Tissue-Type Plasminogen Activator-A296–299 Prevents Impairment of Cerebral Autoregulation After Stroke Through Lipoprotein-Related Receptor–Dependent Increase in cAMP and p38

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**Background and Purpose**—The sole Food and Drug Administration–approved treatment for stroke is tissue-type plasminogen activator (tPA), but its brief therapeutic window and complications of treatment constrain its use. One limitation may be its potential to exacerbate impairment of cerebral autoregulation after stroke. Vasodilation is maintained by elevations in cAMP. However, cAMP levels fall after stroke because of overactivation of N-methyl-D-aspartate receptors by toxic levels of glutamate, an effect that is exacerbated by tPA. Binding of wild-type (wt) tPA to the low-density lipoprotein–related receptor (LRP) mediates dilation. We propose that binding of wt-tPA to N-methyl-D-aspartate receptor reduces cAMP and impairs vasodilation. We hypothesize that tPA-A296–299, a variant that is fibrinolytic but cannot bind to N-methyl-D-aspartate receptor, preferentially binds to LRP and increases cAMP and p38, limiting autoregulation impairment after stroke.

**Methods**—Stroke was induced by photothrombosis in pigs equipped with a closed cranial window, cerebral blood flow determined by microspheres, and cerebrospinal fluid cAMP and p38 determined by ELISA.

**Results**—Stroke decreased cerebral blood flow. Cerebral blood flow was reduced further during hypotension, indicating impairment of autoregulation. Autoregulation was further impaired by wt-tPA, which was prevented by MK801 and tPA-A296–299. Protection by tPA-A296–299 was blocked by anti-LRP Ab, the LRP antagonist receptor-associated protein, and the p38 inhibitor SB 203580, but not by control IgG. Stroke reduced cerebrospinal fluid cAMP, which was reduced further by wt-tPA, but augmented by tPA-A296–299. Cerebrospinal fluid p38 was unchanged by wt-tPA, increased by tPA-A296–299, and decreased by anti-LRP Ab and receptor-associated protein.

**Conclusions**—tPA-A296–299 prevents impairment of cerebral autoregulation after stroke through an LRP-dependent increase in cAMP and p38. (Stroke. 2016;47:2096-2102. DOI: 10.1161/STROKEAHA.116.012678.)

**Key Words:** plasminogen activators ▪ receptors, n-methyl-D-aspartate ▪ signal transduction ▪ stroke ▪ tissue-type plasminogen activator
this damage are unknown. Although the NMDA-R antagonist MK 801 protects against cerebral dysregulation after traumatic brain injury, its toxicity limits usage in humans indicating the need for novel approaches.

wt-tPA may damage brain tissue through breakdown of the blood–brain barrier and intracranial hemorrhage, in addition to overactivation of NMDA-Rs. In this study, we focused on the contribution of NMDA-Rs to the toxicity of tPA administered after stroke and asked whether this can be mitigated without loss of fibrinolytic activity. We took advantage of our finding that deletion of the docking site in tPA prevents its binding and activation of NMDA-Rs. Based on this, we constructed a variant, tPA-K206A/H207A/R208A/R209A (tPA-A296–299), that prevents the enzyme from binding to NMDA-Rs but fully maintains its fibrinolytic activity. We hypothesize that tPA-A296–299, a variant that is fibrinolytic but cannot bind to NMDA-Rs, preferentially binds to LRP, which increases cAMP and p38, limiting impairment of autoregulation after stroke.

Activation of NMDA-Rs has both vital and neurotoxic effects, but the mechanism(s) involved in this transition are less well understood. Elevation of cAMP and activation of protein kinase A by glucagon prevents upregulation of endogenous tPA, diminishes NMDA-R excitotoxicity, and protects cerebral autoregulation and responsiveness to vasodilator stimuli in the setting of traumatic brain injury in the pig, providing support for the hypothesis that interventions that limit tPA-NMDA-R activation and restore cAMP might improve outcome after thrombotic stroke. Phosphorylation (activation) of the cAMP response element–binding motif can occur by the activation of mitogen activated protein kinase (MAPK). MAPK is a family of at least 3 kinases, extracellular-regulated kinase, e-Jun N-terminal kinase (JNK), and p38; the p38 isoform seems protective in the setting of stroke.

