tPA-S⁴⁸¹A Prevents Impairment of Cerebrovascular Autoregulation by Endogenous tPA after Traumatic Brain Injury by Upregulating p38 MAPK and Inhibiting ET-1

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Abstract

Traumatic brain injury (TBI) is associated with loss of cerebrovascular autoregulation, which leads to cerebral hypoperfusion. Mitogen activated protein kinase (MAPK) isoforms ERK, p38, and JNK and endothelin-1 (ET-1) are mediators of impaired cerebral hemodynamics after TBI. Excessive tissue plasminogen activator (tPA) released after TBI may cause loss of cerebrovascular autoregulation either by over-activating N-methyl-D-aspartate receptors (NMDA-Rs) or by predisposing to intracranial hemorrhage. Our recent work shows that a catalytically inactive tPA variant (tPA-S⁴⁸¹A) that competes with endogenous wild type (wt) tPA for binding to NMDA-R through its receptor docking site but that cannot activate it, prevents activation of ERK by wt tPA and impairment of autoregulation when administered 30 min after fluid percussion injury (FPI). We investigated the ability of variants that lack proteolytic activity but bind/block activation of NMDA-Rs by wt tPA (tPA-S⁴⁸¹A), do not bind/block activation of NMDA-Rs but are proteolytic (tPA-A^{296–299}), or neither bind/block NMDA-Rs nor are proteolytic (tPA-A^{296–299}S⁴⁸¹A) to prevent impairment of autoregulation after TBI and the role of MAPK and ET-1 in such effects. Results show that tPA-S⁴⁸¹A given 3 h post-TBI, but not tPA-A^{296–299} or tPA-A^{296–299}S⁴⁸¹A prevents impaired autoregulation by upregulating p38 and inhibiting ET-1, suggesting that tPA-S⁴⁸¹A has a realistic therapeutic window and focuses intervention on NMDA-Rs to improve outcome.

Key words: cerebral autoregulation; cerebral blood flow; excitatory amino acids; newborn, plasminogen activators; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is the leading cause of injury-related death in children.¹ The effects of TBI have been investigated extensively in adult animal models,² but less is known about outcome in the newborn/infant. TBI can cause uncoupling of cerebral blood flow (CBF) and metabolism, resulting in cerebral ischemia or hyperemia.³ Although hyperemia has been considered the cause of diffuse brain swelling after pediatric TBI,⁴ recent evidence suggests hypoperfusion predominates in childhood injury.⁵ In line with this finding, fluid percussion injury (FPI) constricts pial arteries and reduces CBF in piglets.⁶ One inference from this observation is that piglets offer the advantage of an animal whose size permits pediatric cerebral hemodynamic investigation and a gyrencephalic brain containing substantial white matter, which is more sensitive to ischemic damage, similar to

the human setting. The mechanism underlying uncoupling is not understood, however.

Among the possible mediators, glutamate is known to bind to each of three ionotropic receptor subtypes named after synthetic analogues: N-methyl-D-aspartate (NMDA), kainate, and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). Activation of NMDA receptors (Rs) contributes to excitotoxicity,⁷ but also elicits cerebrovasodilation and represents a mechanism by which local metabolism is coupled to CBF.⁸ Glutamatergic hyperactivity has been implicated in neurotoxicity after TBI, whereas NMDA antagonists are protective in some models.^{9,10} Although CBF is thought to contribute to neurologic outcome, little attention has been given to the role of NMDA-Rs in the regulation or dysregulation of this process. In our studies, NMDA-R-mediated vasodilation is reversed to vasoconstriction after FPI in the piglet.¹¹

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Similarly, upregulation of endogenous wild type (wt) tissue plasminogen activator (tPA) after TBI not only enhances excitotoxic neuronal cell death through excessive activation of NMDA-Rs,^{12,13} but also contributes to impaired NMDA-R-mediated vasodilation, loss of autoregulation during hypotension, and histopathology^{14–17} via mitogen activated protein kinase (MAPK),¹⁸ a family of three kinases (ERK, p38, and JNK) critically important in hemodynamics after TBI.¹⁴ We found that activation of ERK and JNK contributed to impaired NMDA-R cerebrovasodilation after FPI, while p38 was protective.¹⁸ Endothelin-1 (ET-1) is also upregulated and antagonizes NMDA-R-mediated vasodilation after FPI.¹⁹ The link between activation of NMDA-Rs and autoregulation is supported by several interventions at the receptor level. The NMDA antagonist MK 801 protects against cerebral dysregulation after FPI,²⁰ but its toxicity limits use in humans. Alternatively, glucagon minimizes the surge in glutamate after TBI in mice and pigs that is partially responsible for over-activation of NMDA-Rs and preserves autoregulation during hypotension by blunting upregulation of tPA, which prevents tissue damage.¹⁵

Excessive tPA released after TBI may impair cerebral hemodynamics by over-activating NMDA-Rs as well. Post-TBI (30 min) administration of a catalytically inactive tPA variant (tPA-S⁴⁸¹A) that competes with wt tPA for binding to NMDA-R through its receptor docking site but cannot activate it, prevents activation of ERK MAPK and thereby prevents impairment of autoregulation after FPI.²¹ The potential deleterious role of tPA-mediated fibrinolysis and intracerebral hemorrhage (ICH) after TBI, however, may also contribute to adverse outcome. Also, a therapeutic window of greater than 30 min is needed for any agent to have clinical utility for this indication.

