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Nicholas Weinrich March 28, 2023

Plasminogen Activator Inhibitor – 1's Effects on Blood-Brain Barrier Permeability

by

Nicholas Weinrich

Dr. Yoland Smith
Advisor

Biology

Dr. Yoland Smith

Advisor

Dr. Miguel Reyes

Committee Member

Dr. Victor Faundez

Committee Member

2023

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By

Nicholas Weinrich

Dr. Yoland Smith

Advisor

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Abstract

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The blood-brain barrier (BBB) is a semipermeable membrane between the body's circulating blood and central nervous system (CNS) consisting of astrocytes and endothelial cells as well as pericytes on a basement membrane that maintains homeostasis by regulating what molecules can enter the brain. Stroke damages the brain by cutting off its blood supply, usually through a thrombus reaching the brain, and this causes a series of pathological increases to the permeability of the BBB. The clot is broken down by plasmin, which is facilitated by its cleaving from plasminogen by tissue-type plasminogen activator (tPA). Plasminogen activator inhibitor – 1 (PAI-1) inhibits this process, slowing thrombolysis. Previous work has established that tPA is able to mitigate the negative changes seen in the BBB, but no studies have been done on PAI-1's effect on the BBB. It was found that treatment with PAI-1 induced a decrease in the permeability of an *in vitro* model of the BBB. However, PAI-1 knockout mice did not show any significant difference in expression of blood protein IgG in the brain, indicating no change in the permeability of the BBB.

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Introduction

The blood-brain barrier (BBB) is an important dynamic and metabolic interface that precisely regulates homeostasis in the central nervous system (CNS) and protects delicate neural tissue from potentially toxic substances and pathogens[1]. The BBB is comprised of tight junctions between endothelial cells lining blood vessels, pericytes, astrocytic end feet, and a basement membrane that acts as a strict control point for what can enter the brain parenchyma[2]. The BBB only allows the passive transport of small and hydrophobic molecules. For other important molecules such as glucose, amino acids, fatty acids, and others, the transport proteins use energy to facilitate their movement across the barrier[3].

Certain disorders of the CNS are known to cause pathological changes in the permeability of the BBB, leading to unwanted passage of certain molecules through the barrier[4]. Some of these disorders include Alzheimer's Disease, Parkinson's Disease, and epilepsy[4,5,6,7]. Along the same lines, several interesting changes in permeability arise from ischemia in the brain, or stroke[8]. Studies in rodents have shown that stroke alters the BBB and increases its permeability, and biomarkers of blood proteins such as IgG and serum albumin have been found in humans that show similar results[9]. There is a series of changes in the BBB permeability brought on by stroke, correlated with the hyperacute, acute, subacute, and chronic stages of stroke[10]. During the hyperacute phase of 6 hours after the initial ischemic insult, the permeability starts to increase rapidly[10]. Following this, the acute phase of between 6 and 72 hours after the stroke shows a quick decrease followed by another rapid and even greater increase in

permeability[10]. A similar but smaller and slower trend is observed during the subacute phase of the next 6 weeks following the ischemia[10]. Finally, during the chronic phase of stroke and recovery after 6 weeks, the permeability slowly returns to its baseline level[10].

According to a report made in 2022 by the World Stroke Organization, there are over 12.2 million strokes and 6.5 million deaths from stroke each year, with 1 in 4 people predicted to have a stroke in their lifetime[11]. With the average visit to the hospital for stroke treatment costing patients around $20,000 each, the amount of money, time, and resources necessary to address this disease are massive[11]. Therefore, impactful, and cost-effective treatments for stroke are needed for cost reduction and patient outcome improvement, and mitigation of the damage to the BBB is an interesting avenue to explore for new stroke treatments.

There has been previous research into the role of tissue-type plasminogen activator (tPA) and its effect on stroke[12]. tPA, a serine protease enzyme, cleaves plasminogen into its active form, plasmin, to break up clots in the body[13]. Previous studies have tested how the blood-brain barrier was impacted after ischemic conditions and how the change was mitigated by tPA treatment[12]. Regarding blood-brain barrier permeability, it was found that tPA mitigated the damage done to the blood brain barrier by oxygen and glucose deprivation (OGD)[12]. tPA's main inhibitor is plasminogen activator inhibitor 1 (PAI-1) which, among other functions, binds with tPA to prevent it from cleaving plasminogen[13]. This prevents tPA-facilitated fibrinolysis in the brain, the process that degrades clots[14].