Disassociation of tPA can bind to either the lipoprotein-related receptor (LRP), which mediates vasodilation, or the NMDA-Rs. We propose that binding of wt-tPA binds to NMDA-Rs reduces cAMP and impairs ability of cerebral vessels to vasodilate, whereas tPA-A296–299, a variant that is fibrinolytic but cannot bind to NMDA-Rs, preferentially binds to LRP, which increases cAMP and p38 and limits impairment of autoregulation after stroke.

Methods

Anesthetic Regimen, Closed Cranial Window Technique, and Cerebral Photothrombosis

Pigs (4 weeks old) of either sex were studied. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The anesthetic regimen consisted of premedication with dexmedetomidine (20 µg/kg IM), induction with isoflurane (2%–3%), isoflurane taper to 0% after start of total intravenous anesthesia with fentanyl (100 µg/kg per hour), midazolam (1 mg/kg per hour), dexmedetomidine (2 µg/kg per hour), and propofol (2–10 mg/kg per hour), and maintenance of total intravenous anesthesia for the balance of the surgical and experimental portions of the pig preparation. A catheter was inserted into a femoral artery to monitor blood pressure and femoral veins for drug administration. The trachea was cannulated, the animals ventilated with room air, and temperature maintained in the normothermic range (37–39°C), monitored rectally. The closed cranial window technique was used to measure pial artery diameter and collect cerebrospinal fluid (CSF) for ELISA analysis. Hypotension was induced by the rapid withdrawal of either 5 to 8 or 10 to 15 mL blood/kg to induce moderate or severe hypotension (decreases in mean arterial blood pressure of 25% and 45%, respectively). Such drops in blood pressure were maintained constant for 10 minutes by titration of additional blood withdrawal or blood reinfusion. CBF was measured in the cerebral cortex using radioactively labeled microspheres.

Induction of photothrombosis was based on that described for the pig, but in our studies, we used the area of the closed cranial window to expose 2 to 3 main and 1 to 3 smaller arteries supplying the middle cerebral artery territory. Arterial occlusion was achieved by photothrombosis, in which a stable thrombus consisting of aggregating platelets, fibrin, and other blood components is formed in response to endothelial peroxidative damage. The photochemical reaction occurs because of interaction of intravenous photosensitizing dye erythrosine B (20 mg/kg IV) and the focused beam of a solid state laser operated at 532 nm, power of 200 mW, average intensity of 60 to 75 W/cm², and durations of up to 3 to 5 minutes using a Snake Creek minilaser (Hallstead, PA).

Protocol

Pial small arteries (resting diameter, 120–160 µm) were examined and similar-sized pial arteries were used in male and female pigs. For sample collection, 300 µL of the total cranial window volume of 500 µL was collected by slowly infusing artificial CSF into 1 side of the window and allowing the CSF to drip freely into a collection tube on the opposite side. Pigs were randomized to one of the following experimental groups (all n=5): (1) sham control, (2) photothrombosis, (3) photothrombosis+wt-tPA (1 mg/kg IV), (4) photothrombosis+MK 801 (10⁻⁵ M), (5) photothrombosis+tPA-A296–299 (1 mg/kg IV), (6) photothrombosis+tPA-A296–299+IgG (10 µg/mL), (7) photothrombosis+tPA-A296–299+anti-LRP Ab (10 µg/mL), (8) photothrombosis+tPA-A296–299+receptor-associated protein (RAP) (10⁻⁵ M), and photothrombosis+tPA-A296–299+SB 203580 (1 mg/kg IV). The vehicle for all agents was 0.9% saline, except for SB 203580 that used dimethyl sulfoxide (100 µL) diluted with 9.9-mL 0.9% saline. In sham control and photothrombotic animals, responses to hypotension and isoproterenol (10⁻⁶, 10⁻⁴ M) were obtained initially and then again 5 hours later in the presence of vehicle. In drug-treated photothermbotic animals, drugs were administered 4 hours after injury, and the insult protocol followed. Pial artery reactivity was determined in small pial arteries close to the area of injury (peri-ischemic area) using the closed cranial window technique.

