Activation of PDGF-CC by Tissue Plasminogen Activator Impairs Blood Brain Barrier Integrity During Ischemic Stroke

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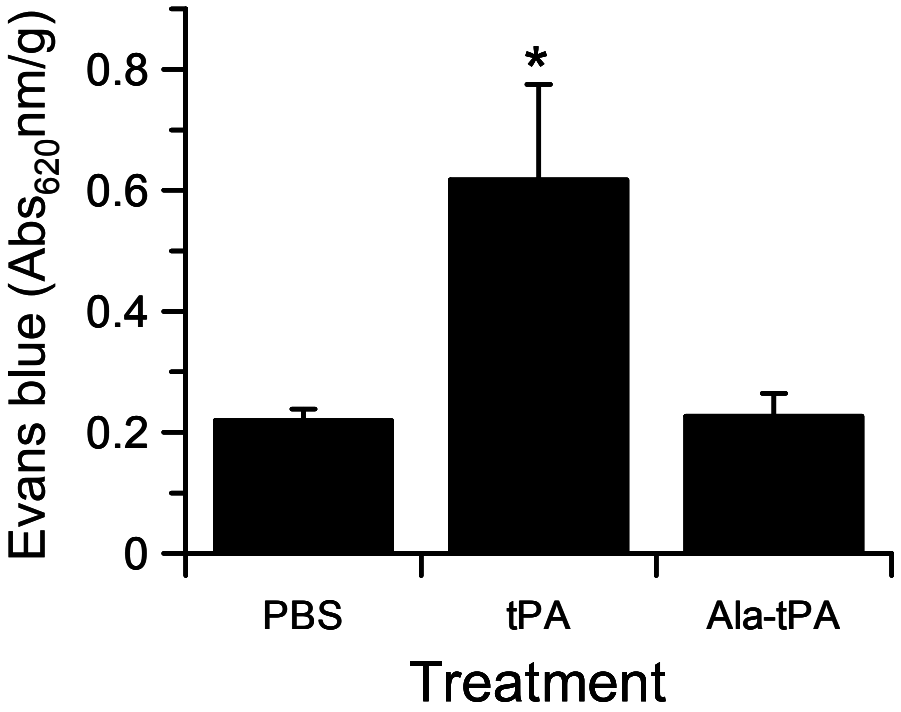
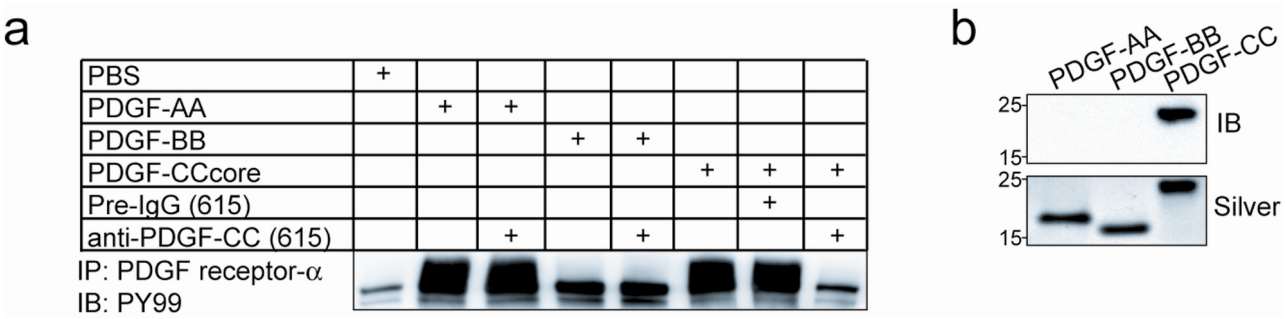


Supplementary Figure 1: Catalytically inactive tPA does not induce cerebrovascular permeability. Intraventricular injection of a catalytically inactive tPA mutant fails to induce cerebrovascular permeability in nonischemic mice. Analysis of EB extravasation 1h after direct intraventricular injection of PBS (PBS), active tPA (tPA), or a mutant form of mouse tPA with its active site serine at position 481 mutated to alanine and which lacks proteolytic function (Ala-tPA) (Molecular Innovations). The final concentrations of each protein is 3 μM. Cerebrovascular permeability was determined 1h after injection from EB extravasation as described in the **Supplementary Methods 1 (**online). Each group contains n = 8–10 and errors represent S.E.M. The asterisks indicate $p<0.05$ vs. animals injected with PBS using Wilcoxon rank sum test.