The general objective of this experiment was to evaluate if PAI-1 impacts BBB permeability. This was accomplished with two specific objectives. First, permeability

quantification was sought by using an *in vitro* model of the BBB adapted from Diaz et al. and comparing control conditions with PAI-1 treatment[12]. To do so, resistance values of semipermeable membranes seeded with endothelial cells and astrocytes were measured and compared based on whether the cells were treated with PAI-1. This indicates changes in the BBB based on solute flow between the two chambers, with a lower resistance signaling that BBB permeability is increased. The second aim was to measure permeability using IGG as a biomarker to detect changes *in vivo*. It was planned for measurements of a blood protein to be collected as an additional method of testing how PAI-1 effects the BBB. To do so, IgG levels in wild type (WT) and PAI-1 knockout (KO) mice were compared. Western blot analysis provides a way to identify protein expression based on molecular weight and signal intensity in cells and tissues by collecting all their proteins and blotting against a given antibody. Immunohistochemistry provides a similar result, but the proteins can be seen in their native locations on the tissue. It was hypothesized that, inversely to its substrate, treatment with PAI-1 will lead to an increase in the permeability of the BBB.

Methods

Animals and Reagents

Animals were either 1 day old (pups for astrocytic culture) or 6 months old (adults for immunohistochemistry and western blot), either genetically deficient in PAI-1 (PAI-$1^{-/-}$ /PAI-1 KO) or WT C57BL/6J mice. Animal procedures were conducted following the guidelines of the Guide for the Care and Use of Laboratory Animals, with the approval

of the Institutional Animal Care & Use Committee (IACUC) of Emory University, Atlanta GA. Reporting adhered to ARRIVE 2.0 guidelines. 12 adult mice and 9 pups were sacrificed for these experiments. Reagents were: Hoechst, BCA, sample buffer 5X, Pierce BCA Protein Assay Kit, trypsin 1x, PBS pH 7.4, recombinant human PAI-1, and ProLong Gold antifade mountant, HBSS, DNAse I (ThermoFisher: Catalog # H3570, 23225, 1859594, 23225, 25200056, 10010023, RP-75686, P36930, 24020117, 18047019. Grand Island, NY), Triton 100X, attachment factor solution, Poly-I-lysine, RIPA buffer, casein, donkey serum, goat serum (Sigma, OmniPur, MilliPore. Catalog # 9400, 123-100, P1399, R0278-500ML, C7078-1KG, 566460-5ML, NS02L-1ML. Burlington, MA), PhosSTOP- Phosphatase inhibitor and protease inhibitors complete tablets EASYpack (Roche: Catalog # 4906837001 and 4693132001. Indianapolis, IN), nitrocellulose membranes, Mini-PROTEAN TGX Gels (Bio-Rad: catalog # 1620112, 4561084. Hercules, CA), and paraformaldehyde (Electron Microscope Sciences: Catalog # 15714. Hatfield, PA). Astrocytes were maintained using media consisting of MEM (GIBCO, Thermofisher: Catalog # 11095080. Grand Island, NY) with sodium pyruvate 1:100 (Quality Biological: Catalog # 118093721. Gaithersburg, MD), Penicillin/Streptomycin (GIBCO, Thermofisher: Catalog # 15140122. Grand Island, NY), MEM non-essential Amino Acids (NEAA) 1:100 (Quality Biological. Catalog # 116079721. Gaithersburg, MD), Fetal Bovine Serum (Corning. Catalog # 35010-CV. Corning, NY), and 20% glucose solution (TEKNOVA. Catalog # G0535 Hollister, CA). Endothelial cells were cultured using rat brain endothelial cell growth medium (Cell Applications, Inc. Catalog # R819-500. San Diego, CA). The antibodies used were against IgG (Cell Signaling Technology: Catalog # 2729. Danvers, MA), beta actin, Goat

anti-rabbit, Alexa Fluor 488 (ThermoFisher: Catalog # H3570, A21207, A11017. Grand Island, NY), and Li-COR secondary antibodies 800 rabbit and 680 mouse as well as blocking buffer (Li-COR: Catalog # 926-32213, 926-68072, 927-60001. Lincoln, NE).

