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ORIGINAL ARTICLE

Release of IL-6 After Stroke Contributes to Impaired Cerebral Autoregulation and Hippocampal Neuronal Necrosis Through NMDA Receptor Activation and Upregulation of ET-1 and JNK

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Abstract

The sole FDA-approved drug treatment for ischemic stroke is tissue-type plasminogen activator (tPA). However, upregulation of JNK mitogen-activated protein kinase (MAPK) and endothelin 1 (ET-1) by tPA after stroke contributes to impaired cerebrovascular autoregulation. Wild-type (wt) tPA can bind to the lipoprotein-related receptor (LRP), which mediates vasodilation, or NMDA receptors (NMDA-Rs), exacerbating vasoconstriction. Elevations in IL-6, a marker of inflammation that accompanies stroke, are reported to be an adverse prognostic factor. We hypothesized that IL-6 released into CSF after stroke by wt-tPA through activation of NMDA-Rs and upregulation of ET-1 and JNK contribute to impairment of cerebrovascular autoregulation and increased histopathology. Results show that IL-6 was increased post stroke in pigs, which was increased further by wt-tPA. Co-administration of the IL-6 antagonist LMT-28 with wt-tPA prevented impairment of cerebrovascular autoregulation and necrosis of hippocampal cells. wt-tPA co-administered with the JNK inhibitor SP 600125 and the ET-1 antagonist BQ 123 blocked stroke-induced elevation of IL-6. Co-administration of LMT-28 with wt-tPA blocked the augmentation of JNK and ET-1 post stroke. In conclusion, IL-6 released after stroke, which is enhanced by wt-tPA through activation of NMDA-Rs and upregulation of ET-1 and JNK, impairs cerebrovascular autoregulation and increases histopathology. Strategies that promote fibrinolysis while limiting activation of NMDA-Rs and upregulation of IL-6 may improve the benefit/risk ratio compared to wt-tPA in treatment of stroke.

Keywords Cerebral circulation · Inflammatory mediators · Plasminogen activators · Signal transduction · Ischemia

Introduction

Cerebrovascular autoregulation is a homeostatic mechanism by which the neurovascular unit (NVU) regulates cerebral blood flow (CBF) across a range of blood pressures. NVU-mediated impairment of autoregulation following traumatic brain injury (TBI) is linked to Glasgow Coma Scale (GCS), with greater autoregulatory impairment associated with worse GCS [1]. We hypothesize that these functions of the NVU link impaired cerebrovascular hemodynamics with impaired cognitive function.

The thrombolytic agent tissue-type plasminogen activator (tPA) remains the only approved medical treatment for ischemic stroke [2]. However, tPA is also directly neurotoxic [3], which may derive in part from its ability to exacerbate uncoupling of CBF and metabolism post stroke through aggravation of pre-existing hyperactivity of *N*-methyl-D-aspartate receptors (NMDA-Rs). Glutamatergic hyperactivity occurs in animal models of stroke even in the absence of exogenous tPA [4], and NMDA-R antagonists improve outcome [5, 6]. Binding of glutamate to NMDA-Rs elicits cerebrovasodilation which couples local metabolism to CBF [6, 7] and thereby maintains cerebrovascular autoregulation, but may also foster excess excitoxicity [8–10]. However, the mechanism underlying the transition of NMDA-R activation from vital to neurotoxic effects [8–10] is uncertain.

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Many studies of cerebral ischemia and stroke have been performed in rodent models. Pigs offer a unique advantage in elucidating pathways involved in CNS ischemic injury by virtue of having a gyrencepahalic brain that contains substantial white matter similar to humans, which is more sensitive to ischemic damage than gray matter.

This study is based on prior findings in pigs after thrombotic stroke in which we have shown that tPA impairs cerebrovascular autoregulation in part by activating NMDA-Rs [8, 11, 12]. Although the NMDA-R antagonist MK 801 protects against cerebrovascular dysregulation after stroke [8, 12], its toxicity limits usage in humans indicating the need for novel approaches. After traumatic brain injury, release of cytokines such as IL-6 has been implicated in neuronal inflammation and cell death [13, 14]. Elevation in these cytokines is also thought to be an adverse prognostic factor in the setting of stroke [15–17]. However, the mechanism by which IL-6 and other cytokines are induced and the mechanism by which they contribute to adverse outcome after stroke are uncertain.

