Supplemental Material

Thrombolytic tPA-Induced Hemorrhagic Transformation of Ischemic Stroke is Mediated by PKCβ phosphorylation of Occludin

Materials and Methods

Animals

All animal procedures were performed in accordance with the local welfare legislation and approved by the Institutional Animal Care and Use Committees at the University of Michigan or from the Swedish National Board for Laboratory Animals and the European Union Directive (2010/63/EU) under ethical permits approved by the North Stockholm Animal Ethics Committee. Mice were housed under a 12-hour light/dark cycle with free access to water and a standard rodent chow. Wild-type C57BL/6 male and female mice were from Jackson Laboratory, USA, or Charles River, Germany. Mice with human occludin Ser490 mutated to Ala (S490A_OCC) under a floxed stop targeted to the ROSA26 locus were recently described in 1. To allow dominant conditional vascular expression of the transgene, mice were crossed with PDGFb-(i)CreER mice, a kind gift from Dr. Fruttiger 2. Induction of Cre was achieved by tamoxifen injection (30 µg in 3 µl volume) at P3 into the milk sac. The tPA deficient (tPA−/−) (Platm1Mlg) mice were a gift from Dr. Carmeliet and have been extensively backcrossed into C57BL/6 background 3.

Middle cerebral artery occlusion (MCAO) ischemic stroke model

Age- and gender matched C57BL/6 wild type mice or transgenic mice, were anesthetized with chloral hydrate (450 mg/kg Fisher Scientific) and placed securely under a dissecting microscope (Leica M30). The left middle cerebral artery (MCA) was exposed and a laser Doppler flow probe (Type N (18 gauge), Transonic Systems) was placed on the surface of the cerebral cortex located 1.5 mm dorsal median from the bifurcation of the MCA as described before 4. The probe was connected to a flowmeter (Transonic model BLF22) and relative tissue perfusion units (TPU) were recorded with a continuous data acquisition program (Windaq, DATAQ Instruments). A 3.5-mW green light laser (540 nm, Melles Griot) was then directed at the MCA from a distance of 6 cm, and the photoactivatable dye Rose Bengal (Fisher Scientific) 10 mg/ml in PBS was then injected intravenously for a final dose of 40 mg/kg. The TPU of the cerebral cortex was recorded, and total MCA occlusion was achieved when the TPU dropped to less than 30% of pre-occlusion levels. For sham operations, animals went through identical procedures except craniotomy and
rose bengal injection. To alleviate surgical pain and distress, Rimadyl Vet.® (50mg/ml, Pfizer) was administered by subcutaneous injection at the start of the surgical procedure (5mg/kg) and 24 hours after. PKCβ inhibitor (LY-333531, Eli Lilly) was administered via IP injection. Animals were treated daily with either saline (vehicle) or the PKC β inhibitor (10 mg/kg) beginning 3 days before MCAO, then 1 hour after MCAO and continued for 2 additional days after MCAO (6 doses total).

For late thrombolysis, experiments were carried out as described 4-6. Briefly, saline or tPA (10 mg/kg) was administered via a 26G Abbocath®-T vascular catheter (Hospira) that was inserted into the tail vein and then connected to a Genie Plus syringe pump (Kent Scientific) via a catheter extension set (Catalog No. IS6003, infusion Devices), trimmed to reduce the priming volume to 200 μl. For tPA thrombolysis, tPA was initiated 5 h after MCAO by slow infusion via the tail vein. All animals were maintained at physiological 37°C during the infusion process. Some animals were also treated with either vehicle or PKC β inhibitor (10mg/kg) daily for 3 days starting 1h or 5h post-MCAO.

**Brain vascular permeability**

For analysis of cerebrovascular permeability after MCAO, stroked mice were injected with 100μl of 4% Evans blue (EB) dye (Sigma-Aldrich) intravenously 1 hour prior to sacrifice. The dye was cleared from vessels by transcardiac perfusion with PBS for 5 min under isoflurane anesthesia and the brains removed and separated into hemispheres. Permeability was determined as described previously 4. Background EB dye in the non-ischemic contralateral hemisphere was subtracted from the ischemic hemisphere. EB dye in each hemisphere was calculated using the following formula:

\[
\frac{(A_{620nm} - \frac{(A_{500nm} + A_{740nm})}{2})}{\text{mg wet weight}}
\]