Cell Culture

Astrocytes were cultured from the cerebral cortex of 1-day old PAI-1 KO and WT mice. The mother was anesthetized using isoflurane, perfused with PBS and paraformaldehyde, and the pups were manually decapitated. The cerebral cortex was surgically separated and transferred into HBSS containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. The cells were then incubated in trypsin containing 0.02 % DNase at 37°C for 30 min. The tissue was triturated, and the supernatant was re-suspended in medium comprised of MEM supplemented with sodium pyruvate 1:100, Penicillin/Streptomycin 1:100, MEM non-essential amino acids 1:100, fetal bovine serum 1:10, and 20% Glucose solution 1:50. The media was filtered through a 70 µm pore membrane. Then, cells were plated into poly-I-lysine-coated T75 flasks. Fourteen days later. astrocytes were plated and used for experiments at DIV 7-14.

Endothelial cells were cultured by first incubating a T75 flask with attachment factor overnight. The following day, adult rat brain microvascular endothelial cells (Millipore: Catalog # R840-05A. St. Louis, MO) were seeded into the flask in rat brain endothelial cell growth media for fourteen days until the cells reached confluence required for experiments.

Both cell types were maintained by regular washes with HBSS followed by changing of their respective media when the old media exhibited a color change toward

yellow. When cells reached confluence before experiments were necessary, they were washed once with HBSS, trypsinized for 5 minutes, resuspended in 6 mL of their respective media, and 1 mL was seeded into a new flask with fresh media.

In Vitro Model of the BBB

To examine the effect of PAI-1 treatment on BBB permeability, an *in vitro* model of the BBB was constructed using a semipermeable membrane (CELLTREAT: Catalog # 230629. Pepperell, MA) seeded with astrocytes and endothelial cells on opposite sides (n = 3). Attachment factor solution was incubated for 1 hour on both sides of a 0.4 µm thick membrane within a hanging cell culture insert within a 24-well plate. Afterwards, astrocytes were trypsinized and suspended in media, and 10,000 cells per cm^2 were seeded and incubated on the bottom of the membrane for 2 hours. Following this, endothelial cells were trypsinized and suspended in media, and 10,000 cells per cm^2 were seeded on the top of the membrane. 1 mL of media was added into the bottom of the well, and 200 µL of media was added into the hanging cell insert to cover both surfaces of the membrane. The cells were then allowed to incubate for 7 days at 37° C with 5% CO_2. Afterwards, 6 of the 12 membranes were treated with 10 nM PAI-1 solution from a 10 ug stock and allowed to incubate for an additional 24 hours.

After the incubation period, an EVOM2 Epithelial Voltohmmeter (World Precision Instruments. Sarasota, FL) was used to measure the resistance between the two chambers containing the different cell types on either side of the membrane, with a higher resistance between the chambers indicating a lower permeability. Measurements were taken 3 times for each of the 12 membranes, and the resistance values were compared between the treatment and control groups.