Supplementary Figure 2: Characterization of PDGF-CC-specific antibodies. (**a**) Antibodies to PDGF-CC specifically neutralize PDGF-CC, but not PDGF-AA or PDGF-BB-induced activation of PDGFR-α. The antibodies were preincubated with PDGF-AA, PDGF-BB or PDGF-CC and the mixtures were applied to cultured porcine aortic endothelial (PAE) expressing human PDGFR-α. Antibodies to PDGF-CC specifically neutralized PDGF-CC-induced receptor activation but showed no effect on either PDGF-AA or PDGF-BB induced activation. Preimmune IgG incubated with PDGF-CC did not interfere with PDGF-CC-induced activation. Untreated cells served as a background control. (**b**) Antibodies to PDGF-CC specifically recognize PDGF-CC in immunoblot experiments. Two hundred ng of either PDGF-AA, PDGF-BB or PDGF-CC were subjected to SDS-PAGE under reducing conditions, followed by immunoblotting using the affinity purified antibodies to PDGF-C (no.615) and visualization by chemiluminiscence. Only PDGF-C was detected (upper panel). Equal loading was confirmed by silver stain (lower panel). Methods**:** Serum-starved PAE cells stably expressing human *PDGFR*-α and grown in 35 mm dishes, were incubated with 80 ng of either PDGF-AA, PDGF-BB (kind gifts from Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala Branch, Uppsala, Sweden) or active PDGF-CC in 1 ml PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 0.1% BSA on ice for 60 min. Prior to addition to the cells, the growth factors were incubated for 1.5 h at room temperature with affinity-purified IgG against PDGF-CC (3 μg/ml) or with rabbit preimmune IgG (3 μg/ml). The cells were lysed as previously described[1] and PDGFR-α was immunoprecipitated using a specific antiserum[2]. Precipitated proteins were separated by SDS-PAGE under reducing conditions. Tyrosine-phosphorylated receptors were detected by immunoblotting using an antibody to phosphotyrosine (PY99, Santa. Cruz).

Reference List

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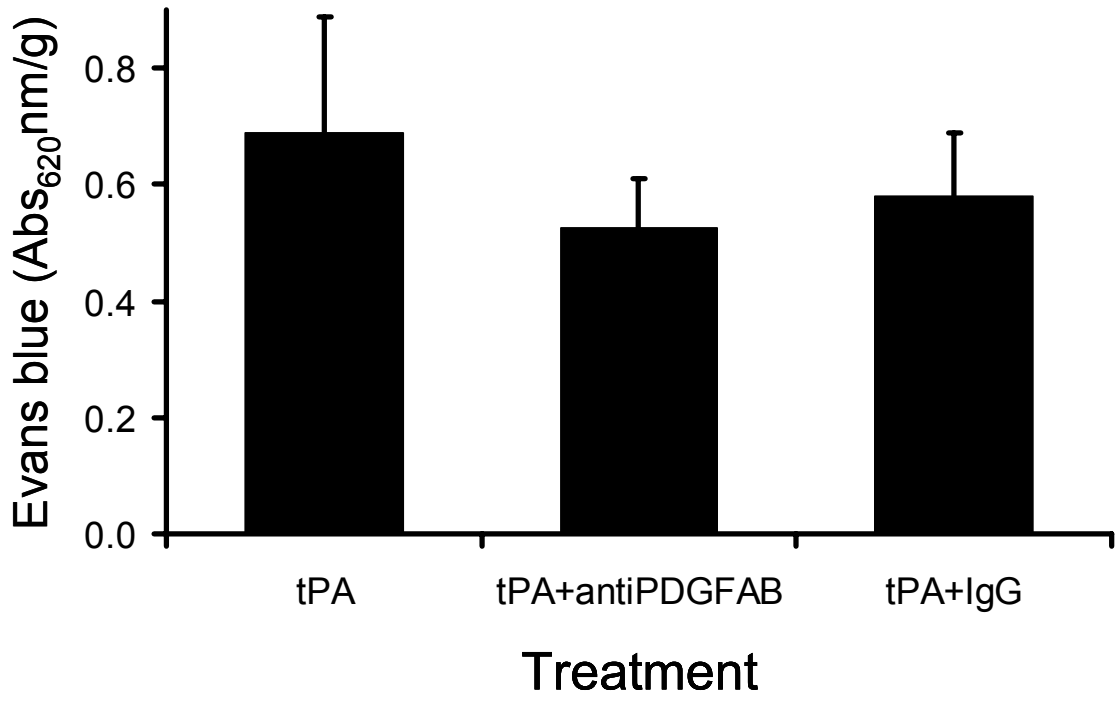