We hypothesize that over-activation of NMDA-Rs is a major contributor to adverse outcome by the rise in endogenous tPA after TBI. To test this hypothesis, we used a model in which ICH does not predominate^{14,17,21} and examined the effect of tPA variants that are either: (1) not proteolytic (do not cleave/activate NMDA-Rs or fibrin) but bind/block activation of NMDA-Rs (tPA-S⁴⁸¹A); (2) do not bind NMDA-Rs but are proteolytic (tPA-A^{296–299}); or (3) neither bind/activate NMDA-Rs nor are proteolytic (tPA-A^{296–299}S⁴⁸¹A), to prevent impairment of autoregulation after TBI, as well as the roles of p38 MAPK and ET-1 as mediators of these effects.

Methods

tPA variants

To generate tPA-S⁴⁸¹A, tPA-A^{296–299} and tPA-A^{296–299}S⁴⁸¹A, mutations were introduced in wt tPA by polymerase chain reaction using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA),

and the complete sequences were verified.^{22–24} Each protein contains two extra amino acids, RS-, at the NH₂ terminus, resulting from the introduction of the Bgl II cloning site. Proteins were expressed in S2 Drosophila Expression System (Invitrogen) according to the manufacturer's protocol and were purified by affinity chromatography using anti-tPA coupled to CN-Br activated Sepharose.

We constructed three tPA variants with mutations in either the NMDA-R binding site, the catalytic triad, or both that were designed to: (1) selectively block proteolytic activation of NMDA-Rs by endogenous wt tPA; (2) selectively block proteolysis; or (3) both (Table 1A). The first variant, tPA-S⁴⁸¹A, has a single mutation in the catalytic site that enables it to bind to but not to cleave and activate NMDA-Rs or fibrin (Table 1A). At therapeutic concentrations, tPA-S⁴⁸¹A will compete with high levels of endogenous wt tPA for binding to NMDA-Rs and fibrin (i.e., tPA binds to fibrin through the finger and K2 domains, outside both the NMDA-R docking site and the catalytic site) and protect both from proteolysis, and it will neither activate NMDA-Rs nor cause fibrinolysis (Table 1).²⁵ The second tPA variant has a mutated docking site, tPA-K²⁹⁶A/H²⁹⁷A/R²⁹⁸A/R²⁹⁹A (tPA-A^{296–299}), that prevents it from binding to NMDA-Rs, while maintaining binding to fibrin, and it will augment the proteolytic activity of endogenous wt tPA (Table 1A). The third is a double-variant tPA-A^{296–299}S⁴⁸¹A that is designed not to bind to NMDA-R and lacks proteolytic activity. The double variant is used to compete with endogenous tPA for binding to fibrin and thereby prevent ICH (Table 1A), but not bind to NMDA-R and therefore not compete with receptor activation by endogenous wt tPA (Table 1B). We hypothesized that tPA-S⁴⁸¹A would protect against the toxicity of endogenous wt tPA in this model by inhibiting the binding and activation of NMDA-Rs without causing ICH (Table 1B). The difference between the protective effect of tPA-A^{296–299} and tPA-A^{296–299}S⁴⁸¹A compared with tPA-S⁴⁸¹A would help to elucidate the relative contribution of the two pathways (Table 1B).

Closed cranial window and brain injury procedures

Newborn pigs (1–5 days old, 1.0–1.4 kg) of both sexes were studied. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animals were anesthetized with isoflurane (1–2 MAC) and maintained with a-chloralose (50–80 mg/kg, supplemented with 5 mg/kg/h intravenously [i.v.]).

The closed cranial window technique for measuring pial artery diameter and collection of cerebrospinal fluid (CSF) for enzyme-linked immunosorbent assay (ELISA) analysis has been described.⁶ This window consisted of three parts: a stainless steel ring, a circular glass cover-slip, and three ports consisting of 17-gauge hypodermic needles attached to three precut holes in the stainless steel ring. For placement, the dura was cut and retracted over the cut bone edge. The cranial window was placed in the opening and

TABLE 1. tPA VARIANT ACTIONS

Action	Wild type tPA	tPA-S ⁴⁸¹ A	tPA-A ^{296–299}	tPA-A ^{296–299} S ⁴⁸¹ A
<i>A. tPA variant physiologic action</i>				
Bleeding	Fibrinolytic	Not fibrinolytic	Fibrinolytic	Not fibrinolytic
NMDA-R activation	Binds excessive activation	Binds but does not activate	Does not bind	Does not bind
<i>B. tPA variant pathologic action after TBI</i>				
Bleeding	4+	–	5+	–
NMDA-R activation	4+	–	–	–
Outcome	–4	5+	–1	–1

tPA, tissue plasminogen activator; NMDA-R, N-methyl-D-aspartate receptors; TBI, traumatic brain injury. Numeric scoring from 0 to 5.

cemented in place with dental acrylic. The volume under the window was filled with a solution, similar to CSF, of the following composition (in mM): 3.0 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO₃. This artificial CSF was warmed to 37°C and had the following chemistry: pH 7.33, pCO₂ 46 mm Hg, and pO₂ 43 mm Hg, which was similar to that of endogenous CSF. Pial arterial vessel diameter was measured with a microscope, a camera, a video output screen and a video microscaler.