ELISA

Commercially available ELISA Kits were used to quantity CSF p38 and cAMP (Assay Designs, Farmingdale, NY; EMD, Billerica, MA; Enzo, Farmingdale, NY) concentration.

Infarct Volume

Staining with 2,3,5-triphenyltetrazolium chloride (TTC) was used to determine infarct volume. The brains of pigs (1–5 days old) were first perfused transcardially with heparinized saline, followed by 2% TTC. Brains were stored in TTC for 20 minutes in the dark, fixed in 4% paraformaldehyde for 48 hours, and serial 5-µm sections were prepared. The area of ischemic damage of the hemispheres was measured for each brain slice using ImageJ software. Complete lack of TTC staining was defined as core and viable tissue stained red. Total infarction volume was taken to be the sum of core and penumbra volumes. Damage volume was calculated as percentage of ipsilateral hemisphere volume (damage/total volume×100). This was done for 3 hemispheric brain slices per animal, and the mean value was taken as the numeric for that individual pig. Animal groups were sham control, photothrombosis, photothrombosis+tPA (1 mg/kg IV) and photothrombosis+tPA-A296–299. The drugs wt-tPA and tPA-A296–299 (1 mg/kg IV) were administered at 4 hours, and TTC perfusion was completed at 5 hours post stroke.
Statistical Analysis
Pial artery diameter, CBF, CSF p38, cAMP, and infarct volume values were analyzed using ANOVA for repeated measures. The order of vascular stimuli application was randomized, and no data were excluded from analysis for statistical difference. The Investigator was blinded to treatment. If the value was significant, the data were then analyzed by Fisher protected least significant difference test. An α level of P<0.05 was considered significant in all statistical tests. A sample size calculation determined that with an n of 5, statistical determination could be made with P<0.05 and power of 0.87 for hemodynamic values and 0.84 for ELISA values. Values are represented as mean±SEM of the absolute value or as percentage changes from control value.

Results
tPA-A296–299 prevents, whereas wt-tPA aggravates, impairment of cerebral autoregulation when administered 4 hours after stroke. CBF was unchanged during hypotension (moderate 25%, severe 45% decrease in mean arterial blood pressure), in sham control animals, indicating cerebral autoregulation was intact (data not shown). Values for mean arterial blood pressure during normotension, moderate, and severe hypotension were 75±9, 56±6, and 42±5 mm Hg, respectively, n=5. CBF was reduced in brain tissue surrounding the infarct at 5 hours post thrombotic stroke and reduced further during hypotension, indicating impairment of autoregulation (Figure 1). Administration of wt-tPA (at 4 hours post stroke) elicited a short-lived normalization of CBF, but blood flow returned to a level below that observed in untreated pigs by 5 hours post stroke (Figure 1). In fact, administration of tPA at 4 hours further aggravated reductions in CBF during normotension and hypotension compared with vehicle at 5 hours post stroke (Figure 1). In contrast, administration of tPA-A296–299 prevented impairment of autoregulation when administered at 4 hours post stroke (Figure 1). CBF was unchanged by stroke or drug treatment in the contralateral cortex, demonstrating the focal effects of stroke induction and limiting the possibility that drug-induced effects were an epiphenomenon (Figure 1). There was no significant effect of either wt-tPA or tPA-A296–299 on mean arterial blood pressure (78±10 versus 76±9 mm Hg and 74±9 versus 78±10 mm Hg, respectively, n=5).