Based on our finding that the "docking site" in tPA is required to activate NMDA-Rs [18], we constructed a variant, tPA-K^{296}A/H^{297}A/R^{298}A/R^{299}A (tPA-$A^{296–299}$) that prevents the enzyme from binding to these receptors while retaining fibrinolytic activity. We recently found that tPA-$A^{296–299}$ prevents acute impairment of cerebrovascular autoregulation when given in a therapeutically relevant timeframe after ischemic stroke in pigs [12, 19] by blocking further upregulation in the synthesis of endothelin-1 (ET-1) and the c-Jun-terminal kinase (JNK) isoform of mitogen-activated protein kinase (MAPK) after stroke by endogenous tPA [12, 19]. tPA-$A^{296–299}$ limits both infarct volume and neuronal cell necrosis in the hippocampus, a brain region recognized as important in learning and memory, by inhibiting upregulation of JNK and ET-1 [12, 19]. Because activation of the extracellular signal-related kinase (ERK) isoform of MAPK increases the CSF concentration of IL-6 after TBI in the pig [14], we wondered if JNK could contribute to release of IL-6 after stroke.

The present study is predicated on the hypothesis that IL-6 released into the CSF and brain tissue after stroke by wild type (wt-tPA) through activation of NMDA-Rs and upregulation of ET-1 and JNK contributes to impairment of cerebral autoregulation and increased histopathology.

Materials and Methods

Anesthetic Regimen, Closed Cranial Window Technique, and Cerebral Photothrombosis

Yorkshire pigs (1.1–1.6 kg; 2–7 days 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, Orion Finland), induction with isoflurane (2–3%; Abbott North Chicago IL), isoflurane taper to 0% after start of total intravenous anesthesia (TIVA) with fentanyl (200 μg/kg/h; Hospira Lake Forest, IL), midazolam (1 mg/kg/h; Hospira), dexmedetomidine (2 μg/kg/h), and propofol (2–10 mg/kg/h; Zoetis Kalamazoo MI), and maintenance of TIVA 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 CSF for ELISA analysis [8]. 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). Such drops in blood pressure were maintained constant for 10 min by titration of additional blood withdrawal or blood reinfusion.

Induction of photothrombosis was based on a modification of that previously described for the pig [20]. In our studies, we used the area of the closed cranial window to expose two to three main and one to three smaller arteries supplying the territory of the middle cerebral artery. 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 peroxidative endothelial damage. The photochemical reaction results from the interaction of the iv photosensitizing dye erythrosine B (20 mg/kg iv) with the focused beam of a solid-state laser operated at 532 nm, power of 200 mW, average intensity of 60–75 W/cm^2, and durations of up to 3–5 min using a Snake Creek minilaser (Hallstead, PA).

Protocol

Pial small arteries (resting diameter, 120–160 μm) were studied. For sample collection, 300 μl from the total cranial window volume of 500 μl 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.

Pigs were randomized to one of the following experimental groups (all $n = 5$): (1) sham control, (2) photothrombosis (PTI), (3) PTI + wt-tPA (1 mg/kg iv), (5) PTI + tPA-$A^{296–299}$, (6) PTI + wt-tPA + BQ 123 (1 mg/kg), (7) PTI + wt-tPA + SP 600125 (1 mg/kg iv), and (8) PTI + wt-tPA + LMT-28 (1 mg/kg iv). The vehicle for all agents was 0.9% saline. In sham control animals and those studied post photothrombotic injury, responses to hypotension and isoproterenol (10^{-8}, 10^{-6} M) were obtained initially and then again 5 h later in the presence of vehicle. In treated animals, drugs

were administered 4 h after photothrombotic 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 [8].

Histologic Preparation

The brains were prepared for histopathology at 6 h post stroke. The brains were perfused with heparinized saline, followed by 4% paraformaldehyde. For histopathology, staining was performed on paraffin-embedded slides and serial sections were cut at 30-μm intervals from the front face of each block and mounted on microscope slides. The sections (6 μm) were stained with hematoxylin and eosin (HE) for histopathology and NeuN to identify the cells as being neurons. The mean number of necrotic neurons (± SEM) in CA1 and CA3 hippocampus in vehicle control, stroke, stroke + tPA, and stroke + tPA + LMT-28-treated animals was determined, with data displayed for the sides of the brain both ipsilateral and contralateral to the site of injury. Morphologic criteria for a necrotic neuron are (1) pyknosis, (2) granulation of the cytoplasm, and (3) the emergence of an unstained area between the nucleus and the cytoplasm. The investigator was blinded to treatment group. Neuronal pathology scoring was described based on damaged neurons/1.2 mm^2 of a specific anatomic region as either mild (1–5), moderate (6–15), or severe (> 15).

ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to quantity CSF JNK-MAPK and ET-1 (Assay Designs, Farmingdale, NY) and IL-6 (Abcam) concentration.

Statistical Analysis

Pial artery diameter, CSF ET-1, JNK MAPK, IL-6, and histopathologic values were analyzed using ANOVA for repeated measures and consideration given towards parametric analysis. If the value was significant, the data were then analyzed by Fishers protected least significant difference test. An α level of $p < 0.05$ was considered significant in all statistical tests. Values are represented as mean ± SEM of the absolute value or as percentage changes from control value. All data were included in analysis. Power analysis from prior studies shows that a sample size of 5 for hemodynamic data sets will yield statistical significance at the $p < 0.05$ level with power of 0.84. Similar analyses for histopathology and biochemical indices (JNK MAPK) have powers of 0.83 and 0.86, respectively.

Results

Induction of IL-6 by wt-tPA Contributes to Impairment of Cerebral Autoregulation After Thrombotic Stroke

This model of thrombotic stroke in the pig caused moderate levels of injury that was exacerbated by the administration of wt-tPA administration [12, 19]. The concentration of IL-6 in the CSF was increased at 5 h following the onset of stroke. The increase in IL-6 was enhanced by administration of wt-tPA but blocked by tPA-$A^{296–299}$ at 4 h post insult (Fig. 1). In sham control animals, pial arteries dilate during hypotension, indicating intact cerebrovascular autoregulation (Fig. 2). However, vasodilation was blunted after stroke, indicating that autoregulation was disturbed (Fig. 2). Vasodilation in response to isoproterenol was unchanged after stroke, indicating

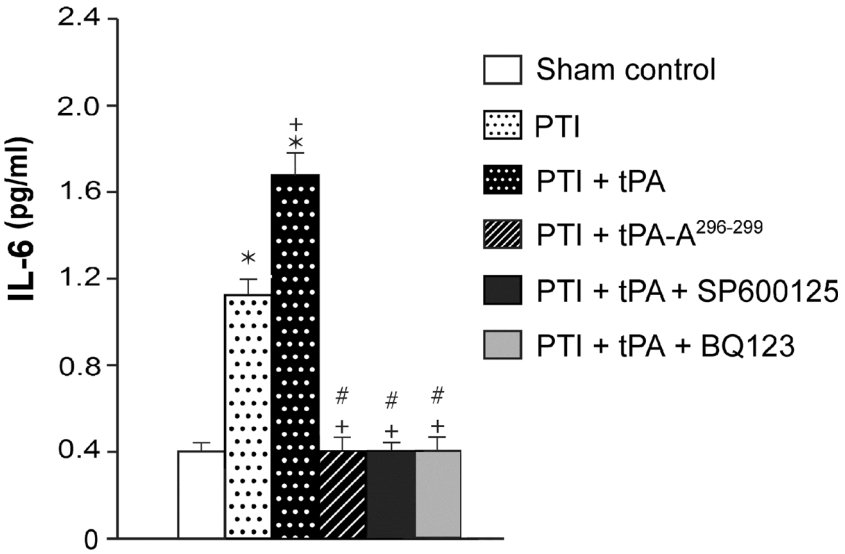

Fig. 1 CSF IL-6 (pg/ml) prior to thrombotic stroke (sham control), or 4 h after stroke (PTI), or after 4 h after administration of wt-tPA (1 mg/kg iv), tPA-$A^{296–299}$ (1 mg/kg iv), tPA + SP 600125 (1 mg/kg iv), or tPA + BQ 123, $n = 5$. $^{*}p < 0.05$ versus corresponding sham control value; $^{+}p < 0.05$ versus corresponding untreated PTI value; $^{\#}p < 0.05$ versus PTI + tPA value