The method used to induce brain FPI has been described.¹⁵ A device designed by the Medical College of Virginia was used. A small opening was made in the frontoparietal skull contralateral to the cranial window. A metal shaft was sealed into the opening on top of intact dura and fluid coupled to the brain injury device; i.e., the shaft was connected to the transducer housing, which was in turn connected to the fluid percussion device. The device itself consisted of an acrylic plastic cylindrical reservoir 60 cm long, 4.5 cm in diameter, and 0.5 cm thick. One end of the device was connected to the transducer housing, whereas the other end had an acrylic plastic piston mounted on O-rings. The exposed end of the piston was covered with a rubber pad. The entire system was filled with 0.9 % saline. The percussion device was supported by two brackets mounted on a platform.

FPI was induced by striking the piston with a 4.8 kg pendulum. The intensity of the injury (usually 1.9–2.3 atm. with a constant duration of 19–23 ms) was controlled by varying the height from which the pendulum was allowed to fall. The pressure pulse of the injury was recorded on a storage oscilloscope triggered concurrently with the fall of the pendulum. The amplitude of the pressure pulse was used to determine the intensity of the injury.

Protocol

Pial small arteries (resting diameter, 120–160 μ m) were examined. Typically, 2–3 mL of artificial CSF were flushed through the window over 30 sec, and excess CSF was allowed to run off through one of the needle ports. For sample collection, 300 μ L from the total cranial window volume of 500 μ L was collected by slowly infusing artificial CSF into one side of the window and allowing the CSF to drip freely into a collection tube on the opposite side.

Seven experimental groups were studied (all $n=5$): (1) sham control, (2) FPI, vehicle treated, (3) FPI treated with tPA (1 mg/kg i.v.), (4) FPI treated with tPA-S⁴⁸¹A (1 mg/kg i.v.), (5) FPI treated with tPA-A^{296–299} (1 mg/kg i.v.), (6) FPI treated tPA-A^{296–299}S⁴⁸¹A (1 mg/kg i.v.), and (7) FPI treated with tPA-S⁴⁸¹A + the p38 MAPK antagonist SB 203580 (1 mg/kg i.v.). Hypotension was induced by the rapid withdrawal of either 5–8 or 10–15 mL blood/kg to induce moderate or severe hypotension (decreases in mean arterial blood pressure of 25 and 45%, respectively).¹⁵ Such decreases in blood pressure were maintained constant for 10 min by titration of additional blood withdrawal or blood reinfusion.¹⁵ The vehicle for all agents was 0.9% saline, except for the MAPK inhibitor, which used dimethyl sulfoxide (100 μ L) diluted with 9.9 mL 0.9% saline. In sham control animals, responses to hypotension (moderate, severe), papaverine, NMDA (10^{–8}, 10^{–6} M), and PGE₂ (1, 10 ng/mL) were obtained initially and then again 4 h later in the presence of the agent vehicle. In animals treated post-FPI, tPA variants were administered 3 h post-injury, and responses to vasoactive stimuli were measured both before and 4 h post-injury.

CBF was measured in the cerebral cortex and hippocampus using radioactively labeled microspheres.¹⁵ Briefly, neutron activated microspheres (Biopal; 15 μ m diameter; 300,000–800,000 spheres) were injected into the left ventricle. After each experiment, the piglet was sacrificed and the brain removed and weighed. The energy from each nuclide was separated by differential spectroscopy. Aliquots of the actual microsphere solutions injected were used for overlap calculations. The count in each milliliter per min of blood flow was determined by dividing the

counts in the reference withdrawal by the rate of reference withdrawal. Thus, blood flow can be calculated as $Q = C \times R \times CR^{-1}$, where Q is brain blood flow (in mL/min), C is counts per min (cpm) in the tissue sample, R is the rate of withdrawal of reference blood sample (in mL/min), and CR is the total counts in the reference blood sample. CBF so determined reflects flow to the cerebral cortex and hippocampus both ipsilateral and contralateral to the injury site. Previous studies have shown that multiple injections of microspheres in similar quantities (300,000–800,000 spheres per nuclide) do not cause significant detectable microvessel plugging, thereby limiting this as an experimental caveat for data interpretation.^{6,15}

ELISA and radioimmunoassay (RIA)

Commercially available ELISA kits were used to quantify CSF p38 MAPK (Assay Designs) concentration, while RIA kits were used to quantify ET-1 (Phoenix Pharmaceuticals). Values for phosphorylated p38 MAPK enzyme were normalized to total form and then expressed as percent of the control condition.

Statistical analysis

Pial artery diameter, CSF p38 MAPK, and ET-1 values were analyzed using analysis of variance for repeated measures. 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. Values are represented as mean $\pm$ standard error of the mean of the absolute value or as percentage changes from control value.