Pial artery dilation in response to hypotension is reproducible over time in sham control animals (data not shown), indicating intact autoregulation. However, dilation of pial arteries during hypotension was blunted at 5 hours after stroke (Figure 2), indicating disturbed autoregulation. Administration of wt-tPA 4 hours post insult reversed pial artery dilation to vasoconstriction, whereas tPA-A296–299 protected autoregulatory vasodilation during hypotension (Figure 2). Because isoproterenol-induced dilation was unchanged by thrombotic stroke and by wt-tPA or by tPA-A296–299, these data show that impairment of autoregulation after stroke is not an epiphenomenon.

Binding of tPA to NMDA-R Versus LRP Alters Outcome of Impairment of Cerebral Autoregulation After Stroke
MK 801 (10⁻⁷ M) blocked tPA-mediated impairment of pial artery dilation during hypotension to the same extent as tPA-A296–299 (Figure 2). On the contrary, coadministration of anti-LRP antibody (10 µg/mL) or the LRP antagonist RAP (10⁻⁷ M), but not control IgG (10 µg/mL), along with tPA-A296–299 blocked its ability to protect pial artery dilation during hypotension after stroke (Figure 2).

Thrombotic stroke in the pig reduces CSF cAMP, reduced further by wt-tPA, but prevented by tPA-A296–299. cAMP and p38 MAPK are augmented by tPA-A296–299 in an LRP-dependent manner.

We next explored the intracellular signaling pathways activated by wt-tPA via LRP. Drugs were administered 4 hours post stroke as above, and the concentration of CAMP and p38 MAPK in CSF was measured 1 hour later. The concentrations of substances in CSF reflect those in brain parenchyma.4,5 Thrombotic stroke reduced the concentration of CAMP, which was further reduced by administration of wt-tPA, but CAMP was augmented above levels in sham (control) animals after treatment with tPA-A296–299 (Figure 3). In contrast, p38 MAPK was robustly augmented in animals given tPA-A296–299 (Figure 3). wt-tPA administered after stroke modestly, although not significantly, decreased CAMP compared with poststroke pigs not receiving tPA. Coadministration of

Figure 1. Effect of photothrombosis (PTI) on cerebral blood flow (CBF; mL/min 100 g) during normotension and hypotension (moderate, severe) at 5 h post insult in the ipsilateral and contralateral cortex in the absence and presence of wild-type-tissue-type plasminogen activator (tPA) and tPA-A296–299 (1 mg/kg IV), administered at 4 h post insult, n=5. *P<0.05 vs corresponding PTI-alone value #P<0.05 vs corresponding normotension value.
anti-LRP Ab or RAP but not of control IgG with tPA-A296–299 blocked upregulation of cAMP and p38, indicating that the tPA variant modulates cAMP and p38 concentration in an LRP-dependent manner (Figure 3).

**Figure 3.** Cerebrospinal fluid cAMP and phosphorylated p38 mitogen activated protein kinase before thrombotic stroke (sham control), after stroke (photothrombosis [PTI]), or treated at 4 h post injury with wild-type-tissue-type plasminogen activator (tPA; 1 mg/kg IV), tPA-A296–299 (1 mg/kg IV), tPA-A296–299+IgG (10 µg/mL), tPA-A296–299+anti–low-density lipoprotein-related receptor (LRP) Ab (10 µg/mL), tPA-A296–299+receptor-associated protein (RAP) (10−7 M), or tPA-A296–299+SB203580 (1 mg/kg IV), n=5. *P<0.05 vs corresponding sham control value, +P<0.05 vs corresponding untreated PTI value, #P<0.05 vs tPA-A296–299 alone.