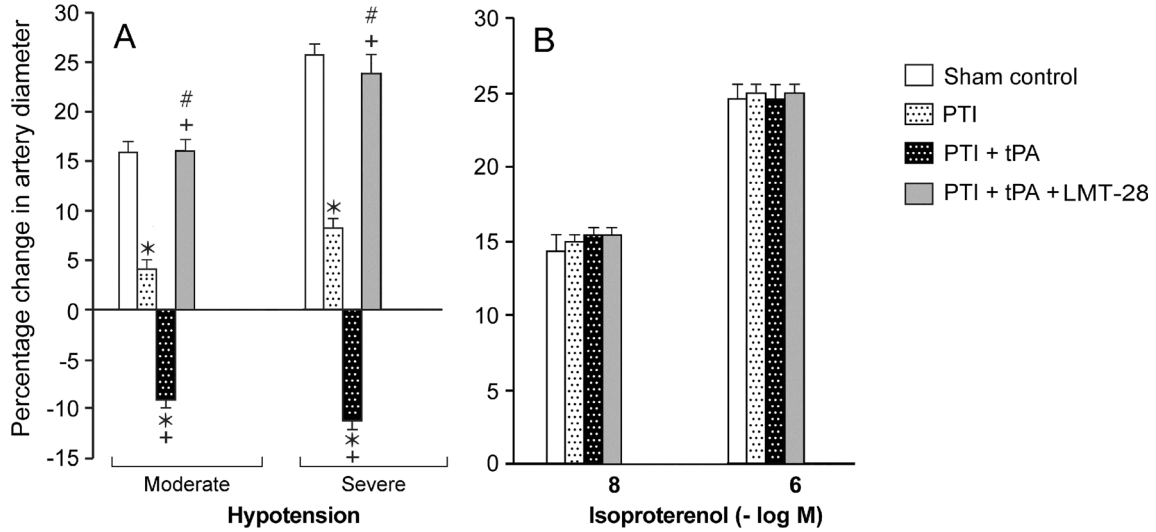

Fig. 2 Influence of hypotension (moderate, severe) and isoproterenol (10^{-8}, 10^{-6} M) on pial artery diameter in pigs before (sham control), 4 h after photothrombotic injury (PTI), or 4 h after administration of wt-tPA (1 mg/kg iv), or wt-tPA + LMT-28 (1 mg/kg iv), $n = 5$. $^{*}p < 0.05$ versus corresponding sham control value; $^{+}p < 0.05$ versus untreated PTI value; $^{\#}p < 0.05$ versus PTI + wt-tPA value

that this response was not an epiphenomenon (Fig. 2). Administration of wt-tPA (1 mg/kg iv) reversed pial artery dilation to vasoconstriction (Fig. 2). Prior studies have shown that wt-tPA produced a transient increase in CBF after stroke [12, 19], indicating effective recanalization. Co-administration of the IL-6 antagonist LMT-28 [21] with wt-tPA prevented impairment of autoregulatory pial artery dilation, suggesting a role for IL-6 in vascular derangement post stroke (Fig. 2).

IL-6 Contributes to Hippocampal Histopathology After Stroke

To investigate the implications of IL-6 upregulation after stroke, we investigated neuronal cell integrity in the hippocampus. Stroke increased the number of necrotic neurons in the CA1 and CA3 regions of the hippocampus in the ipsilateral, but not in the contralateral hemisphere, demonstrating the focal nature of the injury (Fig. 3). Administration of wt-tPA (4 h post stroke) increased the number of necrotic neurons in CA1 and CA3 (Fig. 3). In contrast, co-administration of LMT-28 with wt-tPA attenuated CA1 and CA3 neuronal cell loss and the brains looked remarkably similar to sham controls (Fig. 3).

Reciprocal Relationships Between IL-6, JNK MAPK, and ET-1 Leading to Impaired Cerebral Vascular Reactivity After Stroke

Co-administration of either the JNK inhibitor SP600125 or the ET-1 antagonist BQ 123 blocked the potentiation in the rise of IL-6 levels in the CSF induced by wt-tPA following stroke (Fig. 1). Stroke elevated the CSF concentrations of ET-1 and phosphorylated (activated) JNK MAPK (Fig. 4), which was also potentiated by administration of wt-tPA, consistent with previous findings [12, 19]. Co-administration of LMT-28 with wt-tPA blocked elevations of CSF JNK and ET-1 (Fig. 4). These data indicate that IL-6 release by stroke and the augmentation seen after administration of wt-tPA are mediated through activation of JNK and ET-1. All three mediators (IL-6, JNK, and ET-1) are increased after administration of wt-tPA and likely contribute to the impairment of cerebrovascular autoregulation after stroke possibly through a reciprocal feed-forward pathway (Fig. 2)[12, 19].