Results

Wild type tPA aggravates, tPA-S⁴⁸¹A mitigates, and tPA-A^{296–299} and tPA-A^{296–299}S⁴⁸¹A have little effect on impaired cerebral autoregulation during hypotension post-FPI

CBF was unchanged during hypotension (moderate 25%, severe 45% reduction in mean arterial pressure) in sham control animals, indicating cerebral autoregulation remained intact (Fig. 1A, 1B). CBF was reduced after FPI and reduced further during hypotension, which was aggravated by administration of exogenous wild type (wt) tPA (1 mg/kg iv) 3 h post-injury (Fig. 1A, 1B). Similarly, FPI reduced pial artery diameter, which was reduced further by wt tPA (Fig. 1C). In contrast, administration of tPA-S⁴⁸¹A (1 mg/kg i.v.) 3 h post-FPI, prevented injury-induced pial artery constriction and hypoperfusion in the parietal cortex and hippocampus monitored 1 h later (4 h post-injury) (Fig. 1). Further reductions in CBF during hypotension (at 4 h post-injury) were also blunted by tPA-S⁴⁸¹A (1 mg/kg i.v.), administered at 3 h post-FPI (Table 1), similar to the effect of tPA-S⁴⁸¹A administered 30 min post-injury.²¹ Aggravation of hypoperfusion, impairment of cerebral autoregulation during hypotension, and reductions in pial artery diameter by exogenous wt tPA after FPI was blocked by co-administration of tPA-S⁴⁸¹A (Fig. 1). Neither tPA-A^{296–299} nor tPA-A^{296–299}S⁴⁸¹A, however, caused a significant reduction in pial artery vasoconstriction, hypoperfusion, or impaired cerebral autoregulation after FPI (Fig. 1).

FPI blunts pial artery dilation during hypotension.¹⁵ This response was reversed to vasoconstriction when wt tPA was administered after FPI (Fig. 2). In contrast to wt tPA, administration of tPA-S⁴⁸¹A (1 mg/kg i.v.) 3 h post-FPI blocked injury-induced pial artery constriction and almost fully restored pial artery dilation monitored 1 h later (4 h post-injury) (Fig. 2). In contrast, neither

FIG. 1. Effect of fluid percussion injury (FPI) on cerebral blood flow (CBF) in the parietal cortex (A) and hippocampus (B) and on pial artery diameter (C) during control (C), moderate and severe hypotension (MH and SH) in sham, 4 h post-FPI + vehicle, FPI + tPA, FPI + tPA-S⁴⁸¹A, FPI + tPA-S⁴⁸¹A + tPA, FPI + tPA-A²⁹⁶⁻²⁹⁹, and FPI + tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A pigs, $n = 3-5$. All drugs (1 mg/kg i.v.) administered at 3 h post-FPI. * $p < 0.05$ compared with corresponding sham value in panels A and B and compared with the non-drug-treated post-FPI value in panel C; # $p < 0.05$ compared with the corresponding control value in panels A and B; + $p < 0.05$ compared with corresponding non-drug-treated post-FPI value in panels A and B.

tPA-A²⁹⁶⁻²⁹⁹ nor tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹ protected pial artery dilation during hypotension (Fig. 2). Papaverine-induced pial artery dilation was unchanged after FPI,¹⁵ and none of the tPA variants significantly affected its cerebrovasodilator effect either before or after brain injury (Fig. 2). Amelioration of pial artery vasoconstriction associated with FPI by tPA-S⁴⁸¹A occurred within minutes and was similar when administered 30 min or 3 h post-injury (Fig. 3).

Wild type tPA aggravates, tPA-S⁴⁸¹A mitigates, and tPA-A²⁹⁶⁻²⁹⁹ and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A have no effect on impaired cerebrovasodilation mediated by activation of NMDA-Rs and PGE2

NMDA-R-mediated pial artery dilation was reversed to vasoconstriction after FPI (Fig. 4A).²⁰ Wild type tPA (1 mg/kg i.v.) administered 3 h post-insult aggravated NMDA-R-mediated pial artery vasoconstriction (Fig. 4A), whereas tPA-S⁴⁸¹A (1 mg/kg i.v., 3 h post-insult) did not. Rather, vasodilation was observed (Fig. 4A), similar to that observed when tPA-S⁴⁸¹A was administered

at 30 post-injury.²¹ tPA-S⁴⁸¹A (1 mg/kg i.v., 3 h post-insult) also prevented impairment of PGE2-mediated cerebrovasodilation (Fig. 4B), similar to the protection observed when the tPA variant was administered 30 min post-injury.²¹ Neither tPA-A²⁹⁶⁻²⁹⁹ nor tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A, however, prevented the reversal of NMDA-R-mediated dilation to vasoconstriction after FPI (Fig. 4A), consistent with their inability to bind to NMDA-Rs. Similarly, in piglets treated with tPA-A²⁹⁶⁻²⁹⁹ and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹, impairment of PGE2 dilation was no different in animals given vehicle post-FPI (Fig. 4B).

tPA-S⁴⁸¹A prevents impaired autoregulation and NMDA-R-mediated pial artery dilation after FPI by upregulating p38 MAPK and downregulating ET-1

tPA augments reversal of NMDA-R-mediated dilation to vasoconstriction and contributes to impairment of autoregulation after FPI by upregulating ERK and JNK MAPK.^{18,20} As upregulation of p38 MAPK by wt tPA appears to limit impairment of

FIG. 2. Influence of A. hypotension (moderate, severe) and papaverine (10^{-8} , 10^{-6} M) on pial artery diameter before (control), 4 h after FPI, and after FPI in animals post-treated (3 h) with tPA, tPA-S⁴⁸¹A, tPA-A²⁹⁶⁻²⁹⁹, and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A (1 mg/kg i.v.), $n = 5$. Baseline pial artery diameters were: 130 ± 14 , 102 ± 10 , 94 ± 8 , 122 ± 13 , 111 ± 10 , and 108 ± 8 μ m, respectively for the six animal groups, control, FPI, FPI + tPA, FPI + tPA-S⁴⁸¹A, FPI + tPA-A²⁹⁶⁻²⁹⁹, and FPI + tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A. * $p < 0.05$ compared with corresponding control; + $p < 0.05$ compared with FPI alone.