### tPA-A296–299 Reduces Infarct Volume After Thrombotic Stroke

The amount of brain tissue stained with TTC was reduced after stroke ipsilateral, but not after contralateral, to the insult consistent with the ability of this stain to differentiate healthy from damaged brain tissue (Figure 4). On a percentage basis, the volume of damaged tissue increased after stroke to the same extent in vehicle- and wt-tPA (1 mg/kg IV)–treated animals, but was reduced to a value statistically no different than control value in animals treated with tPA-A296–299 (Figure 4).

### Blood Chemistry

Blood chemistry values were collected before and after all experiments. There were no statistically significant differences between sham control, tPA, and tPA-A296–299–treated animals. Specifically, the values were 7.4±0.05, 38±4, and 94±11 and 7.43±0.06, 37±5, and 95±11 mm Hg for pH, Pco2, and Po2 at the beginning and end of the experiments in sham controls, whereas pH, Pco2, and Po2 values were 7.45±0.04, 37±5, and 91±11 and 7.43±0.03, 39±5, and 93±11 mm Hg for pH, Pco2, and Po2 at the beginning and end of the experiments in tPA-A296–299–treated animals, respectively.

### Discussion

An important new finding of translational relevance in this study is that the tPA variant tPA-A296–299 prevents reductions in CBF and impairment of cerebral autoregulation when administered in a clinically relevant timeframe (4 hours) after thrombotic stroke. In contrast, standard-of-care administration of wt-tPA in the same timeframe only transiently restores CBF, and blood flow values are in the ischemic range when determined at 1 hour post drug administration. Furthermore, wt-tPA did not prevent impairment of cerebral autoregulation after stroke.
that infarct volume was reduced by tPA-A296-299 to a value statistically no different than sham control indicates that this tPA variant both protects cerebral hemodynamics and prevents histopathology associated with the development of stroke.

wt-tPA can bind to either LRP, important in mediating vasodilation to vasoactive stimuli, or NMDA-Rs (Figure 5). We propose that after stroke, the pathway shown on the right side predominates based on release of endogenous tPA and is markedly accelerated by therapeutic administration of wt-tPA. This directly impacts the mechanisms responsible for compensatory vasodilation. For example, elevation of cAMP and the cAMP response element–binding motif is an important mechanism contributing to vasodilation. Phosphorylation (activation) of cAMP response element-binding can occur by activation of MAPK.16,17 However, toxic levels of glutamate and overactivation of NMDA-Rs in the setting of stroke activate phosphodiesterases that decrease cAMP (Figure 5). MAPK is a family of at least 3 isoforms: extracellular-regulated kinase, p38, and JNK. The JNK isoform is upregulated robustly and contributes to tPA-mediated impairment of autoregulation after thrombotic stroke in the pig.6,7 Indeed, results of the present study show that coadministration of the NMDA-R antagonist with wt-tPA prevented impairment of cerebral autoregulation after stroke, supporting the predominance of the pathway shown on the right side in Figure 5, wherein wt-tPA acts primarily on NMDA-Rs to contribute to damage after stroke, presumably, in part, via upregulation of JNK MAPK.6,7

Additional mechanistic studies were designed to address our hypothesis that the administration of tPA-A296-299 will drive the pathway depicted in Figure 5 to the left, which would protect autoregulation. We decided to investigate the p38 isoform of MAPK because previous studies showed that interventions that selectively upregulate this isoform protect autoregulation in the setting of thrombotic stroke in the pig.15,16 Indeed, we observed that protection by tPA-A296-299 was blocked by anti-LRP Ab, the LRP antagonist RAP,20,21 the p38 inhibitor SB 203580, but not by control IgG, supportive of activity mediated primarily through LRP rather than NMDA-Rs.