Supplementary Figure 3: PDGF-AB-specific antibodies do not block tPA-induced cerebrovascular permeability. To confirm that the effects of tPA on cerebrovascular permeability were specifically dependent on PDGF-CC and not PDGF-AA or PDGF–BB, a polyclonal antibody[1] that neutralizes PDGF-AA, PDGF-BB, and the PDGF-AB heterodimer was tested for its ability to block the effects of tPA injection into the CSF. This antibody which was raised against PDGF-AB (Millipore catalogue # 06-127) failed to inhibit tPA-induced cerebrovascular permeability after intraventricular injection. This is in contrast to the results with anti-PDGF-CC antibodies, and indicates that tPA acts specifically through PDGF-CC and PDGFR-α signaling to induce cerebrovascular permeability. Methods: Injections contained 3 μl of either active tPA (3 μM), tPA (3 μM) plus anti-PDGF-AB antibody (0.4 mg/ml), or tPA (3 μM) plus control IgG (0.4 mg/ml). Cerebrovascular permeability was then determined 1h later from EB extravasation as described in the **Supplementary Methods 1** (online). Each group contains n = 5-6 and errors represent S.E.M.

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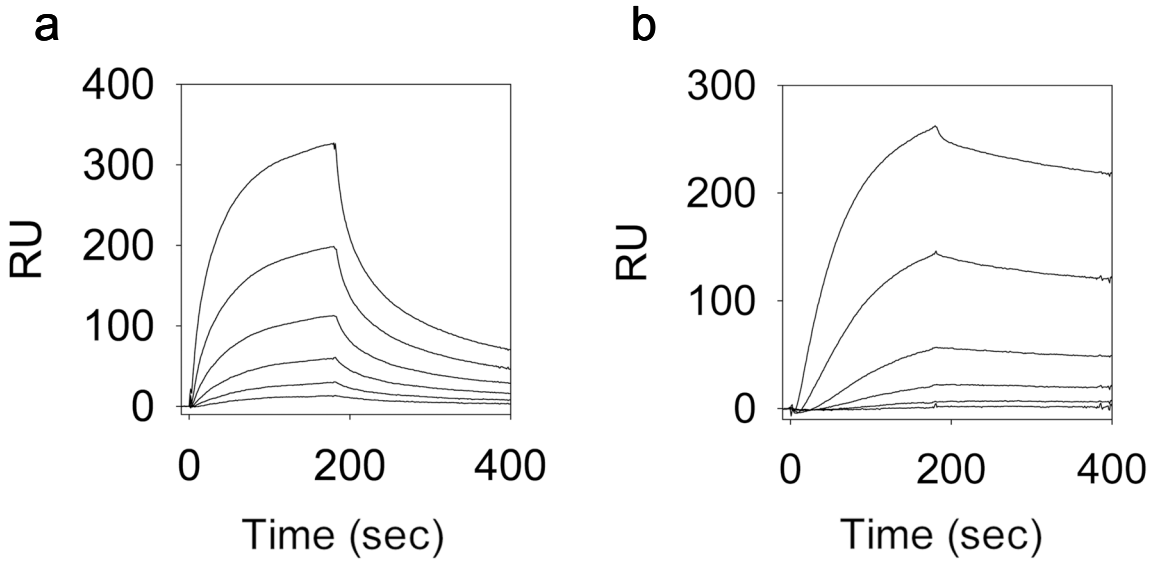
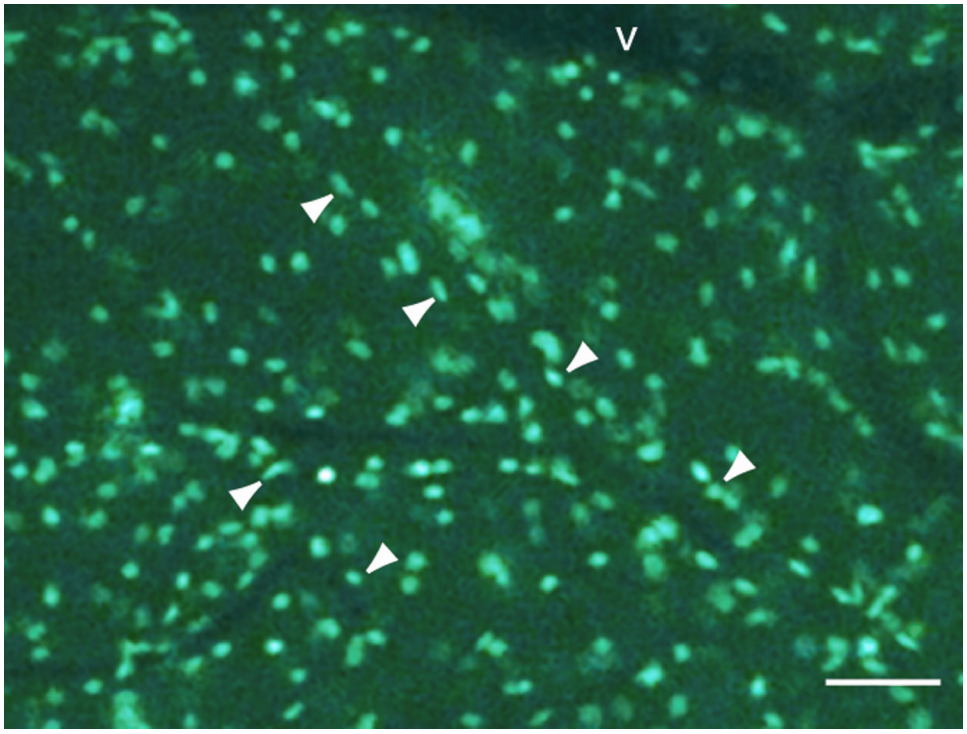