CSF IL-6 Released After Stroke by wt-tPA Acts Through NMDA-Rs to Impair Cerebrovascular Autoregulation

Elevation of CSF IL-6 after stroke is augmented by exogenous wt-tPA (Fig. 1). This suggests that endogenous wt-tPA released in the setting of stroke is mediated through NMDA-Rs. Administration of tPA-$A^{296–299}$ also prevents stroke- and wt-tPA-associated elevations in CSF ET-1 and JNK, along with providing protection against stroke-associated increases in infarct volumes and hippocampal cell histopathology [12, 19]. These data indicate that IL-6 acts through NMDA-Rs to impair cerebrovascular autoregulation and increase hippocampal cell necrosis after stroke (Figs. 1 and 3).

Blood Chemistry

Blood chemistry values were collected before and after all experiments. There were no statistical differences between sham control, photothrombosis, and photothrombosis antagonist-treated animals. There were no differences in mean arterial blood pressure among groups.

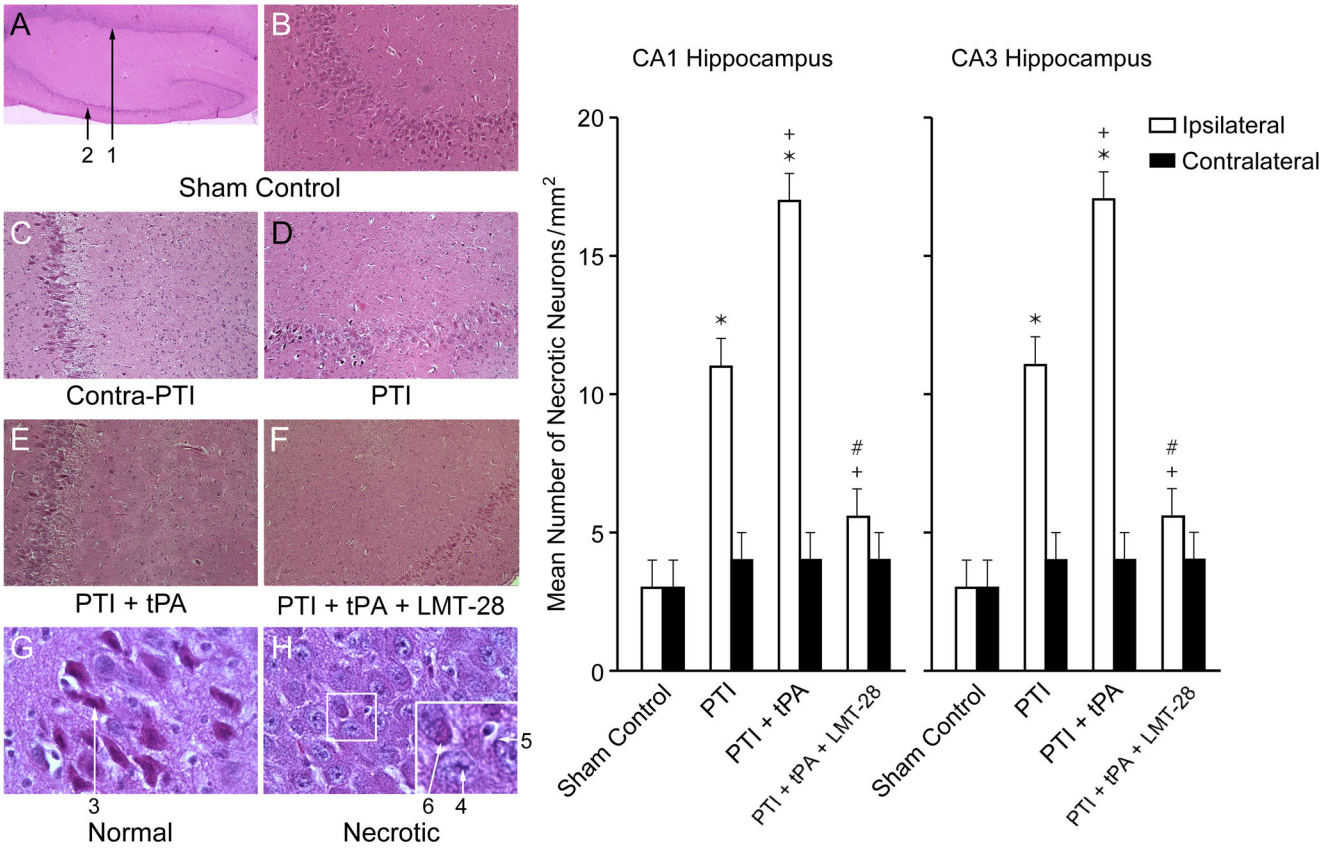

Fig. 3 **a** Low magnification (40×) showing CA1 (#1) and CA3 (#2) hippocampal regions from typical sham control. **b** Higher magnification (100×) of typical sham control CA3 hippocampus. **c** Typical appearance of CA3 hippocampus contralateral to stroke (100×). **d** Typical appearance of CA3 hippocampus ipsilateral to stroke (100×). **e** Typical appearance of CA3 + tPA ipsilateral to stroke (100×). **f** Typical appearance of CA3 + tPA + LMT-28 ipsilateral to stroke (100×). **g** High magnification (600×) of typical viable sham control neurons (#3) with intact cytoplasm and darkly stained nucleus. **h** High magnification (600×) of typical post stroke necrotic neurons, showing pyknotic nucleus of small neuron (#4), accompanied by neuronal cytoplasm shrinkage (#5) and granulated eosinophilic characteristics (#6) associated with cell death ("red dead" neuron). Summary data showing mean number of necrotic neurons (**i**) in CA1 and CA3 hippocampus ipsilateral and contralateral to stroke in newborn pigs under conditions of sham control, stroke (PTI), PTI + tPA, and PTI + tPA + LMT-28, $n = 5–6$. $^{*}p < 0.05$ compared to corresponding sham control value; $^{+}p < 0.05$ compared to corresponding PTI alone value; $^{\#}p < 0.05$ versus PTI + tPA value