Brain vascular permeability was also assessed in frozen cross-sections. Twenty-three hours after MCAO, animals were injected intravenously with 50 mg/kg BW 70kDa Dextran-TR (Molecular Probes) that was allowed to circulate for 1h. Animals were then perfused with PBS transcardially and brains were removed, processed and embedded in OCT blocks. Coronal brain sections (10 μm) were obtained and imaged for extravasated dextran using a Leica DM 6000 fluorescence microscope. For each animal, images from five randomly chosen fields in the peri-infarct area and in a similar region of the contralateral cortex were acquired and analyzed by an
investigator blinded to the study group. The average of the fluorescence intensity for the five regions was determined using Imaris software (Bitplane).

**Infarct volume measurement**

For assessment of infarct volume, mice were sacrificed at 72 h or 7 days after stroke and brains were removed and cut into 2-mm thick coronal sections and stained with 4% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS for 20 min at 37°C, and then fixed in 4% paraformaldehyde solution for 10 min. Five brain slices/mouse were analyzed using the Image J software (NIH) by an investigator blind to the treatments. The following formula was used to calculate infarct volume:

Equations 1: \( V_{\text{stroke}} = \frac{\sum (\text{Areas of lesion})}{\sum (\text{Areas of ipsilateral hemisphere})*100} \)

Infarct volume (V\%stroke) was calculated as percent of the ipsilateral hemisphere in order to avoid an artifact due to brain edema.

**Hemorrhagic volume measurement**

Assessment of hemorrhagic volume in the brain was performed as described. Briefly, digital images of coronal sections were analyzed using Image J software (NIH) for the area of hemorrhage on both sides of the section, by an investigator unaware of the treatment administered. The two sides were then averaged for the area of each section and the sum of all sections was calculated and multiplied by the thickness of region analyzed, 2mm, giving the hemorrhagic volume (Equations 2). The sections were then processed for infarct volume as above.

Equations 2: \( V_{\text{ICH}} = \frac{(\sum (\text{Areas of ICH mm}^2))/2}{2} \times 2\text{mm} \)

Where \( V_{\text{ICH}} \) is ICH volume calculated in cubic millimeters.

**Corridor task**

Lateralized sensory-motor integration was measured using a corridor task adapted from 7,8. Briefly, a long narrow rectangular plexiglass corridor (L=60 cm x W=4 cm x H=15 cm) with 10 pairs of adjacent Eppendorf caps placed at 5-cm intervals containing 4-5 sugar pellets (20 mg; TestDiet) was used as the testing corridor. A corridor with the same dimensions but without adjacent Eppendorf caps was used as the habituation corridor. Twenty-four hours before MCAO mice were habituated to the corridor by scattering sugar pellets along the corridor floor and
allowing them to freely explore for 10 min. Laterized sensory-motor integration was tested 7 days after MCAO. On the testing day, mice were placed in the habituation corridor for 5 min in the absence of sugar pellets, then mice were transferred to one end of the testing corridor containing sugar pellets and video recorded for 5 min. The video recordings were analyzed by a blinded investigator. The number of ipsilateral and contralateral explorations relative to the stroked hemisphere were counted until the mouse made a total of 40 investigations or the video ended. An investigation was defined as a nose-poke into an Eppendorf cap, whether the sugar pellet was poked or eaten, and a new investigation was only counted by exploring a new cap. Data is expressed as a percentage of bias lateralized explorations, calculated as:

\[
\text{Bias (\%)} = 100 \times \frac{\text{ipsilateral} - \text{contralateral}}{\text{ipsilateral} + \text{contralateral}}
\]

