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RBC-coupled tPA Prevents Whereas tPA Aggravates JNK MAPK-Mediated Impairment of ATP- and Ca-Sensitive K Channel-Mediated Cerebrovasodilation After Cerebral Photothrombosis

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Abstract

The sole Food and Drug Administration-approved treatment for acute stroke is tissue-type plasminogen activator (tPA), but tPA aggravates impairment of cerebrovasodilation during hypotension in a newborn pig photothrombotic model of stroke. Coupling to carrier red blood cells (RBC) enhances thrombolytic effects of tPA, while reducing its side effects. ATP- and Ca-sensitive K channels (K_{atp} and K_{ca}) are important regulators of cerebrovascular tone and mediate cerebrovasodilation during hypotension. Mitogen-activated protein kinase, a family of at least three kinases, ERK, p38, and c-Jun-N-terminal kinase (JNK), is upregulated after photothrombosis. This study examined the effect of photothrombosis on K_{atp}- and K_{ca}-induced cerebrovasodilation and the roles of tPA and JNK during/after injury. Photothrombosis blunted vasodilation induced by the K_{atp} agonists cromakalim, calcitonin gene-related peptide, and the K_{ca} agonist NS 1619, which was aggravated by injection of tPA. In contrast, both pre- or post-injury thrombosis injection of RBC-tPA and JNK antagonist SP 600125 prevented impairment of K_{atp}- and K_{ca}-induced vasodilation. Therefore, JNK activation in thrombosis impairs K channel-

mediated cerebrovasodilation. Standard thrombolytic therapy of central nervous system ischemic disorders using free tPA poses the danger of further dysregulation of cerebrohemodynamics by impairing cation-mediated control of cerebrovascular tone, whereas RBC-coupled tPA both restores reperfusion and normalizes cerebral hemodynamics.

Keywords

Cerebral circulation; Newborn; Plasminogen activators; Signal transduction; Ischemia

Introduction

Neonatal stroke may occur in as many as 1 in 4,000 births [1]. Maternal and perinatal coagulopathy predispose to perinatal stroke [2, 3], with 30% of neonatal strokes being due to thrombosis [4]. A better understanding of the pathophysiologic responses that occur in children after ischemic stroke is needed to develop mechanism-based approaches to therapy. One contributor to neurological damage after ischemic stroke may be cerebrovascular dysfunction. Hypotension leads to loss of cerebrovascular regulation promoting tissue ischemia, while cerebrovaso-constriction associated with hypocapnia contributes to periventricular leukomalacia in the perinate [5]. We have shown that pial artery dilation in response to hypotension and hypercapnia is blunted in a piglet photothrombotic model of ischemic stroke [6, 7].

Relaxation of blood vessels can be mediated by several mechanisms, including cGMP, cAMP, and K^+ channels [8]. Membrane potential of vascular muscle is a major determinant of vascular tone, and activity of K^+ channels is a major regulator of membrane potential [8]. Activation or opening of these channels increases K^+ efflux, producing hyperpolarization of vascular muscle. Membrane hyperpolarization closes voltage-dependent calcium channels and causes relaxation of vascular muscle. Direct measurements of membrane potential and K^+ current in vitro indicate that several types of K^+ channels are present in cerebral blood vessels. In addition, a number of pharmacological studies using activators and inhibitors have provided functional evidence that K^+ channels, especially ATP-sensitive (Katp) and calcium-sensitive (Kca) channels, regulate cerebrovascular tone [8]. Cromakalim and calcitonin gene-related peptide (CGRP) are prototypical Katp agonists; NS 1619 a Kca agonist. Vasodilation induced by these drugs is as an index of the intactness of K channel function after traumatic brain injury and cerebral ischemia [9, 10]. Pial artery dilation in response to hypotension is due to activation of Katp and Kca channels [11], giving functional significance to intactness of K channel function.

Vascular injection of recombinant tissue-type plasminogen activator (tPA) is the only Food and Drug Administration-approved treatment for stroke [12]. However, tPA exhibits deleterious as well as beneficial effects that profoundly constrain its clinical utility. In addition to its salutary effect of reperfusion mediated by dissolution of intravascular thrombi, tPA contributes to bleeding, excito-toxic neuronal cell death [13], and increases stroke infarct volume in mice [14]. Systemic administration of tPA within a clinically relevant timeframe after photothrombotic vascular injury in piglets aggravates impairment of cerebrovasodilation [6, 7], which is blocked by co-administration of a PA inhibitor-1-derived peptide [6].