The functional significance of these observations is that cognitive outcome (Glasgow coma scale) and risk of death have been shown to correlate with the severity of cerebral autoregulatory impairment in other CNS ischemic conditions, such as human traumatic brain injury.25 Because isoproterenol-induced dilation was unchanged by thrombotic stroke and by tPA or tPA-A296-299, these data show that impairment of autoregulation after stroke is not an epiphenomenon. In addition, the finding

Figure 4. A. Representative sham control brain slice stained with 2,3,5-triphenyltetrazolium chloride (TTC), B) corresponding typical brain slice from a photothrombotic stroke (PTI) pig (left hemisphere) treated with vehicle and (C) typical brain slice from a pig post stroke (left hemisphere) treated with tissue-type plasminogen activator (tPA)-A296–299, distance marker is in mm. D. Summary data showing on a percentage basis the volume of damaged tissue volume in sham control, PTI stroke, and animals post stroke treated with tPA-A296–299 (1 mg/kg IV), n=4 to 5. *P<0.05 vs corresponding sham control value, +P<0.05 vs corresponding untreated PTI value.

Figure 5. Proposed signaling pathways and outcomes after stroke and administration of wild-type-tissue-type plasminogen activator (tPA) or tPA-A296-299. wt-tPA drives pathway toward the right leading to damage, whereas tPA-A296-299 drives pathway toward the left and providing protection.

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Historically, the neurotoxic effects of wt-tPA in the brain have received considerable attention. tPA activity increases rapidly in ischemic tissue after middle cerebral artery occlusion and mice genetically deficient in tPA (tPA−/−) have reduced infarct volumes after middle cerebral artery occlusion. More recently, studies have focused on mechanisms that link the neurotoxic effects of wt-tPA to NMDA-R signaling. For example, tPA may enhance NMDA-R-mediated calcium influx and activate the extracellular-regulated kinase isofrom of MAPK to cause neurotoxicity.

It is possible that the activation of NMDA-R subunits within the synapse may support neuronal survival, whereas high or sustained levels of activation of extrasynaptic NMDA-Rs may promote cell death. This, in turn, may depend on the expression level or distribution of NR2A-D subunits that must interact with NR1 for NMDA-Rs to be functional. The level and subcellular localization of wt-tPA may also help to determine whether it promotes cell death or survival via a plasminogen-independent proteolytic cleavage of an NR subunit or nonproteolytic signaling through LRP via activation of mTOR (mechanistic target of rapamycin), accumulation of HIF-1α (hypoxia inducible factor 1-alpha), recruitment of glucose GLUT3 (glucose transporter 3) to the plasma membrane, and GLUT3-mediated uptake of glucose by neurons in the ischemic brain.

Finally, there are a few experimental caveats that should be discussed about our data in the present study. Additional work is needed to identify the anatomic location of tPA and NMDA-R and their interaction observed in the present study. Others have observed the presence of tPA in cerebrovascular endothelial cells and oligodendrocytes, but not in pericytes, microglial cells, or astrocytes. NMDA-Rs have been identified on microglia in murine and human CNS. We have evidence that the NR1 subunit of the NMDA-R is expressed in brain vascular smooth muscle cells (unpublished observations), but more rigorous testing is needed. In addition, the mechanism linking binding to LRP to elevation of CSF cAMP and p38 concentration will need to be investigated. We speculate that p38 may increase cAMP, which may, in turn, cause further phosphorylation of p38 in a feed forward cycle. The opposite sequence (p38 causing decrease in cAMP) may occur via activation of NMDA-Rs by wt-tPA. It is known that the JNK isoform of MAPK is upregulated after stroke and further upregulated by wt-tPA in the setting of stroke, contributing to impairment of cerebral autoregulation. However, mechanism(s) that mediate interactions between p38 and JNK and alter their profile in the setting of stroke to foster protection or damage will also require additional study. Furthermore, there may be additional downstream mechanism(s) that link p38 to protection and JNK to damage after stroke (and their manipulation by wt-tPA and tPA-A296–299) that have not been identified. Future experiments will be conducted to address these and other unresolved relevant issues.

Conclusions
The results of the present study show that tPA-A296–299 blocks the increase in TTC staining associated with infarct volume generation and prevents impairment of cerebral autoregulation after stroke through an LRP-dependent increase in cAMP and p38.

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Disclosures
None.

References


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