Immunohistochemistry of Mouse Brain Slices

To study the effect of PAI-1 on BBB permeability, sections of cerebral cortex of both WT and PAI-1 KO mice brains were cut for staining using a freezing microtome (GMI: Microm HM 440E. Ramsey, MN) (n = 3 WT and 3 PAI-1 KO brains from 6 month old mice). WT and PAI-1 KO mice were deeply anesthetized using pure isoflurane, perfused with 1x PBS, and the brains were then extracted. They were fixed by immersion in 4% paraformaldehyde overnight, transferred into 30% sucrose solution, and were frozen using dry ice. The brains were sliced into 35 µm-thick sections, and 10 slices were analyzed per brain. The slices were rinsed in tris-buffered saline (TBS), permeabilized for 1 hour using 0.1% triton and 0.25% casein in TBS, and rinsed 3 times in TBS for 5 minutes. The brains were then blocked for 1 hour using 0.25% casein, 0.25% goat serum, and 0.25% donkey serum, and incubated in normal rabbit IgG primary antibody 1:200 overnight. The next day, the brains were rinsed 3 times in TBS for 5 minutes and incubated with goat anti-rabbit IgG 488 nm fluorescent secondary antibody as well as Hoescht for nucleus staining and verification of cell presence. Finally, the slices were rinsed 3 final times with TBS for 5 minutes and mounted onto glass slides using 20 µL of ProLong Gold Antifade mounting medium. Slices were imaged at 4x magnification using an immunofluorescent microscope (Olympus. Catalog # IX83. Waltham, MA), and IgG intensity was measured by quantifying the 488 nm fluorescence in each brain slice using image analysis software CellSens Dimension 3.2 (Olympus. Waltham, MA). Intensities were collected by outlining the brain slices and measuring the mean grey intensity value and compared between WT and PAI-1 KO groups for final measurements.

Western Blot of Cerebral Cortex Lysate

To study the effect of PAI-1 on BBB permeability, brain lysates of WT and PAI-1 KO mice were used for western blot analysis (n = 3 WT and 3 PAI-1 KO brains from 6 month old mice). Both WT and PAI-1 KO mouse brains were deeply anesthetized pure isofluorane before being perfused with PBS, upon which the left and right cortexes of both brain types were collected. Following this, tissues were pulverized in RIPA buffer with protease and phosphatase inhibitors and sonicated (Sonics: VibraCell. Newtown, CT) at 30% amplitude to release intracellular proteins. The pulverized tissue mixture was then centrifuged for 14 minutes at 14,000 RPM to separate protein from cellular debris. The protein-filled supernatant was collected and quantified using a Pierce BCA Protein Assay. After quantification, 20 µg of protein were loaded onto a 4-15% TGX Gel and transferred onto a nitrocellulose membrane. The membrane was then blocked using LI-COR proprietary blocking buffer. The membrane was then incubated with normal rabbit IgG primary antibody 1:1,000 in blocking buffer. The following day, the membrane was washed with tris-buffered saline with tween (TBST) 3 times for 5 minutes and incubated with donkey anti-rabbit 800 nm wavelength fluorescent secondary antibody 1:10,000. After 3 final 5-minute washes with TBST, the membrane was developed using the LI-COR Odyssey Fc reader (LI-COR Biosciences. Lincoln, NE) for the 800 nm wavelength corresponding to the secondary antibody, and the band intensities were quantified. To normalize band intensity to the overall protein concentration by using a loading control, the preceding primary and secondary antibody incubations were repeated for the β-Actin structural protein 1:50,000 with donkey anti-mouse 680 nm fluorescent secondary antibody and imaged. The concentration of the

IgG band was then normalized with the same sample's β-Actin band. The normalized data were then compared based on whether the brain was WT or PAI-1 KO. In order to perform these analyses, Image Studio Lite Verison 5.2 was used (LI-COR Biosciences. Lincoln, NE).

Statistical Analyses

For analysis between the two groups found in each experiment (control vs PAI-1 treatment in the *in vitro* model, WT vs PAI-1 KO in immunohistochemistry and western blot), GraphPad Prism software was used (Dotmatics. Boston, MA). For each comparison, a two-sample unpaired t-test was used. In order to verify homogeneity of variance and normal distribution of the data sets, Levene and Shaprio-Wilk tests were applied, respectively.

Results

PAI-1 treatment induces an increase in the membrane resistance of the *in vitro* BBB model.

To determine whether direct treatment with PAI-1 affects the permeability of the BBB, a model was used in which astrocytes and endothelial cells were seeded on the bottom and top of a 0.4 μm thick semi-permeable membrane, respectively, and resistance values were measured between the two chambers in the presence or absence of 10 nM PAI-1 incubated for 24 hours (Figure 1a). The results showed that treatment with PAI-1 caused a significant increase in the resistance values between the two chambers separated by the membrane (Figure 1b, $p < 0.0001$). This indicates that

PAI-1's effects on the membrane cause a decrease in the permeability, allowing fewer solutes and less current to flow through the semipermeable membrane than preceding treatment.

