SUPPLEMENTAL MATERIAL

INHIBITION OF PAI-1 IMPROVES BRAIN COLLATERAL PERFUSION AND INJURY AFTER ACUTE ISCHEMIC STROKE IN AGED HYPERTENSIVE RATS

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Materials and Methods

Animals

Aged male spontaneously hypertensive rats (SHR, Charles River, Kingston, NY, USA), averaged 50-52 weeks old and their young control male SHR (averaged 17-18 weeks old) were used in this study (n=4-12 per experimental group). Assignment of animals to experimental groups was not completely randomized due to availability of aged animals and additional treatment groups added after the completion of the initial study compared aged to young SHR. Treatment to either young or aged SHR was randomized by using an online tool (Random.org). Animals were housed on a 12-hour light/dark cycle and allowed free access to food and water in the Animal Care Facility at the University of Vermont, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Model of Transient Focal Ischemia

A proximal middle cerebral artery occlusion (MCAO) filament model was used. After anesthetizing with inhaled isoflurane (2.5-3%) in oxygen, both femoral arteries were cannulated for monitoring blood pressure and obtaining blood gas samples. The femoral vein was cannulated for chloral hydrate infusion. After instrumentation, chloral hydrate (200-300 mg/kg, i.v.) anesthesia was used in place of isoflurane because of its potent vasodilator effect in the brain. Toe pinch was performed and blood pressure regularly checked for depth of anesthesia. Body temperature of the animal was monitored by anal temperature probe and heating pad throughout the experiment. The MCA was occluded for 2 hours of ischemia and reperfusion was allowed for 2 hours by removal of filament. We selected this acute phase time point because we were interested in brain injury that may be caused by a mechanism related to vascular dysfunction, e.g., reduced collateral perfusion and/or worse incomplete reperfusion caused by the combination of aging and hypertension. Animals were excluded if the drop in cerebral perfusion during ischemia was <70% from baseline.

Treatment with PAI-1 Inhibitor

Young (n=6) and aged (n=4) SHR were treated with the PAI-1 inhibitor TM5441 (Tocris, Minneapolis, MN, USA; 5 mg/kg, i.v. dissolved in DMSO diluted in saline) 10 minutes before reperfusion. This dose of TM5441 was used in vivo in mice to reduce Nω-nitro-L-arginine methyl ester (L-NAME)-induced hypertension.[1] Plasma half-life of TM5441 at 5 mg/kg is 2.3 hours in rats, therefore only 1 dose was administrated for the rest of the experiment (2 hour 10 minutes post-treatment period).[1] All results obtained from these treatment groups were compared to the untreated young (n=12) and aged (n=8) SHR.

Stroke Outcome Determinations

Rats were decapitated and brains removed. Brains were sliced to 2 mm coronal sections and observed for apparent pink coloration within the brain injury area for hemorrhagic transformation. Brains were then incubated for 30 min at 37°C in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffered saline. Brain sections were then fixed in 3.7% formalin in PBS at 4°C for 45 min, followed by imaging using a digital scanner. Brain injury was identified as

white area of each section and measured with ImageJ software (NIH, Bethesda, MD, USA). Edema was calculated by subtracting the area of contralateral from the ipsilateral hemisphere. Measurements were made by investigators who were blinded to experimental groups. Further microscopic observation for hemorrhagic transformation was performed using brain coronal sections stained with hematoxylin and eosin.

Immunohistochemistry of Aβ42, PAI-1, Hemoglobin, Collagen IV

Formalin fixed, paraffin embedded brain coronal sections were cut to 5 μm thin sections. Antigen retrieval was performed using Dako Target Retrieval Solution (Agilent, Santa Clara, CA, USA). Slides were then blocked in 10% goat serum diluted with PBS/5.0% BSA/0.1% Triton X-100. Primary antibodies for Aβ42 (1:100, Bioss, Woburn, MA, USA), PAI-1 (1:500, Bioss), hemoglobin (1:100, abcam, Cambridge, MA, USA) and collagen IV (1:1000, Sigma, St Louis, MO, USA) were incubated overnight at 4 °C. Presence of bound primary antibody was detected using Alexa Fluor secondary antibodies for Aβ42 (goat anti-mouse IgG, Invitrogen, Carlsbad, CA, USA), PAI-1 (goat anti-rabbit IgG, Invitrogen), hemoglobin (goat anti-rabbit IgG, Invitrogen) and collagen IV (goat anti-mouse IgM, Invitrogen) at 4 μg/ml. Specific binding was evaluated by secondary antibody only controls for Aβ42 (Figure IIA), PAI-1 (Figure IIB) and hemoglobin (Figure IIC). Samples were imaged on a Zeiss LSM 510 Meta laser scanning confocal microscope. Two images were taken on each animal at the brain injury area. Analysis of images was performed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) by investigator who was blinded to experimental groups. The number of capillaries in an image was determined by counting collagen IV positive staining and confirmed by blood vessels that were <10 μm in diameter. Percent expression of Aβ42 or PAI-1 in brain capillary was calculated by the number of capillaries with positive staining divided by total amount of capillaries in the image. Percentage of Aβ42 PAI-1, or hemoglobin positive staining in the whole brain, regardless of tissue type, was calculated by pixels with positive staining divided by total number of pixels in an image (Figure IID, E, F).