Supplementary Figure 4: TPA and PDGF-CC bind to the LDL-R family member LRP. Real time binding curves for the binding of tPA (**a**) or PDGF-CC (**b**) to LRP is shown. The tPA concentrations were 3.8, 7.5, 15, 30, 60 and 120 nM. The PDGF-CC concentrations were 3, 6.25, 12.5, 25, 50 and 100 nM. Methods: Binding of mouse tPA or active human PDGF-CC to full length LRP was measured using a Biacore® 3000 (Biacore). For these studies, a Biacore CM5 sensor chip was activated with a 1:1 mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 0.05 M N-hydroxysuccimide in water as described by the manufacturer. Purified human LRP was immobilized at the level of 3000 response units (RU), by passing a working solution of 10 µg/ml in 10 mM sodium acetate, pH 4.0 through the Biacore flow cell at a rate of 5 µl/min. Remaining binding sites were blocked by 1 M ethanolamine, pH 8.5. A flow cell with immobilized ovalbumin at the level of 500 RU was used as a control for nonspecific protein binding and bulk refractive index subtraction. All binding reactions were performed in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20; (HBS-P buffer Biacore). Binding of tPA and PDGF-CC to LRP was measured at 25°C at a flow rate of 30 µl/min for 3 min, followed by 3 min of dissociation. Chip surfaces were regenerated with a 1 min pulse of 20 mM HCl. All collected data were analyzed with BIAevaluation software (Biacore).



Supplementary Figure 5: PDGFR-α expression in mouse brain. PDGFR-α expression in brain was investigated using GFP in transgenic heterozygous mice expressing a nuclear targeted histone H2B-GFP fusion protein inserted in the *Pdgfrα* locus[1] (*Pdgfrα*$^{+/GFP}$). This analysis demonstrated that cells expressing PDGFR-α were abundant in brain and that they represented diverse cellular populations. One population expressing GFP intensely was closely associated with a subset of arterioles (arrowheads). GFP-positive cells were also observed around veins (v) but the fluorescence was generally weaker and the labeled cells were not as closely associated with the vessel structure as seen with arterioles. Other cell types, presumably oligodendrocytes[2], also expressed GFP but were not found in association with vessel structures. Scale bar = 500 μm.