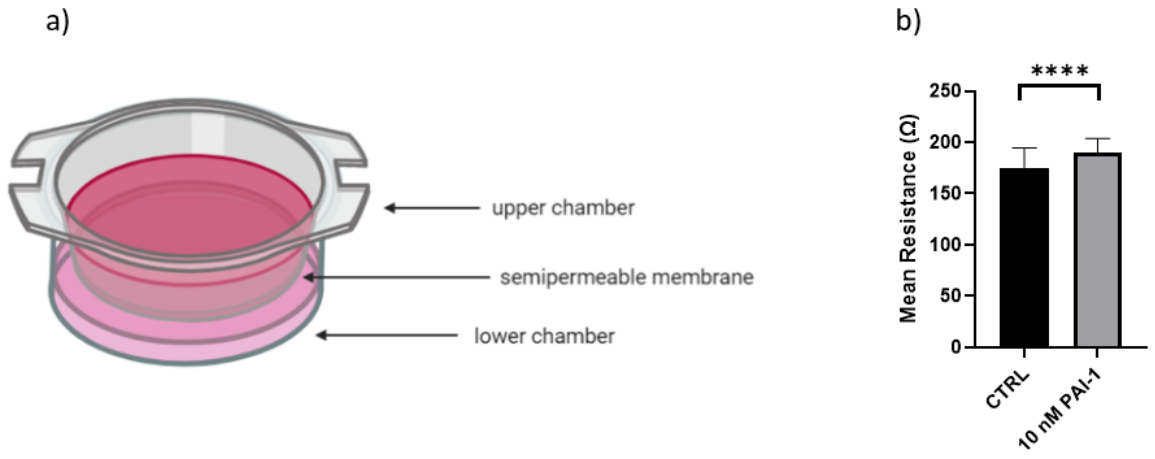

Figure 1: PAI-1 treatment induces an increase in the membrane resistance of the *in vivo* BBB model

a) Representative diagram of the cell culture insert, showing both chambers and the semipermeable membrane separating them (figure made using Biorender (Toronto, Canada)) b) Quantification of resistance values between the two chambers of the hanging cell insert membrane for both control and 10 nM PAI-1 treatment groups ($n = 3$, $p < 0.0001$)

Immunohistological analysis shows no significant difference between IgG expression between WT and PAI-1 KO mice.

To study BBB permeability between WT and PAI-1 KO mice, 35 µm-thick cortex slices from both mouse types were collected and treated with IgG primary antibody followed by fluorescent secondary antibody (Figure 2a/b). The results showed that there

was no significant difference in the level of fluorescent labeled IgG in the brain slices, indicating that IgG in the brain is not affected based on PAI-1 accumulation (Figure 2c, p = 0.6743).

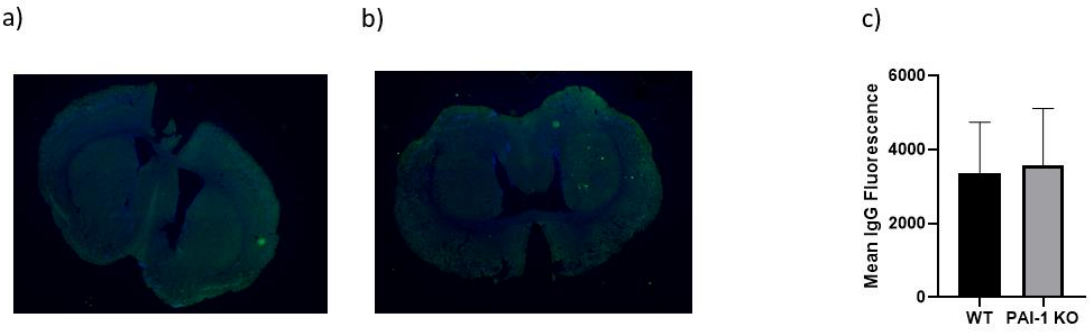

Figure 2: Immunohistological analysis shows PAI-1 KO mice exhibit increased IgG levels in the brain.

a) Representative image of a WT brain labeled with fluorescent antibodies for IgG (green) and Hoescht (blue) b) Representative image of a PAI-1 KO brain labeled with fluorescent antibodies for IgG and Hoescht c) Quantification of mean IgG fluorescence between WT and PAI-1 KO brain slices (n = 3 WT and 3 PAI-1 KO brains, p = 0.6743)

Western blot analysis shows no significant difference between WT and PAI-1 KO brains.