**Intracerebroventricular injection for immunoblot analysis**

To perform intracerebroventricular (ICV) injection of PBS, tPA (Alteplase, Genentech, Lot#3087948 dialyzed into 0.4M HEPES/0.1M NaCl pH7.4), ANGPTL4 (R&D Systems), Imatinib (Selleck Chemicals), PKCβ inhibitor (LY-333531, Eli Lilly), and VEGFR2 kinase inhibitor I (SU 5408 Cayman Chemicals), wild type C57BL/6 mice were anesthetized with isoflurane, placed on a stereotactic frame, and injected at bregma – 2, mediolateral 0, and dorsoventral 2. Injections contained 3 µl of either PBS, active tPA (3 µM), or active tPA plus ANGPTL4 recombinant protein (3 µM), PKC β inhibitor (3 µM), Imatinib (3 µM) or VEGFR2 inhibitor (3 µM). Cerebrovascular permeability was then determined 6 h later from Evans Blue extravasation in one hemisphere as described above. Western blotting of brains was carried out in the other hemisphere using the previously characterized occludin anti-phospho-Ser490 (anti-pS490) specific antibody at 1:500, described in §. Other antibodies used were: occludin (1:1000, Thermo Fisher), anti-β-actin (1:10000, Cell Signaling). After washing with 0.1% Tween-20 in TBS membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and developed using ECL substrate (Lumigen) and the luminescence signal was acquired on a FluorChem E (Cell Biosciences). The intensity of the bands was quantified using ImageJ software (NIH).

**Immunohistochemistry**

Immunostaining was performed in 10 µm brain coronal sections. Sections were incubated with mouse Alexa Fluar 488-conjugated anti-occludin antibody (1:100, Thermo Fisher), rat anti-
ZO-1 antibody (1:100, Millipore Sigma), a rabbit polyclonal antibodies specific to occludin pS490 site (1: 100 dilution), FITC-conjugated anti-Ly6G antibody (1:50, eBioscience), rat anti-CD45 antibody (1:100, Novus Biologicals) or Alexa Fluor 647-conjugated IB4 overnight at 4°C. Staining of phospho-occludin was restricted to the first 24 hours after MCAO since occludin phosphorylation leads to its ubiquitination and trafficking of occludin to the cytoplasm where it is degraded. Primary antibodies were detected with Alexa Fluor 488-, 594- or 647-conjugated anti-rabbit or anti-rat IgG secondary antibodies (Life Technologies) incubated overnight at 4°C. Brain sections were imaged using a Leica DM 6000 fluorescence microscope. Image intensity of the anti-occludin or anti-pS490-occludin or anti-ZO-1 antibodies staining in the brain vessels were quantified for total image intensity using Imaris software (Bitplane). For Ser490 occludin phosphorylation, image intensity co-localizing with ZO-1 was quantified. For each animal, the blood vessel border intensity from five images was averaged in the peri-infarct area and in a similar region in the contralateral cortex. The total number of positive inflammatory cells were counted using a particle analyzer plugin for ImageJ (NIH) in stitched images of each hemisphere, obtained on a DM6000 (Leica Microsystems) fluorescence microscope and normalized per mm². Representative images were obtained on a Leica SP8 confocal microscope (Leica Microsystems).

**Vascular fragment isolation**

Active PDGF-CC protein (PDGF-CCa), (3 µL, 3 µM (4µL for the array analysis)) produced in Sf9 cells 10, active tPA (3 µL, 3 µM) or PBS was stereotactically injected into the left lateral ventricle (bregma -0.6, mediolateral -1.2, and dorsoventral 2) of C57BL/6 mice. Four hours later the mice were anesthetized with isoflurane and perfused with Hanks’ balanced salt solution (HBSS). Brains were subsequently rapidly dissected out and placed into ice-cold HBSS. The left-brain hemispheres were used for vascular fragment isolation and subjected to protease digestion, passed through 100µM and 40µM mesh filters, and a CD31 (BD Biosciences) antibody was used to capture vascular fragments by magnetic sorting as described 11. Vascular fragment isolations from C57BL/6 mice 24h after MCAO or sham operation were also performed. Quality control of the isolation using cell–specific markers showed an approximately 80-fold enrichment of endothelial cells (Pecam1), an ~25-fold enrichment of pericytes (Pdgfrb), an ~13-fold enrichment of perivascular astrocytes (Aqp4), and an ~90-fold reduction in neurons (Dlg4) in the magnetically sorted tissue compared to whole brain tissue. Total RNA was extracted and RNA quality was
assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was subsequently either used for expression array analysis or cDNA generation for qPCR analysis. cDNA was prepared using the iScript kit (Bio-Rad, Hercules, CA, USA).