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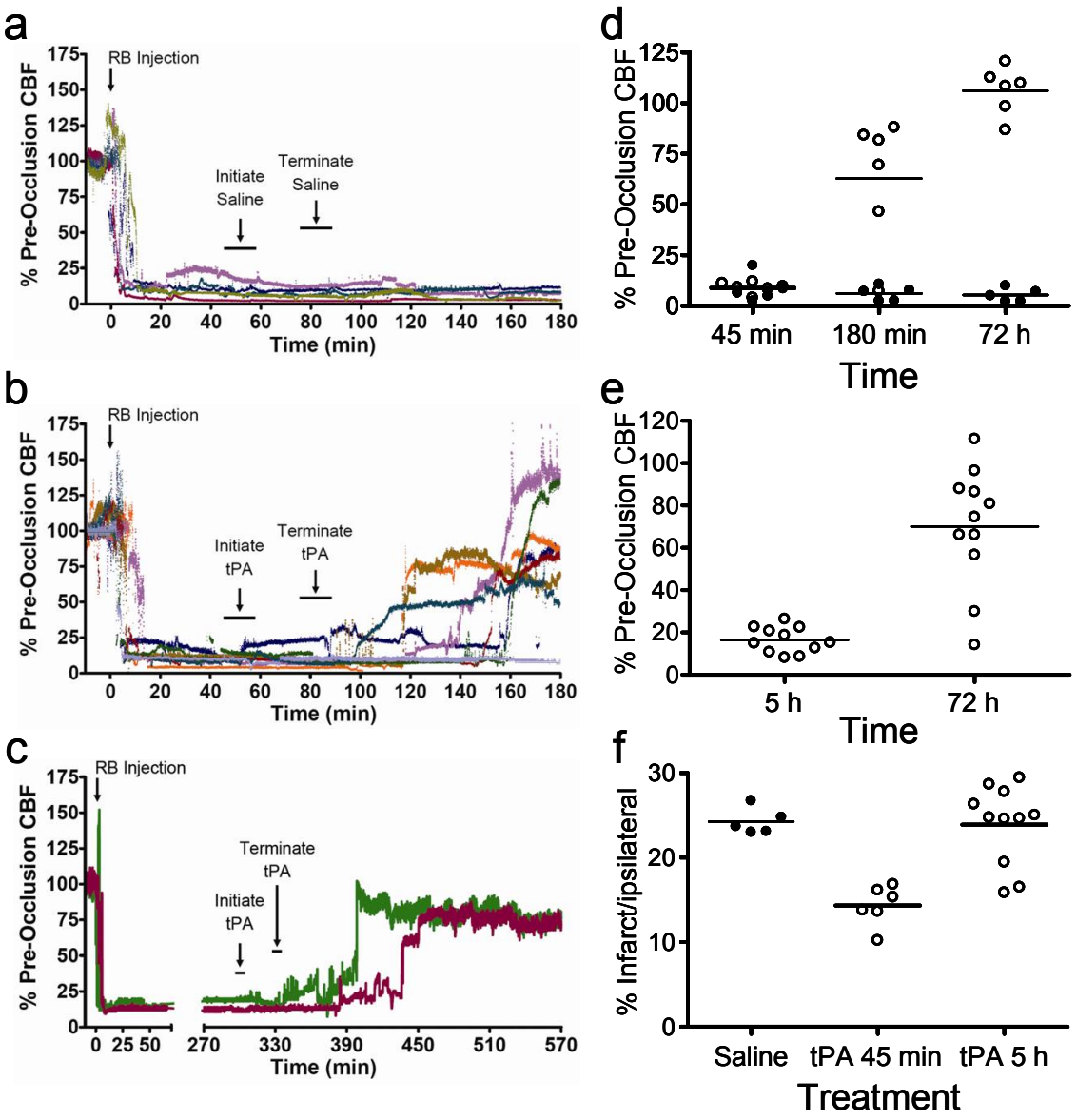

Supplementary Figure 6. Characterization of photothrombotic stroke in mice. Measurement of cerebral blood flow (CBF) by laser-Doppler flowmetry in mice subjected to photothrombotic stroke. Ten week old wild type C57BL/6J mice were divided into three groups: (**a**) Control mice underwent photothrombotic stroke as described in the **Supplementary Methods 1** (online) and were treated with I.V. saline initiated 45 min after occlusion of the MCA (MCAO); (**b**) Mice that underwent photothrombotic stroke and were treated with thrombolytic tPA initiated 45 min after MCAO; (**c**) Mice that underwent photothrombotic stroke and were treated with thrombolytic tPA 5 h after MCAO. Since MCA occlusion occurred at slightly different times after Rose Bengal