In a separate set of experiment, collagen IV staining was used for determining capillary density. Immunohistochemistry procedures of collagen IV were similar except using Vectastain ABC peroxidase mouse IgM secondary antibody kit (Vector Labs, Burlingame, CA, USA) and imaged with Olympus BX50 microscope. Two images were taken in each animal and positive staining of collagen IV was counted twice in each image. Capillaries were confirmed by blood vessels with diameter of <10 μm. In each animal, data were an average of two images and were presented as capillaries/mm^2.

Determination of Circulating Oxidative and Inflammatory Markers

Blood sample from each rat was collected and serum was stored at -80 °C until use. Commercially available ELISA kits were used for determining circulating levels of Aβ42 (Immuno-Biological Lab., Gunma, Japan), PAI-1 (Abcam, Cambridge, MA, USA), oxidized low-density lipoprotein (oxLDL, Biomatik, Wilmington, DE, USA) and tumor necrosis factor-α (TNFα, R&D Systems, Minneapolis, MN, USA). Manufacturers' instructions were followed. Serum samples were undiluted for determination of Aβ42, PAI-1, and TNFα; and diluted 1:10 for oxLDL.

Statistical Power Calculation

Power calculation was based on difference between means of the two groups and standard deviation. Statistical power (1-β) was set at 80 % as probability of type I error (α) was 0.05 and that of type II error (β) was 0.2. Statistical power in determining difference in brain injury volume between aged and young SHR was 82.0 % at n=8, calculated based on similar data from a previous study (mean difference in brain injury volume between groups: 12.2, standard deviation: 12.0).[2] Statistical power in determining difference in brain injury volume after TM5441 treatment was 86.5 % at n=5, calculated based on similar data from a previous study (mean difference in brain injury volume between groups: 10.0, standard deviation: 7.3).[3]

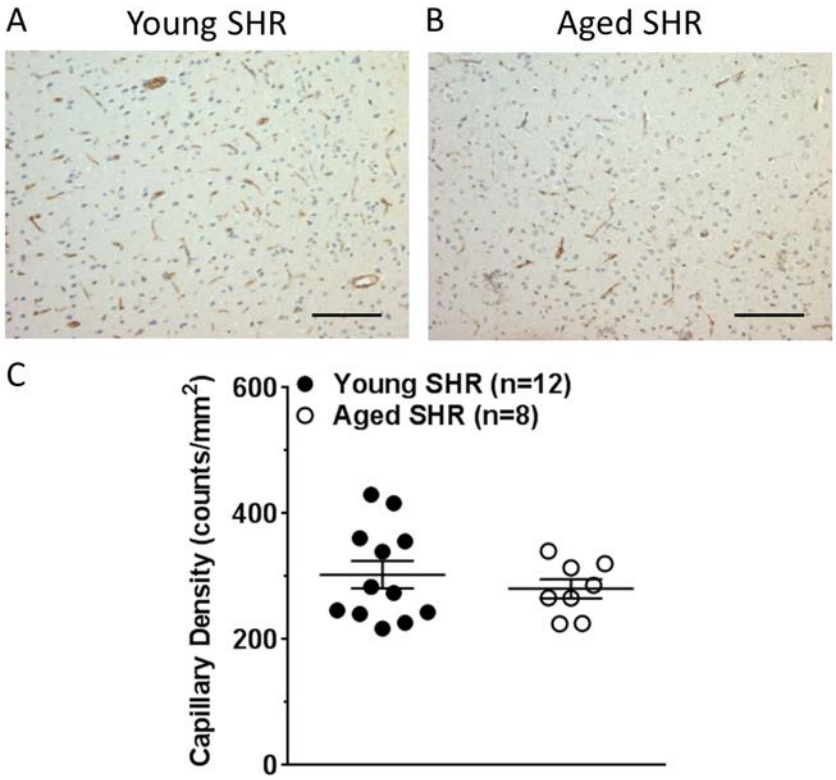

Figure I. Quantification of capillary density using immunohistochemistry of collagen IV in rat brain coronal sections. Representative images showing positive collagen IV staining in young (A) and aged (B) SHR. Graph showing quantification of collagen IV staining in young and aged SHR (C). Scale bar: 100 µm