Contemporaneous studies demonstrate that anchoring tPA on carrier red blood cells (RBC) endows the resultant complex, RBC-tPA, with dramatically prolonged circulation time enabling thromboprophylaxis to be attained at relatively low doses compared with its soluble counterpart [15–17]. In rodent models of thrombosis and traumatic brain injury and

a piglet model of photothrombosis, RBC-tPA provided effective thromboprophylaxis, rapid reperfusion, neuroprotection, and reduction in mortality, all without associated hemorrhage [7, 18, 19].

Mitogen-activated protein kinase (MAPK), a family of at least three kinases (extracellular signal-related kinase—ERK-, p38, and c-Jun-N-terminal kinase—JNK), is upregulated after cerebral ischemia [20–22]. tPA impairs dilation during hypotension following photothrombosis through upregulation of JNK MAPK [7]. This study more fully characterized this observation by examining the effects of tPA and RBC-tPA after photothrombosis on Katp- and Kca-induced pial artery dilation and JNK and p38 MAPK activation in K channel-mediated cerebrovasodilation.

Materials and Methods

Closed Cranial Window Technique and Cerebral Hypoxia/ Ischemia

Newborn pigs (1–5 days, 1.2–1.6 Kg) of either sex 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), maintained with a-chloralose (30–50 mg/kg supplemented with 5 mg/kg/h iv). A catheter was inserted into a femoral artery to monitor blood pressure. The trachea was cannulated and the animals were ventilated with room air. A heating pad was used to maintain the animals at 37–39°C, monitored rectally.

A cranial window was placed in the parietal skull of these anesthetized animals. This window consisted of three parts: a stainless steel ring, a circular glass coverslip, 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 cemented in place with dental acrylic. The volume under the window was filled with a solution, similar to cerebrospinal fluid (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.

Induction of photothrombosis was based on that described for the newborn pig [23] but 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 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 due to interaction of iv 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 60–75 W/cm², and durations of up to 3–5 min using a Snake Creek minilaser (Hallstead, PA, USA).

Protocol

Two types of pial vessels, small arteries (resting diameter, 120–160 µm) and arterioles (resting diameter, 50–70 µm) were examined to determine whether segmental differences in the effects of H/I could be identified. Typically, 2–3 ml of artificial CSF were flushed through the window over a 30-s period and excess CSF was allowed to run off through one of the needle ports.

Twelve experimental groups of animals were studied (all $n=5$): (1) sham control, vehicle pre-treated; (2) photothrombosis, vehicle pre-treated; (3) photothrombosis, pre-treated with free tPA (2 mg/kg iv); (4) photothrombosis, pre-treated with RBC-tPA conjugate (0.1 mg/kg iv); (5) photothrombosis, pre-treated with the JNK MAPK antagonist, SP 600125 (1 mg/kg iv); (6) photothrombosis, pre-treated with the p38 MAPK antagonist SB 203580 (1 mg/kg iv); (7) sham control, vehicle post-treated; (8) photothrombosis, post-treated with vehicle; (9) photothrombosis, post-treated with tPA; (10) photothrombosis, post-treated with RBC-tPA; (11) photothrombosis, post-treated with SP 600125; and (12) photothrombosis post-treated with SB 203580. The vehicle for all agents was 0.9% saline, except for the MAPK antagonists, which was dimethyl sulfoxide (stock) diluted with saline, with a maximal ratio of 1:1,000. These two types of vehicle–CSF controls had no significant effect on pial artery diameter. In sham control animals, responses to cromakalim, CGRP, and NS 1619 (10^{-8} , 10^{-6} M) were obtained initially and again 2.5 h later in the presence of agent vehicle. In photothrombosis vehicle animals, responses to vasoactive stimuli were obtained initially and then again 2.5 h post-insult in the presence of vehicle. In drug-treated animals, drugs were administered either 30 min before photothrombosis (pre-treatment) or 2 h after photothrombosis (post-treatment).