(RB) injection, the initiation of saline or tPA injections were begun at slightly different times as well and this is indicated by the short horizontal bars beneath the arrows. The termination of injections is likewise represented by short horizontal bars. Occlusion of the MCA began shortly after RB injection and was stabilized between 5 and 10 min after injection in all mice. In the saline treated mice the occlusion remained stable for the duration of the monitoring in all mice. The injection of saline did not significantly alter the CBF in any of the animals. CBF tracings of 8 mice treated with tPA 45 min after MCAO (**b**) were similar to the saline treated mice in their initial CBF tracings but differ after tPA delivery, where they began to show restoration of CBF at approximately 1 h after the initiation of tPA administration, and by approximately 2 h after the initiation of treatment only one animal still showed MCAO. CBF tracings for 2 mice who received tPA 5 h after MCAO are shown (**c**), a third animal died during the second phase of the CBF monitoring procedure in this group and is not shown. For this group the initial CBF tracings continued for approximately 60 min after MCAO after which the animals were allowed to recover from anesthesia. At 4.5 h after MCAO the mice were again anesthetized and the laser Doppler flow probe was replaced at the same position as during the occlusion phase and the CBF tracing was restarted. Both animals showed signs of reperfusion after tPA delivery and the increased flow persisted during the remainder of the tracing period.

Quantitative analysis of CBF relative to the pre-MCAO values was also performed (**d–e**). Mice that received saline (●) or tPA (○) 45 min after MCAO were also analyzed for CBF relative to the pre-MCAO values at 45 min, 180 min, and 72 h after MCAO (**d**). These data demonstrate that nearly full reperfusion was obtained in all of the mice treated with tPA early after MCAO, whereas none of control group showed any restoration of CBF. Mice that received late treatment with tPA (●) 5 h after MCAO were also analyzed for CBF relative to the pre-MCAO values at 5 h, and 72 h after MCAO (**e**). These data demonstrate that unlike the response in mice treated with tPA early, the late administration of tPA yields a wide range of CBF at 72 h with one animal still fully occluded and another with only approximately 30% CBF compared to its pre-occlusion level. All remaining animals showed 50% or greater CBF compared to their pre-occlusion levels. All three groups were also analyzed for stroke volumes at 72 h after MCAO (**f**). These data demonstrate that early reperfusion with tPA yields a significant reduction in infarct volume compared to, un-reperfused control mice, whereas late administration of tPA shows a

more mixed outcome with no overall improvement in infarct size. Statistical analysis of infarct volumes indicates that there is a highly significant difference between control and early tPA administration ($p < 0.01$) but that there is no significant difference between control and late tPA administration ($p > 0.05$).

For all mice the quantitative analysis of CBF relative to the pre-occlusion values were calculated by averaging 5 min of CBF data taken immediately before the time point indicated and dividing by the pre-occlusion CBF. For the analysis of CBF performed 5 h and 72 h after MCAO the animals were anesthetized and the laser Doppler flow probe was placed on the surface of the cerebral cortex, at the same position that the original tissue perfusion was measured, and a continuous measurement of CBF was taken for 5 min. For the determination of stroke volumes mice were euthanized after the 72 h CBF measurements and the brains were processed for TTC analysis as described in the **Supplementary Methods 2**. Note that 8 animals are shown in the CBF tracings of mice treated with tPA 45 min after MCAO in panel **b**, but that only 6 mice are shown in the quantitative analysis of these mice in panels **d** and **f**. This is because two mice were excluded from the quantitative analysis of this group due to their death between days 2 and 3 after surgery. No deaths occurred in the control group and five mice are shown for each analysis of this group. Eleven mice are shown in the quantitative analysis of mean CBF in mice treated with tPA at 5 h after MCAO (○) compared to only two mice shown in the CBF tracings of this group (**c**). This is because mice subjected to extended CBF analysis (**c**) immediately after the 5 h tPA administration were not further studied due to potential complications associated with the extended anesthesia necessary to obtain the real-time CBF tracings shown in (**c**). The overall mortality in these studies was 0% (0/5) in the saline treated group and 14% (3/22) in the tPA treated mice. However, combining the control data from these studies with previous control experiments performed under identical conditions yields an overall mortality rate for control mice of 5% (1/20). These data suggest that there is an increased mortality associated with tPA treatment in this model; however, statistical analysis of survival using a Log Rank test (Prism 4, GraphPad, Inc) indicated that there was no significant difference in survival between the control and tPA treated groups ($p = 0.34$). Methods: Saline or tPA 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. All CBF tracings were started 10 min before injection of RB and the average CBF over this time was considered 100% and used to normalize all CBF measurements taken at later time points. Time zero was set at the initiation of RB injection. Control animals (n = 5) received 200 μl of saline 45 min after occlusion of the MCA, of which 100 μl was given via a bolus and the remaining 100 μl infused at 3μl/min for 30 min. For tPA thrombolysis, animals were divided into two groups, an early treatment group where tPA was initiated 45 min after MCAO (45 min tPA, n = 8), and a late treatment group where tPA was initiated 5 h after MCAO (5 h tPA, n = 14). In both treatment groups tPA was given at 10 mg/kg in 200 μl saline, of which 100 μl was given as a bolus and remaining half was infused at 3 μl/min for 33 min. In the late treatment group 3 out of the 14 animals were randomly selected for extended CBF monitoring while the remaining 11 animals were selected for infarct volume analysis and CBF was monitored for 30 min during the occlusion phase, again for 5 min before tPA injection and again for 5 min before sacrifice at 72 h after MCAO.