Discussion

Several new findings emerged from this study. First, IL-6 levels in the CSF rose after stroke and contributed to impairment of cerebral autoregulation. Soluble mediators of inflammation, and IL-6 in particular, have been considered to be predictors of mortality in long-term survivors after stroke, although the mechanism by which they impair neuronal function is only partially understood [15–17]. IL-6 plays an important role in host defense. However, overexpression of

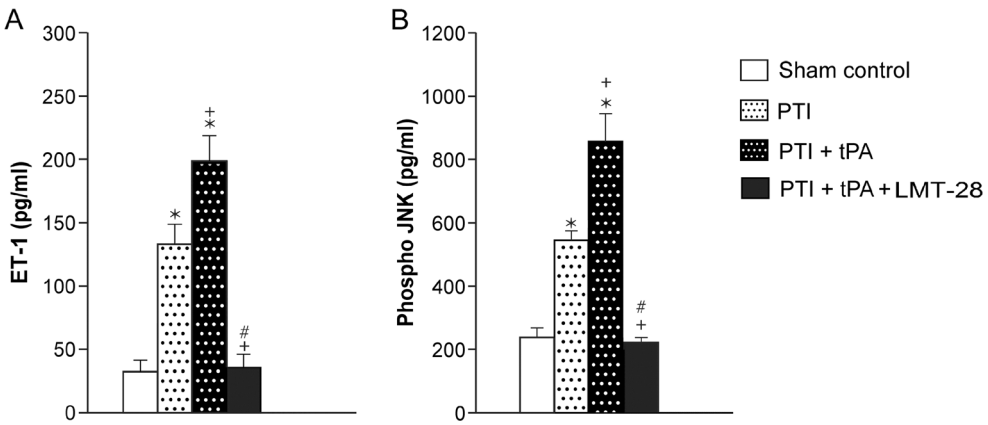

Fig. 4 CSF ET-1 and phosphorylated JNK MAPK prior to thrombotic stroke (sham control), 4 h after stroke (PTI), or 4 h after administration of wt-tPA (1 mg/kg iv), or tPA + LMT-28 (1 mg/kg iv), $n = 5$. $^{*}p < 0.05$ versus corresponding sham control value; $^{+}p < 0.05$ versus corresponding untreated PTI value; $^{\#}p < 0.05$ versus PTI + tPA value

IL-6 also contributes to host injury in the setting of inflammation, autoimmunity, or cancer [21, 22], among others. Blockade of IL-6 by antibody has been investigated as an adjunct to treat inflammatory diseases that are refractory to conventional therapy [21]. This study is the first to our knowledge to describe an effect of IL-6 on brain histopathology by impeding CBF.

Cerebrovascular autoregulation is a homeostatic mechanism by which the NVU regulates CBF across a range of blood pressures. Failure to match blood flow to the metabolic needs of neuronal tissue contributes to cell death after stroke. In our study, the rise of IL-6 after stroke impaired cerebrovascular autoregulation and led to hippocampal neuronal cell necrosis that were prevented by the IL-6 antagonist LMT-28. Similar NVU-mediated impairment of autoregulation following TBI has been linked to GCS, i.e., greater autoregulatory impairment associated with worsening GCS [1]. While many areas of the brain are affected, it is generally accepted that damage to the hippocampus impairs learning and memory. Based on this, we speculate that these NVU functions link cerebrovascular hemodynamics with cognitive dysfunction and that attenuation of IL-6-mediated impaired CBF may mitigate cognitive dysfunction after stroke.