FIG. 3. Influence of tPA-S⁴⁸¹A (1 mg/kg iv) on pial artery diameter as a function of time when administered either at 30 min (A) or 180 min (B) post-FPI. **p* < 0.05 compared with fluid percussion injury (FPI) value before administration.

FIG. 4. Influence of (A) N-methyl-D-aspartate (NMDA) (10^{-8} , 10^{-6} M, and (B) PGE2 (1, 10 ng/mL) on pial artery diameter before (control), 4 h after fluid percussion injury (FPI), and after FPI in animals post-treated (3 h) with tissue plasminogen activator (tPA), tPA-S⁴⁸¹A, tPA-A²⁹⁶⁻²⁹⁹, and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A (1 mg/kg i.v.), *n* = 5. Baseline pial artery diameters were: 133 ± 15 , 101 ± 11 , 93 ± 8 , 124 ± 13 , 112 ± 10 , and 106 ± 9 μ m, respectively, for the six animal groups, control, FPI, FPI + tPA, FPI + tPA-S⁴⁸¹A, FPI + tPA-A²⁹⁶⁻²⁹⁹, and FPI + tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹A. **p* < 0.05 compared with corresponding control; + *p* < 0.05 compared with FPI alone.

NMDA-R-mediated pial artery dilation after FPI,¹⁸ we speculated that a change in the MAPK isoform profile toward further increasing p38, concomitant with diminished ERK might serve as the mechanism underlying the vasoprotective effect of tPA-S⁴⁸¹A after TBI. Indeed, the increase in CSF p38 MAPK post-FPI was augmented mildly by wt tPA but was elevated more robustly by tPA-S⁴⁸¹A (Fig. 5A).

We previously observed that tPA-S⁴⁸¹A blocked the rise in phosphorylated ERK in the CSF seen after FPI.²¹ Co-administration of the p38 MAPK antagonist SB 203580 (1 mg/kg i.v.) with the tPA-S⁴⁸¹A 3 h post-FPI blocked upregulation of phosphorylated p38 MAPK (Fig. 5A) and the ability of the variant to prevent hypoperfusion and the further reduction in CBF during hypotension (Fig. 5B, 5C). Together, these biochemical and pharmacological data suggest that tPA-S⁴⁸¹A prevents impairment of cerebral autoregulation after FPI both by augmenting p38 and by inhibiting upregulation of ERK MAPK.²¹

We also observed that ET-1 is upregulated and antagonizes NMDA-R-mediated dilation after FPI.¹⁹ Therefore, we examined the effect of wt tPA and tPA-S⁴⁸¹A on ET-1 levels in the CSF after FPI. CSF ET-1 was elevated after FPI and was increased further by exogenous wt tPA, but both the total upregulation and that portion augmented by wild type tPA were both blocked by tPA-S⁴⁸¹A administered at 3 h post-FPI (Fig. 6). Co-administration of SB 203580 with tPA-S⁴⁸¹A modestly reversed the total blockade of ET-1 upregulation observed with tPA-S⁴⁸¹A alone (Fig. 6). These data suggest that tPA-S⁴⁸¹A-mediated protection of autoregulation after TBI may result from the capacity of p38 to block upregulation of ET-1 post TBI.

Blood chemistry and ICH

Blood chemistry values were collected before and after all experiments. There were no statistically significant differences between sham control, FPI, and FPI and drug-treated animals. FPI is reported to produce little ICH,²⁶ and we did not observe hemorrhage after FPI in the present studies, consistent with previous studies using this model in the piglet.^{14,15,17}

Discussion

The toxicity caused by increased endogenous tPA has been well studied in animal models of stroke and in humans. TBI is not stroke, however: the etiology, pathophysiology, and the critical care

FIG. 5. A. Influence of fluid percussion injury (FPI) (4 h) on (A) phosphorylated p38 mitogen activated protein kinase (MAPK) in the absence and presence of tissue plasminogen activator (tPA), of tPA-S⁴⁸¹A, and SB 203580 (1 mg/kg i.v., treatment 3 h after FPI). Influence of FPI on pial artery diameter (B), cerebral blood flow (CBF) in the parietal cortex (C), and hippocampus (D). Pial artery diameter and CBF were obtained during control (C), moderate and severe hypotension (MH and SH) in sham, FPI, FPI + tPA, FPI + tPA-S⁴⁸¹A, and FPI + tPA-S⁴⁸¹A + SB 203580 pigs, $n = 3-5$. * $p < 0.05$ compared with corresponding pre-injury (0 time) in A, control in B, and sham in C, D. + $p < 0.05$ compared with corresponding FPI alone value in A, B and control normotensive value (C) in C and D. # $p < 0.05$ compared to FPI + tPA and FPI + tPA-S⁴⁸¹A value in B and corresponding vehicle FPI value in C and D. & $p < 0.05$ compared with FPI + tPA-S⁴⁸¹A value in C and D.