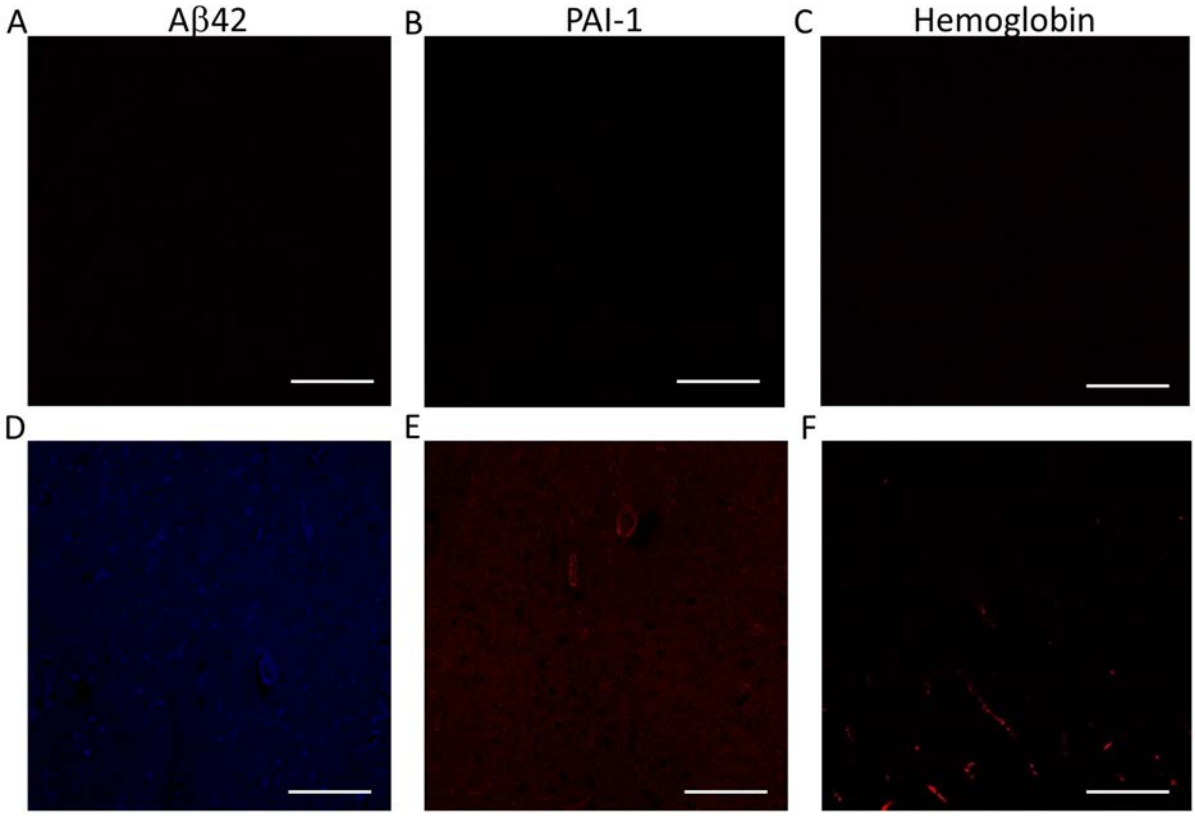

Figure II. Representative images of secondary antibody only control for immunohistochemistry of (A) Aβ42, (B) PAI-1 and (C) hemoglobin. Representative images for quantification of percent positive staining of (D) Aβ42 (blue), (E) PAI-1 (red) and (F) hemoglobin in the whole brain. Images were taken without the green channel (collagen IV). Scale bar: 100 µm.

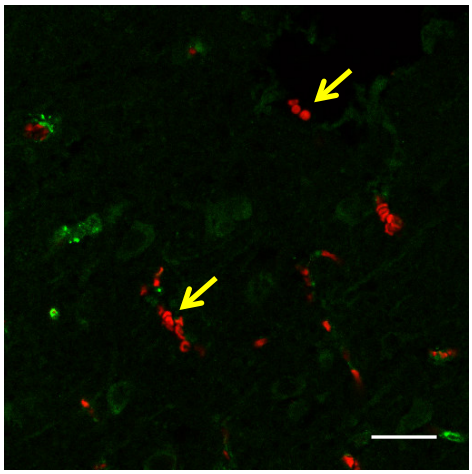

Figure III. Representative image of hemoglobin (red) located outside blood vessels that were identified by collagen IV (green), highlighted by yellow arrows. Scale bar: 50 µm.