Supplementary Methods 1:

Mouse model of ischemic stroke. Male C57BL/6J mice (age 10 weeks) were anesthetized with chloral hydrate (450 mg/kg, I.P., Morton Grove Pharmaceuticals, Inc.). Mice were then placed securely under a dissecting microscope (Nikon SMZ-2T). The left middle cerebral artery (MCA) was exposed as described before[1], 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 MCA. The probe was connected to a flowmeter (Transonic model BLF21) and relative tissue perfusion units (TPU) data was recorded with a continuous data acquisition program (Windaq, DATAQ Instruments). Rose Bengal (Fisher Scientific) was diluted to 10 mg/ml in PBS and then injected into the tail vein with the final dose of 50 mg/kg. A 3.5-mW green light laser (540 nm, Melles Griot) was directed at the MCA from a distance of 6 cm at the onset of the injection, and the TPU of the cerebral cortex was recorded (**Supplementary Figure 5a–c** online). Total occlusion was achieved when the TPU dropped to less than 30% of pre-occlusion levels. Treatment of mice with neutralizing antibodies against PDGF-CC following MCAO was carried out immediately after MCAO by intraventricular injection, as described in Methods section, with 3 μl of either preimmune IgG (0.4 mg/ml) or neutralizing antibody (0.4 mg/ml). To block PDGFR-α activation after stroke, mice were treated with Imatinib (200 mg/kg, p.o.) 1 h and 8 h after MCAO during the first day and then twice daily for the duration of the experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of Unit for Laboratory Animal Medicine at University of Michigan.

The assessment of stroke volume by TTC was performed essentially as described[2]. Briefly, animals were anesthetized with chloral hydrate (450 mg/kg, I.P., Morton Grove Pharmaceuticals, Inc.) and euthanized by exsanguination. The brains were then removed and cut into 2-mm-thick coronal sections in a matrix (Harvard Apparatus), 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. Infarcted tissue showed no reaction to TTC while healthy tissue turned a brick red color. Images of the four central sections of each brain were recorded with an Olympus digital C-3030 color camera attached to an SZ-60 Olympus microscope, and the captured images were analyzed with an image analysis system (NIH Image J) to calculate the percent hemispheric lesion volume with the following formula:

$V\%_{stroke} = \sum(\text{Areas of lesion}) / \sum(\text{Areas of ipsilateral hemisphere}) * 100.$

For analysis of cerebrovascular permeability after MCAO, mice were injected with 100 µl of 4% EB (Sigma-Aldrich) 23 h after MCAO. One h later, animals were perfused with PBS for 4 min and the brains were removed and separated into hemispheres ipsilateral and contralateral to the MCAO. Each hemisphere was then homogenized in N,N-dimethylformamide (Sigma-Aldrich) and centrifuged for 45 min at 25,000 rcf (Eppendorf centrifuge, model 5417R). The supernatants were collected and quantitation of EB extravasation performed as described[3]. Briefly, EB levels in each hemisphere were determined from the formula:

$(A_{620nm} - ((A_{500nm} + A_{740nm}) / 2)) / \text{mg wet weight}.$

Background EB levels in the non-ischemic hemisphere was subtracted from the ischemic hemisphere ipsilateral to the MCAO.