Preparation of RBC-tPA

RBCs were isolated by centrifugation from fresh anti-coagulated (heparin, 1,000 U/Kg) animal blood. Biotinylated tPA was coupled to biotinylated RBC via streptavidin, producing RBC-tPA complexes possessing 5×10^4 tPA molecules per RBC, as described previously [15, 16]. A vehicle control with RBCs in heparin plus uncoupled tPA has previously been observed to have no effect on pial artery diameter [24].

ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to quantify CSF p38, and JNK MAPK (Assay Designs) concentration. Phosphorylated MAPK isoform enzyme values were normalized to total form and then expressed as percent of the control condition.

Statistical Analysis

Pial artery diameter values were analyzed using analysis of variance for repeated measures. If the value was significant, the data were then analyzed by Fisher's protected least significant difference test. The sample populations studied were normally distributed and the power was 0.89. An α level of $p < 0.05$ was considered significant in all statistical tests. Values are represented as mean \pm SEM of the absolute value or as percentage changes from control value.

Results

tPA Aggravates, but RBC-tPA Prevents, Impairment of Pial Artery Dilation in Response to Katp and Kca Channel Agonists After Photothrombosis

Cromakalim, CGRP, and NS 1619 (10^{-8} , 10^{-6}) elicited reproducible dilation of pial small arteries. Vasodilation in response to all 3 K channel agonists was blunted after photothrombosis, and the impairment was aggravated by pre- and post-injury treatment with tPA (2 mg/kg iv) (Figs. 1, 2, and 3). Pre-treatment was 30 min prior to whereas post-treatment was 2 h after injury. In contrast, pre- and post-treatment with RBC-tPA (0.1 mg/kg iv) prevented impairment of pial artery dilation in response to cromakalim, CGRP, and NS 1619 (Figs. 1, 2, and 3). Similar observations were made in pial arterioles. For example, cromakalim (10^{-8} , 10^{-6}) dilated pial arterioles by $17 \pm 1\%$ and $32 \pm 2\%$, $6 \pm 1\%$ and $8 \pm 1\%$,

1±1% and 2±1%, 1±1% and 2±1%, 14±1% and 24±2%, and 9±1% and 14±1%, *n*=5, for sham control, photothrombosis, photothrombosis+ tPA pre-injury treatment, photothrombosis+ tPA post-injury treatment, photothrombosis+RBC-tPA pre-injury treatment, and photothrombosis+RBC-tPA post-injury treatment, respectively.

The JNK MAPK Antagonist SP 600125 Prevents but the p38 MAPK Antagonist SB 203580 Aggravates Impairment of Pial Artery Dilation in Response to Katp and Kca Channel Agonists after Photothrombosis

Pre- and post-injury treatment with SP 600125 (1 mg/kg iv) prevented impairment of dilation of small pial arteries in response to cromakalim and CGRP, but not NS 1619 after photothrombosis (Figs. 1, 2, and 3). This dose of SP 600125 inhibited JNK MAPK upregulation after photothrombosis [7]. In contrast, SB 203580 aggravated impairment of all 3 K channel agonists after photothrombosis (Figs. 1, 2, and 3) at a dose that inhibited p38 MAPK upregulation [7]. Similar observations were made in pial arterioles. For example, cromakalim (10^{-8} , 10^{-6}) dilated pial arterioles by 18±1% and 32±2%, 4±1% and 7±1%, 1±1% and 2±1%, 1±1% and 2±1%, 14±1% and 24±2%, and 11±1% and 14±1%, *n*=5, for sham control, photothrombosis, photothrombosis+tPA pre-injury treatment, photothrombosis+tPA post-injury treatment, photothrombosis+SP 600125 pre-injury treatment, and photothrombosis+SP 600125 post-injury treatment, respectively.

RBC-tPA Blocks JNK but Potentiates p38 MAPK Upregulation After Photothrombosis

Photothrombosis increased CSF JNK MAPK, which was aggravated by tPA (105±3%, 236±12%, and 347±20%, *n*=5 for sham control, photothrombosis and photothrombosis+tPA, respectively). Pre-and post-injury treatment with RBC-tPA blocked increases in CSF JNK MAPK (103±3%, 108±5%, and 116±5%, *n*=5, for sham control, photothrombosis +RBC-tPA pre-injury, and photothrombosis+ RBC-tPA post-injury treatment, respectively). On the other hand, p38 MAPK was elevated by photothrombosis, modestly elevated further by tPA, but potentiated by RBC-tPA pre-injury treatment (102±2%, 180±17%, 204±19%, and 302±13%, *n*=5). Similar observations were made for tPA and RBC-tPA post-injury treatment.