To study BBB permeability between WT and PAI-1 KO mice, lysates of the cerebral cortex were used for western blot analysis against IgG primary and fluorescent secondary antibodies. The results showed that there was no significant difference between the two types of brains, indicating that leakage of IgG into the cortex is not affected based on PAI-1 accumulation (Figure 3b, p = 0.7211).

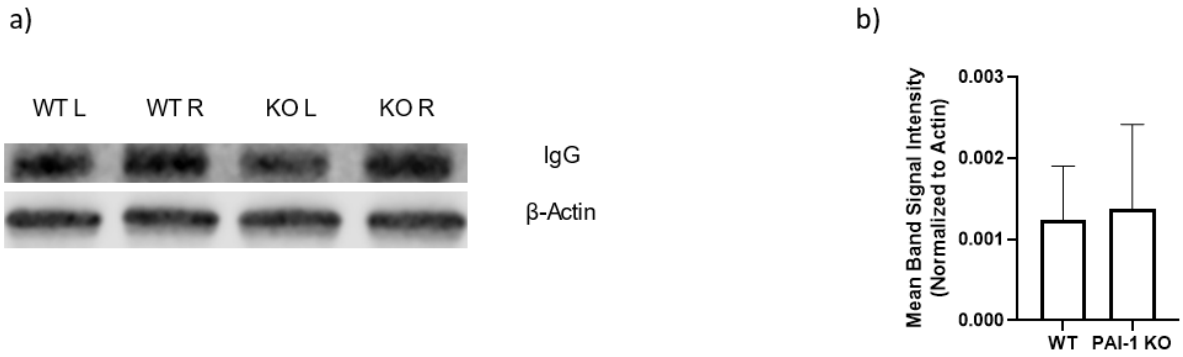

Figure 3: Western blot analysis shows no significant difference between WT and PAI-1 KO brains.

a) Western blot of WT and PAI-1 KO brains for IgG and β-Actin b) Quantification and comparison of the average band intensities, normalized to β-Actin (n = 3 WT and 3 PAI-1 KO brains, p = 0.7211)

Discussion

Because both the immunohistological and western blot data show no significant difference in brain IgG levels between WT and PAI-1 KO groups, PAI-1 expression is shown to have no effect on BBB permeability in these experiments. The results from this experiment regarding resistance values in the *in vitro* model of the BBB show that PAI-1 has some effect on the semi-permeable membrane within the cell culture inserts. This could be an indication that PAI-1 is able to influence the astrocytes and endothelial cells to allow for smaller pores within the membrane, supporting the idea that the protein is able to have an independent effect on the BBB[15]. However, the other results indicate that the change exhibited by PAI-1 treatment could have been caused by its influence on solute concentrations, which could cause a raising or lowering of the conduction in the circuit that exists within the well, in turn affecting the resistance values[16].

PAI-1 is a serine protease inhibitor (serpin) that plays large roles in many biological processes within the body, often having a large impact because of its role in inhibition of tPA[17]. The process of thrombolysis by the tPA-mediated cleaving of plasminogen into plasmin is of vital importance for many normal and pathological processes, and its inhibition by PAI-1 can have major positive and negative effects[18]. For example, PAI-1 has largely negative effects on most cardiovascular outcomes due to its clot-breaking inhibition but seems to facilitate neural protection[18,19]. In stroke, PAI-1's role is more complex, as the anti-thrombolytic properties of PAI-1 are obviously not beneficial to a thrombosis within the brain, but its neuroprotective factors cannot be overlooked[20].