pathways for treatment are related, but differ in important ways, including the role of endogenous wt tPA and the risk of hemorrhage. Endogenous wt tPA appears to be especially important in determining the outcome of TBI, whereas in stroke, the concentration of exogenous tPA administered as a therapeutic predominates. Excessive endogenous tPA released after TBI can cause tissue damage by over-activating NMDA-Rs or by exacerbating ICH, depending in part on the nature and force of the impact. To delineate the role of each pathway in TBI and to block whichever is/are operative in this model, the approach we used is based on our recently published finding that the docking site in tPA is required to bind and cleave and thereby activate NMDA-Rs.²²⁻²⁴ Previous work by others indicates that tPA cleaves the NR-1 subunit of NMDA-Rs, which contributes to excitotoxicity by increasing the influx of calcium,^{12,27} by signaling through an interaction with the NR2B subunit.²⁸

Human TBI is heterogeneous, limiting the ability of animal models to mimic the complexity of the human situation. FPI is thought to be a good mimic of TBI caused by shaken impact and motor vehicle accidents.²⁶ We also chose to study FPI to isolate the

effect of NMDA-R occupancy/cleavage/excitotoxicity by endogenous tPA from its role in causing ICH. FPI is reported to produce little ICH,²⁶ and we did not observe hemorrhage after FPI in the piglet.^{14,15,17} This allowed us to explore the role of NMDA-R in tPA-mediated excitotoxicity post-FPI and to use visible ICH as an adverse end-point from intervention.

We recently reported that administration of tPA-S⁴⁸¹A 30 min post-injury prevents loss of cerebrovascular autoregulation during hypotension and limits histopathology produced by FPI in the piglet.²¹ We therefore hypothesized that tPA-S⁴⁸¹A provides a novel approach toward limiting neuronal injury by blocking excessive activation of NMDA-Rs caused by the robust increase in glutamate and tPA within the brain after TBI (Table 1). Whether tPA-S⁴⁸¹A works by blocking NMDA-R-mediated signaling through non-proteolytic pathways and/or prevents diffuse microvascular bleeds in this model, however, could not be determined using this single variant because it acted on both pathways. Comparative study of the three mutants in the present study helped to define the contribution of each pathway to negative outcome after TBI.

FIG. 6. Influence of fluid percussion injury (FPI) (4 h) on endothelin-1 (ET-1) (pg/mL) in FPI, FPI + tPA, FPI + tPA-S⁴⁸¹A, and FPI + tPA-S⁴⁸¹A + SB 203580 (1 mg/kg i.v.) treated (3 h) pigs, $n = 5$. * $p < 0.05$ compared with FPI; + $p < 0.05$ compared with FPI + tPA.

Results of the present study show that FPI blunts pial artery dilation during hypotension,¹⁵ and this response is reversed to pial artery vasoconstriction when wt tPA was administered. Although our previous studies had suggested that wt tPA inhibits autoregulation,²¹ these data are the first direct demonstration. In contrast, administration of tPA-S⁴⁸¹A 3 h post-FPI blocked injury-induced pial artery constriction and hypoperfusion in the parietal cortex and hippocampus monitored 1 h later (4 h post-TBI). These data are very similar to that reported for tPA-S⁴⁸¹A administered at 30 min post-injury, with hemodynamics monitored at 1 h post-insult.²¹ Amelioration of pial artery vasoconstriction associated with FPI by tPA-S⁴⁸¹A occurred within minutes and was similar when administered 30 min or 3 h post-injury. This is important because it means that tPA-S⁴⁸¹A acts on its target tissues within a realistic therapeutic time frame. Previously, we observed that the NMDA-R antagonist MK801 prevented impairment of cerebral autoregulatory pial artery dilation after FPI.²⁰ This outcome suggested that the protection of cerebral autoregulation by tPA-S⁴⁸¹A post-TBI likely results from ameliorating toxicity induced by the joint action of tPA and glutamate signaling through NMDA-Rs. In support of this, papaverine-mediated vasodilation was unchanged after FPI and tPA-S⁴⁸¹A, indicating the specificity of the variant on endogenous tPA as a mediator.

Our previous studies did not exclude the possibility that tPA-S⁴⁸¹A prevents microhemorrhaging, which in turn impairs autoregulation and CBF. We did not, however, observe any hemorrhages in our animals,^{14,15,17} consistent with the previous experience of others using this model.²⁶ Moreover, neither tPA-A²⁹⁶⁻²⁹⁹, which maintains proteolytic activity, nor tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹, which does not, caused a significant reduction of pial artery vasoconstriction and hypoperfusion after FPI. In addition, pial artery dilation during hypotension was protected only modestly after FPI by tPA-A²⁹⁶⁻²⁹⁹ and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹ and neither variant prevented the loss in autoregulation and reduction in CBF that results from hypotension after FPI. Together, these findings focus attention on neurotoxicity mediated through NMDA-Rs.