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.

Discussion

Several new findings emerged from this study. First, pial artery dilation in response to Katp and Kca channel agonists was impaired after photothrombosis which was aggravated by tPA administered either pre- or post-injury. In contrast, pre- or post-injury administration of RBC-tPA prevented impairment of vasodilation in response to both K channel subtype agonists. Since cerebral autoregulation during hypotension is dependent on intact Katp and Kca function [11], these data suggest that current thrombolytic therapy with tPA to treat central nervous system (CNS) ischemic disorders appears to disrupt autoregulation by impairing cation-mediated control of cerebrovascular tone, thereby aggravating cerebral damage. CGRP is an endogenous activator of the Katp channel in the piglet cerebral circulation [8, 9] and its use therefore provides physiological perspective to observations in this study.

Second, administration of the JNK MAPK antagonist SP 600125 either pre- or post-injury prevented impairment of Katp but not Kca-mediated pial artery dilation. The p38 MAPK antagonist SB 203580 aggravated impairment of both Katp and Kca channel agonist-mediated dilation post-injury. Photothrombosis upregulated CSF JNK MAPK, which was aggravated by tPA but blocked by RBC-tPA administered pre- or post-injury, similar to recent studies [7]. The p38 isoform of MAPK was similarly upregulated by photothrombosis, only modestly potentiated by tPA, but robustly increased by RBC-tPA, similar to recent studies [7]. Taken together, these data suggest that upregulation of JNK MAPK appears to be deleterious, whereas p38 MAPK may protect Katp-mediated cerebrovasodilation in the setting of focal thrombotic cerebral ischemia. For Kca-mediated cerebrovasodilation, although tPA similarly aggravates dilation, the mechanism is independent of JNK and is uncertain at present. Nonetheless, p38 protected Kca-mediated cerebrovasodilation, similar to that observed for the Katp channel. Therefore, the mechanism by which RBC-tPA protects Kca-mediated cerebrovasodilation differs from its effect on Katp in that while p38 upregulation appears beneficial in both settings, but there was little evidence of JNK being deleterious. No role for the third MAPK isoform, ERK MAPK, in impaired cerebrovasodilation to hypotension or hypercapnia after photothrombosis has been observed [6, 7]. However, in unrelated studies using a distinct model of injury, global cerebral hypoxia/ ischemia, tPA-impaired Katp and Kca-mediated cerebrovasodilation through upregulation of ERK MAPK [25]. In that injury model, the ERK antagonist U 0126 protected both Katp- and Kca-mediated cerebrovasodilation in an equivalent manner [25]. Taken together with the results of the present study, these data suggest that global and focal CNS ischemia impair cerebral hemodynamics through heterogenous and only partially overlapping pathways. From a physiological perspective, inhibition of the relaxation to K channel agonists could be due to a number of factors, such as: membrane depolarization, changes in density of K channels, membrane internalization of K channels, changes in gating, and/or changes in receptor/ effector coupling. While we were initially surprised to detect a supposedly 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 with a range of stimuli [26–28]. In particular, we documented parallelism of change in response to hypoxia/ischemia using qualitative immunohistochemistry, semi-quantitative Western, and quantitative ELISA of CSF [28].

Many studies of cerebral ischemia have been performed in rodent models. Piglets offer a unique advantage in elucidating pathways involved in CNS ischemic injury by virtue of having a gyrencephalic brain that contains substantial white matter similar to humans, which is more sensitive to ischemic damage than grey matter. On the basis of interspecies extrapolation of brain growth curves [29], the age of the newborn pig used in these studies roughly approximated the newborn infant time period in the human. Although the incidence of cerebral ischemic events in the pediatric population is relatively low compared to the adult [30], the clinical and social impact is amplified by the potential long-term loss of quality of life years for children with CNS ischemic disorders. Many children, however, are receiving tPA based on the assumption that studies in adults can be generalized to the pediatric population. The data from this study provide additional evidence that both safety and efficacy of tPA must be evaluated systematically in children before being widely adopted in clinical care. A potential limitation of the present study is that we did not directly address the issue of whether safety and efficacy differ in neonates and older children, indicating the need for scientific investigation using different maturational ages of pigs and/ or other species.