Thrombolysis and induction of intracerebral hemorrhage in ischemic stroke. Male C57BL/6J mice (aged 10 weeks) were anesthetized with chloral hydrate (450 mg/kg, I.P., Morton Grove Pharmaceuticals, Inc., Morton Grove, IL). In experiments where intracerebral hemorrhage was analyzed, anesthetized mice were injected with D-Glucose (Sigma-Aldrich, 6ml/kg at 50% w/v) intraperitoneally 15 min before photothrombotic induction of MCAO to enhance the likelihood of intracerebral hemorrhage. Glucose was used because previous studies have demonstrated that transient hyperglycemia significantly increases the extent of intracerebral hemorrhage after ischemic stroke[4], and because hyperglycemia is also associated with increase risk for intracerebral hemorrhage in patients receiving tPA[5,6]. Five h after MCAO thrombolysis was initiated by I.V administration of tPA at 10mg/kg in 200 µl of saline, with 100 µl being given as a bolus and the remaining 100 µl infused at 3 µl/min over 33 min. Twenty four h after MCAO, animals were anesthetized with chloral hydrate and the laser Doppler flow probe (Type N, 18 gauge, Transonic Systems) was placed on the surface of the cerebral cortex, at the same position that the original tissue perfusion was measured the day before, 1.5 mm dorsal median from the bifurcation of MCA. Animals with tissue perfusion values that were at least 50% of the

pre-occlusion values were considered lysed and used for analysis of intracerebral hemorrhage. Approximately 90% of animals treated with tPA after MCAO showed this level of reperfusion of the MCA at 24 h (data not shown). In other experiments where tPA was administered 5 h after MCAO and the percent of pre-occlusion CBF was determined at 72 h after MCAO, 82% of the mice showed greater than 50% pre-occlusion CBF (**Supplementary Figure 5e** online).

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Supplementary Methods 2:

Immunofluorescence localization. Localization of PDGFR-α was performed in heterozygous mice carrying a mutated allele with GFP targeted into the *Pdgfrα* locus (*Pdgfrα*$^{+/GFP}$)[1]. Mice were perfused with 4% PFA, the brains removed, and submerged in PBS for viewing in a dissection microscope equipped with fluorescence optics. For whole mount staining of vessel fragments, the brain was postfixed in 2% PFA, and rinsed in PBS. The brain was placed in PBS and cut in small pieces and disintegrated by trituration. Following centrifugation at 1,000 rpm for 5 min, the sample was incubated in 1% Triton X-100 in PBS for 1 h at RT with end over end mixing for one additional h at + 4°C. Following centrifugation as above, the sample was resuspended in 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.5% Blocking Reagent (TNB PerkinElmer, Inc.) and incubated with end over end mixing. The primary antibodies were incubated over night with end over end mixing at 4°C. The samples were then washed in 20 mM Tris pH 7.2 containing 150 mM NaCl and 0.1% Tween 20, followed by incubation with the secondary antibodies for 2 h at RT. The samples were then washed as above and resuspended in PBS, put in a Petri dish, and viewed in an inverted fluorescence microscope. Vascular smooth muscle cells were visualized by using a monoclonal antibody to α-smooth muscle actin directly conjugated to Cy3 (1:300, Sigma). Astrocytes were visualized using a polyclonal rabbit antibody to fibrillary acidic protein (GFAP) (1:1000, Dako), and secondary antibody to rabbit IgG-Alexa 594 (1:1000, Molecular Probes - Invitrogen). For immunofluorescence localization in frozen brain sections (16 μm), sections were post-fixed with 4% PFA, permeabilized with PBS containing 0.1% Tween, and incubated with TNB blocking buffer. Endothelial cells were visualized using antibody to CD31/platelet-endothelial cell adhesion molecule (PECAM) (1:100, Pharmingen) and secondary antibody to Rat IgG-Alexa 594 (1:300, Molecular probes). Vascular smooth muscle cells and astrocytes were visualized with the reagents described above. Slides were mounted with Vectashield mounting media for fluorescence with DAPI (Vector Laboratories). Bound antibodies were visualized using a fluorescence microscope (ZEISS Axiophot).

For co-localization of PDGF-CC expression with PDGFR-α expression the *Pdgfrα*$^{+/GFP}$ mice were crossed with heterozygous *Pdgfc*$^{+/lacZ}$ mice[2] on C57BL/6J genetic background, carrying a mutated PDGF-C allele containing a lacZ reporter. The double heterozygous mice were perfused

with 4% paraformaldehyde (PFA). The brains were removed, post-fixed for 40 min and divided into hemispheres. Lac Z expression was visualized by whole mount staining using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) in 37°C in the dark overnight using standard protocol. The hemispheres were then embedded in OCT and sectioned. Vessels were visualized in the frozen brain sections by staining endothelial cells using CD31/PECAM antibody (1:400, Pharmingen) and secondary antibody to Rat IgG-Alexa 568 (1:200, Molecular probes). The overlay picture was prepared by reducing the opacity of the bright field image.

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