A second important finding relates to the relationship between IL-6 and tPA. Administration of wt-tPA after stroke increased the extent of tissue injury in this model. The concentration of IL-6 in the CSF was increased following stroke, and this increase in IL-6 was enhanced by administration of wt-tPA. In sham control animals, pial arteries dilate during hypotension, indicating intact cerebrovascular autoregulation. However, vasodilation was blunted after stroke, indicating disturbed autoregulation. Administration of wt-tPA reversed pial artery dilation to vasoconstriction but co-administration of the IL-6 antagonist LMT-28 with wt-tPA prevented this impairment of autoregulatory pial artery dilation. As the time window within which LMT-28 was administered (4 h post stroke) is clinically relevant, our data lead us to suggest that therapeutic targeting of IL-6 may afford a new adjuvant treatment paradigm when tPA is given to treat acute ischemic stroke.

A third important finding of this study relates to the intracellular signaling mechanism whereby IL-6 may impair outcome post stroke. We observed a reciprocal relationship between IL-6, JNK MAPK, and ET-1 that contributes to the impairment of cerebrovascular reactivity after stroke. The potentiation in the rise of IL-6 in the CSF by exogenous wt-tPA following stroke was blocked by the co-administration of either the JNK inhibitor SP600125 or the ET-1 antagonist BQ123. Stroke led to an elevation in the concentrations of ET-1 and phosphorylated (activated) JNK MAPK in the CSF, which was potentiated by administration of wt-tPA, as demonstrated previously [12, 19]. Co-administration of LMT-28 with wt-tPA blocked elevations of CSF JNK and ET-1. These data indicate that IL-6 release by stroke, and its augmentation by wt-tPA via JNK and ET-1, can reciprocally lead to further release of ET-1 and enhanced phosphorylation of JNK in a feed-forward cycle. Thus, each of three mediators (IL-6, JNK, and ET-1) contributes to impairment of cerebral autoregulation after stroke, and each is increased following administration of wt-tPA [12, 19]. These observations suggest that inhibiting the action of IL-6 would interrupt this feed-forward cycle and help preserve CBF and limit ischemic damage. These observations further support the therapeutic targeting of this inflammatory mediator as an adjuvant to tPA administration after stroke.

Finally, our data implicate NMDA-Rs in the induction and adverse effects of IL-6. wt-tPA is capable of binding to the lipoprotein-related receptor (LRP), which mediates vasodilation and permeability of the NVU [23], as well as to NMDA-Rs, which inhibits these changes in the behavior of the vasculature [12, 19] (Fig. 5). We propose that after stroke, the NMDA-R-dependent pathway predominates due to release of high local concentrations of endogenous (i.e., wt) tPA and endogenous glutamate and that this pathway is markedly enhanced when wt-tPA is administered as therapy [12, 19] (Fig. 5). This directly impacts the mechanisms responsible for compensatory vasodilation. We have found that administration of wt-tPA stimulates phosphodiesterase activity, which overrides LRP-dependent signaling and leads to a net decrease in cAMP and p38 (both of which promote dilation), and elevation of ET-1, and the JNK isoform of MAPK (both of which promote vasoconstriction), which together impair cerebral vasodilation in the pig [12, 19] (Fig. 5). Based on our finding that the docking site in tPA is required to activate NMDA-Rs [18], we constructed a variant, tPA-A$^{296–299}$, that prevents the

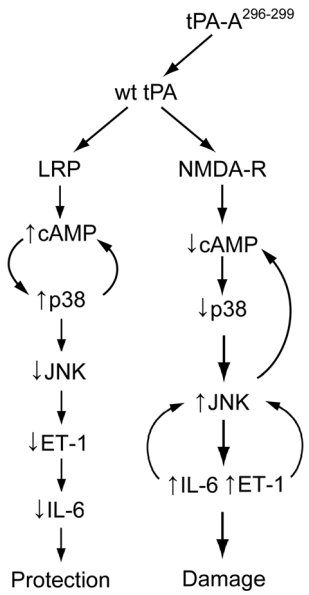

Fig. 5 Proposed signaling pathways and outcomes after stroke and administration of wt-tPA or tPA-A$^{296–299}$. Feed-forward reinforcing pathways and changes in the two pathways following stroke are emphasized. Under physiological conditions, the equilibrium between the LRP and NMDA-R pathways is shifted to the left (LRP), while under pathologic conditions (stroke), the equilibrium is shifted to the right (NMDA-R)