Prior studies of the delivery of plasminogen activators by carrier RBCs [15] provide a logical framework for understanding differences in outcome and signaling from soluble tPA and the implication of these findings for the translational potential of this novel drug delivery strategy. We postulate that the protective effect of RBC-tPA arises from both prolonged duration of RBC-tPA activity, which facilitates thrombolytic reperfusion and intravascular spatial constraints. Anchoring tPA to RBCs does not inhibit its enzymatic activity [16]. However, the biodistribution of radiolabeled RBC-tPA in animals [15] and in vitro studies of binding to vascular cells [31] show that anchoring constrains tPA from exiting the vasculature and inhibits interaction with cellular receptors. These attributes prolong the intravascular activity of tPA, which enables thrombolysis to proceed at lower doses of drug and with less side effects. Moreover, differences in permeation of the vasculature and brain tissue by tPA and RBC-tPA likely account for variation in the MAPK isoforms that were upregulated. This suggests that the location or pattern of activation of these MAPK isoforms by tPA differ within endothelium, vascular smooth muscle, neurons, and glia. From the translational standpoint, it is encouraging that a single IV injection anchors a recombinant protein that fuses a plasminogen activator with single chain fragments of antibodies to circulating RBC and provides prolonged intravascular thromboprophylaxis [32, 33]. This new approach will facilitate the clinical applicability of using RBC carriage of plasminogen activators without the need for ex vivo coupling of tPA to isolated RBCs followed by reinfusion as utilized in this study.

Conclusion

In conclusion, these data indicate that photothrombosis impairs K channel-mediated cerebrovasodilation (Fig. 4). tPA exacerbates the loss of Katp channel function after injury by upregulating JNK MAPK and intensifies the loss of Kca channel-mediated vasodilation through as yet unknown mechanisms. In contrast, RBC-tPA prevents impairment of Katp-mediated cerebrovasodilation after injury through blockade of JNK and upregulation of p38 MAPK. RBC-tPA also prevents impairment of Kca-mediated cerebrovasodilation after injury through upregulation of p38. These data suggest that current thrombolytic therapy for treatment of CNS ischemic disorders dysregulates cerebrohemodynamics by impairing cation-mediated control of cerebrovascular tone (Fig. 4). RBC carriage of tPA preserves adaptive cerebrohemodynamic responses and thereby offers a novel therapeutic approach towards increasing the benefit/risk ratio of thrombolytic therapy for treatment of CNS ischemic disorders (Fig. 4).

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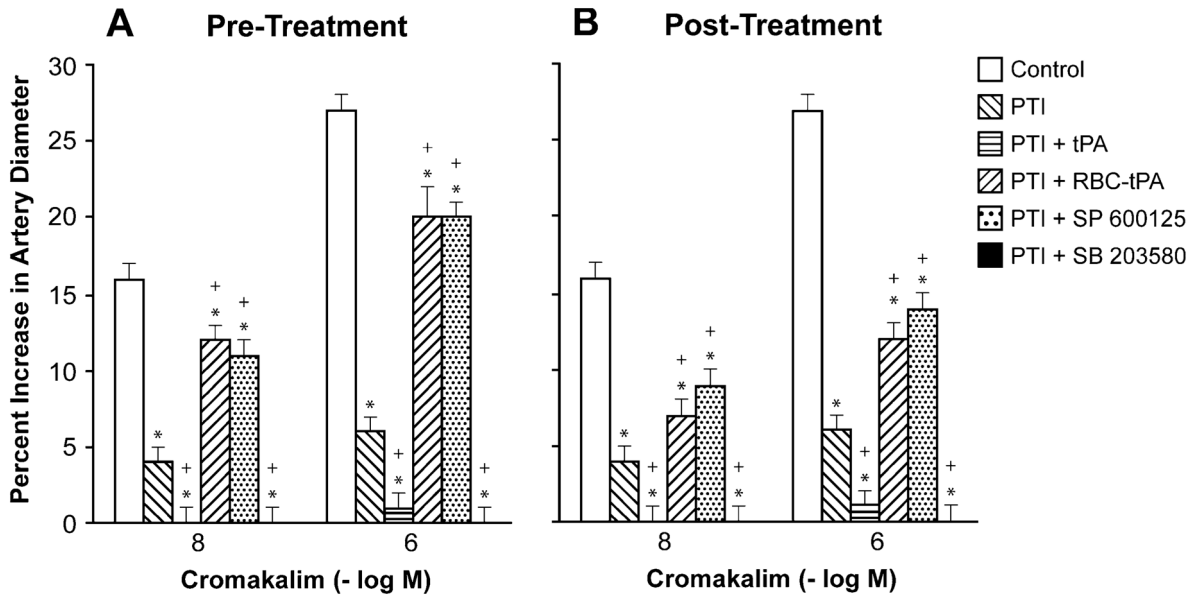