These findings that PAI-1 alone likely has little effect on BBB permeability suggest that tPA is able to exert changes on the BBB without its inhibitor, but there is still a possibility that PAI-1 plays a role in BBB permeability when working in tandem with tPA in the complexed form[12]. For example, tPA and PAI-1 have unique functions individually, but when complexed together they are more than the sum of their parts, with certain inhibitory activity only accessible when the complex itself is available for binding with receptors[21]. This could explain why PAI-1 treatment, but not PAI-1 KO, showed significant differences compared to their controls, as the complete lack of PAI-1 in the KO mice would limit tPA's ability to form complexes and exert its full scope of functionality[21]. However, it has been shown that tPA's effect on the BBB is mediated by its stimulation of astrocytes to release tumor necrosis factor – α (TNF-α), and PAI-1 levels have been shown to rise with TNF-α levels[12,22,23]. With these previous findings in

mind, it is proposed that PAI-1 could possibly mediate changes in the BBB through its interaction with TNF- α.

tPA has been widely used in the hospital setting as one of the few effective drugs to treat stroke, yet related and interacting proteins involved in its pathways are relatively unexplored as treatment options[24]. Further research into alternative treatments for stroke or other pathologies impacting the BBB should be done, although treatment with solely PAI-1 does not seem to be a fruitful route to explore, as its effects are not observable, and many cardiovascular pathologies may experience a worsening due to its antithrombolytic activity before they experience a benefit from its neural protection. However, treatment with TNF-α is a promising avenue to research, as tPA's effects on the BBB seem to result mainly from TNF-α expression[12]. Additionally, PAI-1's interactions with TNF-α should be explored further, as a mechanism in which PAI-1 is able to mediate BBB changes could serve useful for exploration if TNF-α is shown to be the main driver for changes in the permeability of the BBB.

This study has limitations that affect its external validity. The *in vitro* model of the BBB, although used elsewhere, is not a perfect representation of the intricate system found within organisms[12]. It only consists of astrocytes and endothelial cells within media, while the BBB also contains pericytes and exists within a delicate balance of solutes. Additionally, higher n values would be ideal given less of a time constraint. In order to validate these findings, additional repetitions of the experiments as well as an additional BBB are recommended.

Conclusions

PAI-1 treatment induced a significant change in the resistance of a semi-permeable membrane seeded with astrocytes and endothelial cells replicating the BBB *in vitro*. However, PAI-1 KO mice did not exhibit any significant differences in brain IgG levels, a biomarker of BBB permeability increase, in both western blot and immunohistological analysis.

Conflicts of interest

The author has no conflicts of interest to disclose.

References

1) Daneman, R. and A. Prat (2015). "The blood-brain barrier." Cold Spring Harb Perspect Biol **7**(1): a020412.

2) Kadry, H., B. Noorani and L. Cucullo (2020). "A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity." Fluids and Barriers of the CNS **17**(1): 69.

3) Ballabh, P., A. Braun and M. Nedergaard (2004). "The blood-brain barrier: an overview: structure, regulation, and clinical implications." Neurobiol Dis **16**(1): 1-13.

4) Archie, S. R., A. Al Shoyaib and L. Cucullo (2021). "Blood-Brain Barrier Dysfunction in CNS Disorders and Putative Therapeutic Targets: An Overview." Pharmaceutics **13**(11).

5) Cai, Z., P. F. Qiao, C. Q. Wan, M. Cai, N. K. Zhou and Q. Li (2018). "Role of Blood-Brain Barrier in Alzheimer's Disease." J Alzheimers Dis **63**(4): 1223-1234.

6) Bartels, A. L., A. T. M. Willemsen, R. Kortekaas, B. M. de Jong, R. de Vries, O. de Klerk, J. C. H. van Oostrom, A. Portman and K. L. Leenders (2008). "Decreased blood–brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA." Journal of Neural Transmission **115**(7): 1001-1009.

7) van Vliet, E. A., E. Aronica and J. A. Gorter (2015). "Blood–brain barrier dysfunction, seizures and epilepsy." Seminars in Cell & Developmental Biology **38**: 26-34.

8) Jiang, X., A. V. Andjelkovic, L. Zhu, T. Yang, M. V. L. Bennett, J. Chen, R. F. Keep and Y. Shi (2018). "Blood-brain barrier dysfunction and recovery after ischemic stroke." Prog Neurobiol **163-164**: 144-171.