Activation of NMDA-Rs elicits cerebrovasodilation and may represent one mechanism by which local metabolism is coupled to

blood flow.⁸ More recently, it was observed that tPA is critical for the full expression of the flow increase evoked by activation of the mouse whisker barrel cortex.²⁹ Specifically, tPA promoted nitric oxide (NO) synthesis during NMDA-R activation by modulating phosphorylation of nNOS.²⁹ These findings suggest that tPA is a key factor linking NMDA-R activation to NO synthesis and functional hyperemia.

NMDA-R induced pial artery vasodilation is reversed to vasoconstriction by FPI in the newborn pig.¹¹ Release of endogenous tPA in the setting of FPI aggravates NMDA-R-mediated vasoconstriction.^{11,18} The PAI-1 derived hexapeptide EEIIMD blocked reversal of NMDA-R induced dilation to vasoconstriction as well as reductions in baseline pial artery diameter after FPI,¹¹ indicating that the interaction between tPA and NMDA-Rs is altered in the setting of TBI. Because the NMDA-R antagonist MK801 prevents impairment of pial artery dilation during hypotension after FPI,²⁰ these data indicate that reversal of NMDA-R-mediated dilation to vasoconstriction and its aggravation by tPA likely contributes to cerebral dysregulation in the setting of TBI.

Pial artery dilation during hypotension is associated with release of dilator prostaglandins (PGs), such as PGE₂, while the cyclooxygenase inhibitor indomethacin blocks the vascular response, indicating that PGs contribute to autoregulation.³⁰ FPI impairs PGE₂-mediated pial artery dilation,³¹ which probably contributes to disturbed cerebral autoregulation after TBI. This might be better explained by increased superoxide production after FPI,³² which together with increased NO will produce excessive peroxynitrite. Once formed, peroxynitrite might impair dilation of the cerebral vasculature. We, therefore, investigated the relationship between NMDA-R and tPA in the context of impaired PG-mediated vasodilation after TBI.

tPA-S⁴⁸¹A (3 h post-insult) did not cause NMDA-R mediated vasoconstriction. Instead, vasodilation was observed, similar to what we observed when tPA-S⁴⁸¹A was administered 30 min.²¹ tPA-S⁴⁸¹A also significantly prevented impairment of PGE₂-mediated cerebrovasodilation, similar to our findings when that tPA variant was administered 30 min post-injury.²¹ Taken together, these data suggest that tPA-S⁴⁸¹A protects against cerebrovascular dysregulation after TBI in part by preventing impairment of NMDA-R and PGE₂-induced pial artery vasodilation. Neither tPA-A²⁹⁶⁻²⁹⁹ nor tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹ prevented the reversal of NMDA-R-mediated dilation to vasoconstriction after FPI, consistent with their inability to bind to NMDA-Rs. Similarly, in piglets treated with tPA-A²⁹⁶⁻²⁹⁹ and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹, impairment of PGE₂ dilation was no different than in animals given vehicle post-FPI. The findings that tPA-A²⁹⁶⁻²⁹⁹ and tPA-A²⁹⁶⁻²⁹⁹S⁴⁸¹ did not enhance inhibition of responses to PGE₂ and activation of NMDA-Rs like wt tPA further argues against an important role for excessive bleeding in impairing cerebral hemodynamics in this model (Table 1B). Bleeding may contribute to dysregulation in the clinical setting, however, when TBI is more severe.

We next investigated the mechanism by which tPA-S⁴⁸¹A prevented impairment of autoregulation post-TBI. MAPK, a family of kinases (ERK, p38, and JNK), is a post-receptor signaling system that contributes to setting vascular tone and is upregulated after TBI.¹⁴ Release of ERK MAPK after FPI in the piglet contributes to reductions in CBF and worsens histopathology.¹⁴ wt tPA aggravates reversal of NMDA-R-mediated dilation to vasoconstriction and contributes to impairment of autoregulation after FPI by upregulating ERK and JNK MAPK.^{18,21} As upregulation of p38 MAPK by wt tPA appears to limit impairment of NMDA-R-mediated pial artery dilation after FPI,¹⁸ we speculated that

increasing p38 might serve as a mechanism for vasoprotection by tPA-S⁴⁸¹A. In partial support of this hypothesis, the increase in CSF p38 MAPK post-FPI was mildly augmented by wt tPA but more so by tPA-S⁴⁸¹A. We previously observed that tPA-S⁴⁸¹A blocked the increase in phosphorylated ERK in the CSF that occurs after FPI.²¹ The ability of tPA-S⁴⁸¹A to prevent hypoperfusion and further reduction of CBF during hypotension while protecting pial artery dilation after FPI was superimposed was blocked by the p38 MAPK antagonist SB 203580 co-administered with the variant at 3 h post-FPI. SB 203580 blocked the upregulation of phosphorylated p38 MAPK after FPI, demonstrating efficacy of action. These biochemical data support the pharmacological data and suggest that tPA-S⁴⁸¹A prevents impairment of cerebral autoregulation after FPI both by augmenting p38 and by inhibiting upregulation of ERK MAPK.²¹

The severity of constriction after FPI in the presence of exogenous tPA is substantial and probably not the simple result of loss of a dilator, such as NO scavenging by superoxide, but also production of a vasoconstrictor. We previously observed that the spasmogen ET-1 is upregulated and antagonizes NMDA-R-mediated dilation after FPI.¹⁹ Therefore, we investigated the influence of wt tPA and tPA-S⁴⁸¹A on CSF ET-1 levels after FPI. CSF ET-1 was elevated after FPI and increased further by exogenous wt tPA, but upregulation by both wt tPA and FPI was blocked by tPA-S⁴⁸¹A administered at 3 h post-FPI. These data suggest that tPA-S⁴⁸¹A-mediated protection of autoregulation after TBI may result in part from blocking induction of ET-1 post-TBI by endogenous tPA released as a result of injury.