Fig. 1. Influence of cromakalim (10^{-8} , 10^{-6} M) on pial artery diameter before injury (control), 2.5 h after photothrombotic injury (PTI), 2.5 h after PTI treated with tPA (2 mg/kg iv), 2.5 h after PTI treated with RBC-tPA (0.1 mg/kg iv), 2.5 h after PTI treated with SP 600125 (1 mg/kg iv), and 2.5 h after PTI treated with SB 203580 (1 mg/kg iv), $n=5$. **a** Pretreatment 30 min prior to PTI, **b** post-treatment 2 h after PTI. * $p<0.05$ compared with corresponding control value + $p<0.05$ compared with corresponding PTI nontreated value

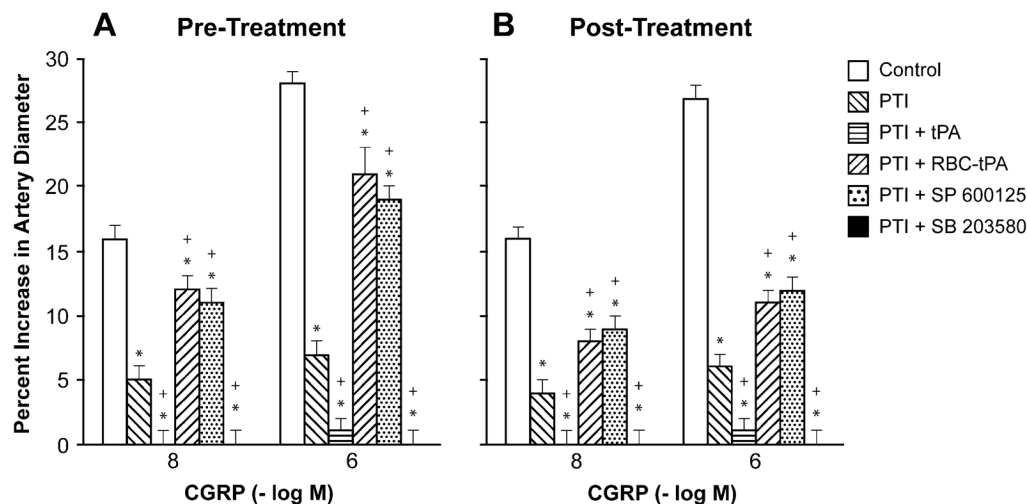


Fig. 2. Influence of calcitonin gene-related peptide (CGRP; 10^{-8} , 10^{-6} M) on pial artery diameter before injury (control), 2.5 h after photothrombotic injury (PTI), 2.5 h after PTI treated with tPA (2 mg/kg iv), 2.5 h after PTI treated with RBC-tPA (0.1 mg/kg iv), 2.5 h after PTI treated with SP 600125 (1 mg/kg iv), and 2.5 h after PTI treated with SB 203580 (1 mg/kg iv), $n=5$. **a** Pretreatment 30 min prior to PTI, **b** post-treatment 2 h after PTI. * $p < 0.05$ compared with corresponding control value + $p < 0.05$ compared with corresponding PTI nontreated value