enzyme from binding to NMDA-Rs but retains fibrinolytic activity. tPA-A$^{296–299}$ prevents acute impairment of cerebrovascular autoregulation when given in a therapeutically relevant timeframe after stroke by increasing cAMP and p38, while simultaneously decreasing JNK and ET-1 [12, 19] (Fig. 5). We have recently shown that administration of tPA-A$^{296–299}$ prevents stroke-associated infarct volume and hippocampal cell histopathology [12, 19]. Data in the present study show that tPA-A$^{296–299}$ blocks stroke-induced increase of IL-6 in the CSF (Fig. 5). In view of this finding in the present study that IL-6 contributes to impaired cerebral autoregulation and hippocampal cell necrosis after stroke, these data together indicate that it does so through an NMDA-R-mediated mechanism.

Study Design Limitations

There are several experimental caveats in the present study that should be considered. First, histology was assessed at a single early time point (6 h post injury). While early neuronal cell death may therefore be induced by activation of NMDA-Rs through endogenous or exogenous wt-tPA, the correlation between normalization of hypoperfusion by tPA-A$^{296–299}$ [19] and prevention of hippocampal neuronal cell necrosis argues against this simple explanation. Rather, these data support an explanation based on hemodynamics as opposed to a direct neurotoxic effect of NMDA-R and wt-tPA in histopathology after stroke. Second, we did not include some measures of neurobehavioral outcome that could be correlated with neuronal injury and hemodynamics. Additional studies will be needed to determine if prevention of NMDA-R-mediated loss of NVU integrity provides durable improvement in cerebral hemodynamics and cognitive function after thrombotic stroke. A third caveat concerns the method we used to test the intactness of autoregulation (hypotension via blood withdrawal), which is not one that occurs commonly in the clinical setting. However, our conclusions are supported by the fact that comparable outcomes are seen when intactness of autoregulation is measured using the transient hyperemic response ratio, a technique used clinically [24], when applied to models of TBI in the piglet [25]. An additional experimental caveat relates to the age of animal studied. Study of 2–7–day-old pigs may limit applicability of our findings to other settings. Most strokes occur in the adult/older human population, although stroke in the pediatric setting may occur in as many as 1 in 4000 births [26]. Similar studies in adult pigs would be cumbersome due to their girth, a problem that might be overcome by the use of mini pigs in future work.

The experimental design used in these studies did not allow us to determine the cellular sources of the CSF IL-6, JNK MAPK, and ET-1. These mediators were assayed in CSF which reflects potential contributions from multiple sites of origin. Such values are therefore used as an indirect index of what may happen to cellular concentrations within brain parenchyma. However, these findings are unlikely to reflect tissue damage because we have detected MAPK in CSF under control conditions and monitored its change with diverse stimuli [27, 28]. Additionally, there are diverse targets downstream of IL-6, JNK, and ET-1 that were not investigated in the present study. Of interest, protein kinase C (PKC) and cyclooxygenase (COX) have been shown by us to mediate release of oxygen free radicals by ET-1 in the setting of TBI [29] and oxygen free radicals activate JNK [30]. Another isoform of MAPK, extracellular signal-related kinase, has been observed to be both activated by and to mediate the release of oxygen free radicals [31, 32]. It is therefore possible that PKC and/or COX-induced upregulation of activated oxygen may be central to the observed ability of ET-1 to activate JNK, which, in turn, appears to reciprocally release ET-1. Relationships between IL-6 and signaling systems such as PKC and COX will be addressed in future experiments.

Lastly, as mentioned above, it will be necessary to develop tests to measure cognitive and other impairments in pigs subjected to stroke to validate the relationship between impaired NVU function, cytotoxicity, and neurologic outcomes.

Clinical Implications and Conclusions

The use of tPA in the treatment of acute ischemic stroke remains challenging, given the narrow therapeutic window, risk of bleeding, and the potential to enhance neuronal excitoxicity. Data from the present study suggest that adjuvant therapy with an inhibitor of IL-6 or its receptor may improve the benefit/risk ratio of therapy with tPA. These data also provide additional support for the use of tPA variants that retain fibrinolytic activity, but do not elicit NMDA-R-mediated excitotoxic neuronal cell death as means to improve outcome in patients with acute ischemic stroke.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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