signaling in injured neurons and vascular endothelium as reported.

In summary, our data suggest that nonanticoagulant 3K3A-APC mutant has important advantages over hyperanticoagulant drotrecogin-alfa activated as a neuroprotective agent in mouse models of stroke, including significantly greater efficacy and no risk for bleeding.

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Disclosures

B.V.Z. is the scientific founder of Socratech LLC and its subsidiary ZZ Biotech, startup biotechnology companies with a focus to develop therapeutics for Alzheimer disease and stroke, including therapies based on APC and its functional mutants. B.V.Z. serves as a Chief Scientific Officer on these 2 companies and owns shares in both Socratech LLC and ZZ Biotech, but does not receive salary support. T.P.D. is CEO of ZZ Biotech and receives financial compensation.

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