ERK MAPK and ET-1 may be linked mechanistically in mediating inhibition of cerebral autoregulation after TBI. ET-1 promotes ERK MAPK upregulation, which contributes to impaired autoregulation after FPI in the piglet,³³ and inhibition of ERK MAPK prevents upregulation of ET receptors and reduction of CBF in rat models of subarachnoid hemorrhage and middle cerebral artery occlusive ischemic stroke.^{34–36} Therefore, it is possible that ERK MAPK may both upregulate ET-1 and contribute to vasoconstriction by this peptide in the setting of TBI, resulting in a feed forward cycle. TBI is not stroke. However, the etiology, pathophysiology, and the critical care pathways for treatment are related, but differ in important ways. In the stroke model used by the Edvinsson group, the ET-B receptor was observed to be the primary ET receptor contributory to vasoconstriction.^{34–36} In contrast, our data show that it is the ET-A receptor that is key to vasoconstriction after FPI in the piglet.^{19,33} Because the p38 inhibitor SB 203580 modestly reversed the total blockade of ET-1 upregulation observed with tPA-S⁴⁸¹A alone, protection of autoregulation by this tPA variant after TBI may result in part from p38 blocking signaling pathways that lead to the induction of ET-1 post-insult. The concomitant block of ERK MAPK and ET-1 may act in concert with the augmentation of p38 MAPK by tPA-S⁴⁸¹A to protect cerebral autoregulation after FPI.

There are several potential clinical implications of the observed relationship between tPA and NMDA-R induced vascular activity. For example, hypotension is an important predictor of poor outcome after TBI in children and adults.^{37,38} Hypotension may be more detrimental to immature than to mature brain.³⁹ Cerebral autoregulation during hypotension is known to be impaired after TBI in the pediatric population.⁴⁰ When autoregulation is impaired, hypotension decreases cerebral perfusion pressure (CPP) and CBF. Decreases in CPP exacerbate NMDA-R induced vasoconstriction, which leads to ischemia, cell swelling,

and edema. The latter leads to increased intracranial pressure (ICP). Increases in ICP decrease CPP further, initiating a vicious cycle.⁴¹

There are also several experimental caveats that may limit data interpretation. First, the focus of this study was on the role of NMDA-R over-activation in impaired cerebral hemodynamics and autoregulation and not histopathology. We reported that tPA-S⁴⁸¹A prevented neuronal cell necrosis in CA1 and CA3 hippocampus when administered 30 min post-TBI.²¹ Future studies will be needed to determine if prevention of disturbed autoregulation when tPA-S⁴⁸¹A is administered at 3 h post-injury (as in this study) prevents histopathology from developing to the same extent. Later time points and varying the dose of tPA-S⁴⁸¹A post-TBI will also need to be investigated to further refine the clinical therapeutic window. Second, the experimental design did not allow us to determine the cellular origin of the MAPK isoforms we detect in CSF, which include neurons, glia, vascular smooth muscle, and endothelial cells. While we were initially surprised to detect the products of an intracellular signaling system in the CSF under sham control conditions, we make the point that this does not reflect damage or pathology. In response to peer review concerns over the years, we have reproducibly detected MAPK in CSF under control conditions and monitored its change in response to diverse stimuli.^{14,18} In particular, we documented parallel changes in tissue and CSF in response to FPI and hypoxia/ischemia using qualitative immunohistochemistry, semi-quantitative Western, and quantitative ELISA of CSF.^{14,42} Therefore, changes in CSF MAPK reflect changes occurring within the tissue. Finally, the use of three tPA mutants does focus attention on glutamate toxicity but did not exclude the possibility that tPA-S⁴⁸¹A also prevents microhemorrhaging. It is unlikely, however, that we overlooked sufficient hemorrhage to explain the changes in autoregulation and CBF we observed.

Conclusion

These studies extend our previous observations that tPA-S⁴⁸¹A provides neuroprotection when given 30 min after TBI in several ways.²¹ First, we found that tPA-S⁴⁸¹A protects cerebrohemodynamics when administered 3 h post-trauma, entering a clinically relevant therapeutic window. Second, it appears likely that a concerted change in MAPK isoform profile contributes to outcome. In addition to blocking upregulation of ERK MAPK, tPA-S⁴⁸¹A upregulates p38 MAPK, which in turn inhibits the upregulation of ET-1 that occurs after TBI, which is known to impair cerebral autoregulation. Third, the use of three tPA variants with separate but overlapping functions focuses attention on glutamate/NMDA-R toxicity as an important mechanism underlying the impairment of cerebral autoregulation after TBI and suggests a potential therapeutic. Additional studies will be needed to delineate the efficacy and safety of tPA-S⁴⁸¹A in the prevention of neurotoxicity post-TBI.

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Author Disclosure Statement

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