9) Zhang, W., L. Zhu, C. An, R. Wang, L. Yang, W. Yu, P. Li and Y. Gao (2020). "The blood brain barrier in cerebral ischemic injury – Disruption and repair." Brain Hemorrhages **1**(1): 34-53.

10) Bernardo-Castro, S., J. A. Sousa, A. Brás, C. Cecília, B. Rodrigues, L. Almendra, C. Machado, G. Santo, F. Silva, L. Ferreira, I. Santana and J. Sargento-Freitas (2020). "Pathophysiology of Blood–Brain Barrier Permeability Throughout the Different Stages of Ischemic Stroke and Its Implication on Hemorrhagic Transformation and Recovery." Frontiers in Neurology **11**.

11) Feigin, V. L., M. Brainin, B. Norrving, S. Martins, R. L. Sacco, W. Hacke, M. Fisher, J. Pandian and P. Lindsay (2022). "World Stroke Organization (WSO): Global Stroke Fact Sheet 2022." Int J Stroke **17**(1): 18-29.

12) Diaz, A., Y. Woo, C. Martin-Jimenez, P. Merino, E. Torre and M. Yepes (2022). "Tissue-type plasminogen activator induces TNF-α-mediated preconditioning of the blood-brain barrier." Journal of Cerebral Blood Flow & Metabolism **42**(4): 667-682.

13) Melchor, J. P. and S. Strickland (2005). "Tissue plasminogen activator in central nervous system physiology and pathology." Thromb Haemost **93**(4): 655-660.

14) Sillen, M. and P. J. Declerck (2021). "A Narrative Review on Plasminogen Activator Inhibitor-1 and Its (Patho)Physiological Role: To Target or Not to Target?" Int J Mol Sci **22**(5).

15) Stamatovic, S. M., A. M. Johnson, R. F. Keep and A. V. Andjelkovic (2016). "Junctional proteins of the blood-brain barrier: New insights into function and dysfunction." Tissue Barriers **4**(1): e1154641.

16) Booth, R. and H. Kim (2012). "Characterization of a microfluidic in vitro model of the blood-brain barrier (μBBB)." Lab Chip **12**(10): 1784-1792.

17) Declerck, P. J. and A. Gils (2013). "Three decades of research on plasminogen activator inhibitor-1: a multifaceted serpin." Semin Thromb Hemost **39**(4): 356-364.

18) Jung, R. G., P. Motazedian, F. D. Ramirez, T. Simard, P. Di Santo, S. Visintini, M. A. Faraz, A. Labinaz, Y. Jung and B. Hibbert (2018). "Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis." Thrombosis Journal **16**(1): 12.

19) Soeda, S., S. Koyanagi, Y. Kuramoto, M. Kimura, M. Oda, T. Kozako, S. Hayashida and H. Shimeno (2008). "Anti-apoptotic roles of plasminogen activator inhibitor-1 as a neurotrophic factor in the central nervous system." Thromb Haemost **100**(6): 1014-1020.

20) Tjärnlund-Wolf, A., H. Brogren, E. H. Lo and X. Wang (2012). "Plasminogen activator inhibitor-1 and thrombotic cerebrovascular diseases." Stroke **43**(10): 2833-2839.

21) Gonias, S. L., M. A. Banki, A. S. Gilder, P. Azmoon, W. M. Campana and E. Mantuano (2018). "PAI1 blocks NMDA receptor-mediated effects of tissue-type plasminogen activator on cell signaling and physiology." J Cell Sci **131**(14).

22) Swiatkowska, M., J. Szemraj and C. S. Cierniewski (2005). "Induction of PAI-1 expression by tumor necrosis factor alpha in endothelial cells is mediated by its responsive element located in the 4G/5G site." Febs j **272**(22): 5821-5831.

23) Samad, F., K. Yamamoto and D. J. Loskutoff (1996). "Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. Induction by tumor necrosis factor-alpha and lipopolysaccharide." J Clin Invest **97**(1): 37-46.

24) Grotta, J. C. (2021). "Fifty Years of Acute Ischemic Stroke Treatment: A Personal History." Cerebrovascular Diseases **50**(6): 666-680.