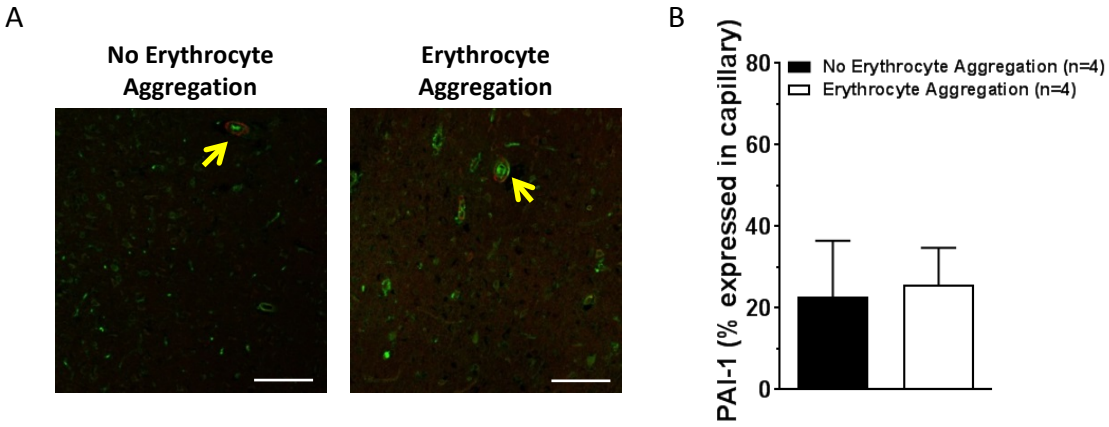

Figure IV. (A) Representative images of brain sections stained for PAI-1 (red) and collagen IV (green) from aged SHR with and without erythrocyte aggregation. (B) Quantification of the percent of microvessels with positive PAI-1 staining showed no difference between young and aged SHR. Scale bar: 100 µm.

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☐ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☑ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.

Table II: Body weights and arterial blood gases of all animals during MCAO surgeries

	Young SHR	Aged SHR	Young SHR + TM5441	Aged SHR + TM5441
n	12	8	6	4
Age (weeks)	16.7±0.2	50.1±2.2	17.8±0.2	51.8±0.2
Weight (g)	322±7	438±11	327±8	444±2
Arterial Blood Gases & Pressure				
Start				
Pressure (mm Hg)	94.5±3.4	95.4±4.5	95.4±4.5	95.4±4.5
pH	7.36±0.02	7.36±0.01	7.44±0.03	7.40±0.01
pCO_2 (mm Hg)	38.5±1.7	38.9±1.4	38.0±2.5	36.7±1.1
pO_2 (mm Hg)	119.9±5.8	103.3±4.9	103.8±6.4	97.5±3.5
Middle				
Pressure (mm Hg)	97.6±3.1	97.1±4.5	97.1±4.5	97.1±4.5
pH	7.35±0.01	7.37±0.01	7.39±0.01	7.36±0.01
pCO_2 (mm Hg)	42.1±1.5	39.9±0.9	40.4±1.2	39.8±2.4
pO_2 (mm Hg)	107.1±4.3	93.0±2.1	102.0±3.6	93.0±4.9
End				
Pressure (mm Hg)	94.1±3.7	98.9±3.4	98.9±3.4	98.9±3.4
pH	7.34±0.01	7.38±0.01	7.38±0.01	7.37±0.01
pCO_2 (mm Hg)	43.2±1.0	39.4±1.0	38.7±1.0	39.3±1.4
pO_2 (mm Hg)	104.5±4.9	98.3±2.3	99.0±5.6	98.8±2.1

References

1. Boe AE, Eren M, Murphy SB, Kamide CE, Ichimura A, Terry D, et al. Plasminogen activator inhibitor-1 antagonist TM5441 attenuates Nomega-nitro-L-arginine methyl ester-induced hypertension and vascular senescence. *Circulation*. 2013;128:2318-2324.
2. Cipolla MJ, Sweet JG, Chan SL. Effect of hypertension and peroxynitrite decomposition with FeTMPyP on CBF and stroke outcome. *J Cereb Blood Flow Metab*. 2017;37:1276-1285.
3. Cipolla MJ, Linfante I, Abuchowski A, Jubin R, Chan SL. Pharmacologically increasing collateral perfusion during acute stroke using a carboxyhemoglobin gas transfer agent (Sanguinate) in spontaneously hypertensive rats. *J Cereb Blood Flow Metab*. 2018;38:755-766.