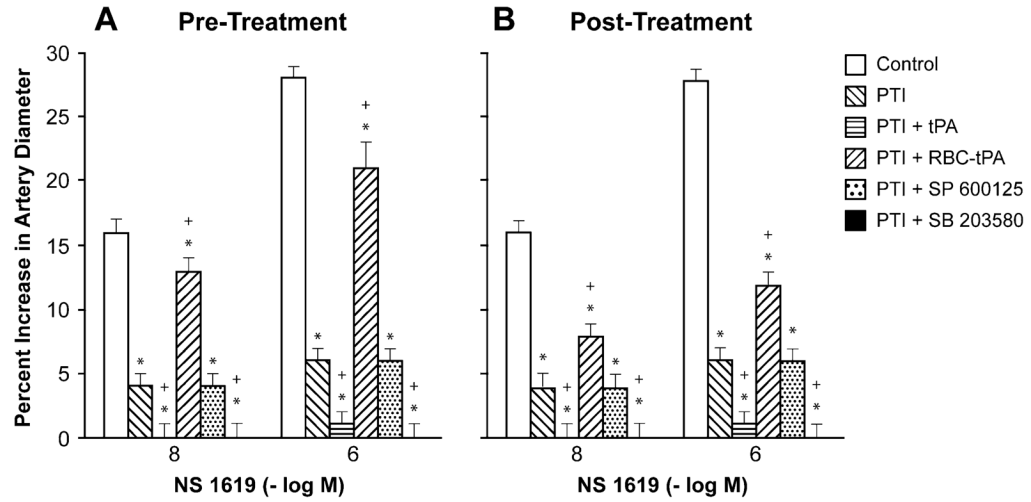


Fig. 3. Influence of NS 1619 (10^{-8} , 10^{-6} M) on pial artery diameter before injury (control), 2.5 h after photothrombotic injury (PTI), 2.5 h after PTI treated with tPA (2 mg/kg iv), 2.5 h after PTI treated with RBC-tPA (0.1 mg/kg iv), 2.5 h after PTI treated with SP 600125 (1 mg/kg iv), and 2.5 h after PTI treated with SB 203580 (1 mg/kg iv), $n=5$. **a** Pretreatment 30 min prior to PTI, **b** post-treatment 2 h after PTI. * $p < 0.05$ compared with corresponding control value + $p < 0.05$ compared with corresponding PTI nontreated value

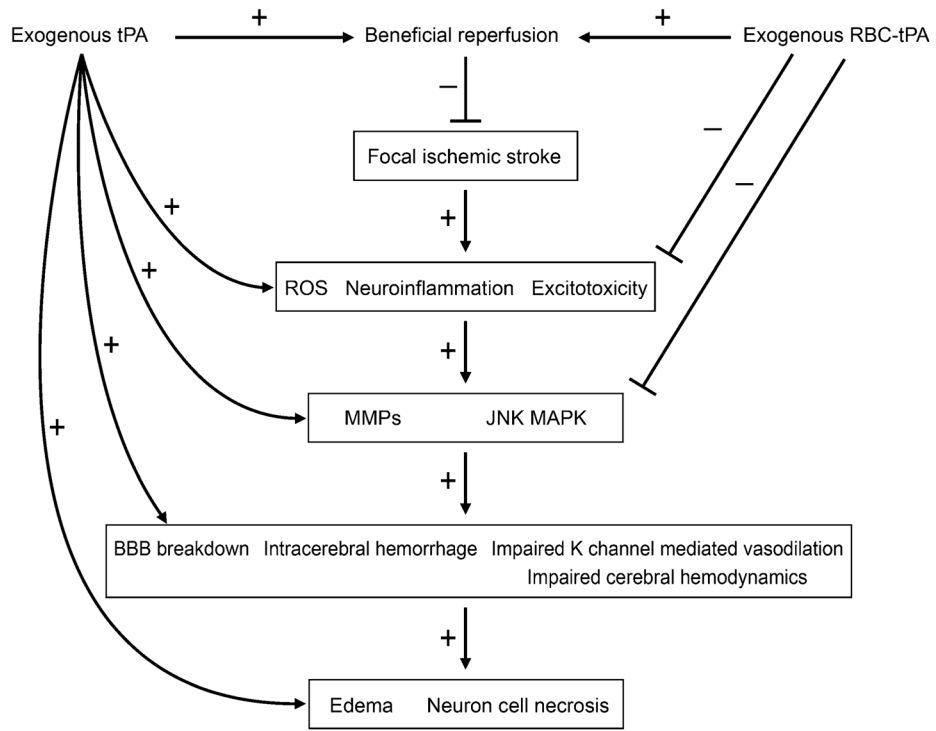


Fig. 4. Schematic diagram illustrating contrasting effects of tPA and RBC-tPA on mediators affecting outcome in the setting of focal ischemic stroke. This figure has been modified from its original published form [34]