**Microarray procedure and data analysis**

Total RNA from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev C 09/2009) and Affymetrix GeneChip WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 6, Affymetrix Inc., Santa Clara, CA). GeneChip ST Arrays (GeneChip Gene 2.0 ST Array) were hybridized, washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip Scanner 3000 7G. The raw data was normalized in the free software Expression Console provided by Affymetrix (http://www.affymetrix.com) using the robust multi-array average (RMA) method. Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). In order to search for the differentially expressed genes between PDGF-CCa-ICV and the PBS-ICV group an empirical Bayes moderated t-test was then applied, using the ‘limma’ package. Heatmaps were generated using heatmapR. Molecules from the data set that met the log2 fold change > or < 0.5 cut off and a P value < 0.05 were uploaded to the David bioinformatics resource (https://david.ncifcrf.gov) for functional annotation and pathway analysis. The full PDGF-CCa and PBS ICV microarray data sets of vascular fragments presented in this publication have been deposited to the GEO expression omnibus database with the accession number GSE176245.

**qPCR method**

Real-time quantitative PCR was performed using KAPA SYBR FAST qPCR Kit Master Mix (2x) Universal (KAPA Biosystems) in Rotor-Gene Q (Qiagen) Real-Time PCR thermal cycler according to the manufacturers’ instructions. Expression levels were normalized to the expression of Rpl19.

Primers: 5’-GGTGACCTGGATGAGAAGGA, 5’-TTCAAGCTTGGATGTGCTC. (Rpl19); 5’-ACCTTAACTGTGCAAGAGCC, 5’-CCCTTTTTTACGCTCCTGCG. (Angptl4).
Statistical Analysis

Results are expressed as Mean ± SEM. Two-tailed student’s t-test was performed to assess the statistical difference between two groups. One-way ANOVA followed by a Holm-Sidak post-hoc test was employed to calculate the statistical difference between 3 or more groups using Prism 9 (Graph Pad Software, San Diego, CA) with value of $P < 0.05$ considered statistically significant.

References

1. Goncalves A, Dreffs A, Lin CM, et al. Vascular expression of permeability-resistant occludin mutant preserves visual function in diabetes. *Diabetes*. 2021.
2. Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis*. 2008;46(2):74-80.
3. Szabo R, Samson AL, Lawrence DA, Medcalf RL, Bugge TH. Passenger mutations and aberrant gene expression in congenic tissue plasminogen activator-deficient mouse strains. *J Thromb Haemost*. 2016;14(8):1618-1628.
4. Su EJ, Fredriksson L, Geyer M, et al. Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. *Nat Med*. 2008;14(7):731-737.
5. Su EJ, Cao C, Fredriksson L, et al. Microglial-mediated PDGF-CC activation increases cerebrovascular permeability during ischemic stroke. *Acta Neuropathol*. 2017;134(4):585-604.
6. Torrente D, Su EJ, Fredriksson L, et al. Compartmentalized Actions of the Plasminogen Activator Inhibitors, PAI-1 and Nsp, in Ischemic Stroke. *Transl Stroke Res*. 2022.
7. Grealish S, Mattsson B, Draxler P, Bjorklund A. Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease. *Eur J Neurosci*. 2010;31(12):2266-2278.
8. Dowd E, Monville C, Torres EM, Dunnett SB. The Corridor Task: a simple test of lateralised response selection sensitive to unilateral dopamine deafferentation and graft-derived dopamine replacement in the striatum. *Brain Res Bull*. 2005;68(1-2):24-30.
9. Sundstrom JM, Sundstrom CJ, Sundstrom SA, et al. Phosphorylation site mapping of endogenous proteins: a combined MS and bioinformatics approach. *J Proteome Res*. 2009;8(2):798-807.
10. Li X, Ponten A, Aase K, et al. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat Cell Biol*. 2000;2(5):302-309.
11. Zeitelhofer M, Adzemovic MZ, Moessinger C, et al. Blocking PDGF-CC signaling ameliorates multiple sclerosis-like neuroinflammation by inhibiting disruption of the blood-brain barrier. *Sci Rep*. 2020;10(1):22383.
12. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4(2):249-264.

13. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.

14. Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res*. 2016;44(W1):W147-153.
Supplemental data

**Supplemental Figure 1:** Mice with the occludin S490A mutant incorporated at the Rosa26 site under a floxed stop were crossed with tamoxifen-inducible endothelial cell specific PDGFb-(i)CreER mice. The transgene was expressed in a vascular endothelial cell restricted manner by tamoxifen injection at P3.