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RESEARCH ARTICLE

Factor VII activating protease (FSAP) inhibits the outcome of ischemic stroke in mouse models

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Abstract

The outcome of ischemic stroke can be improved by further refinements of thrombolysis and reperfusion strategies. Factor VII activating protease (FSAP) is a circulating serine protease that could be important in this context. Its levels are raised in patients as well as mice after stroke and a single nucleotide polymorphism (SNP) in the coding sequence, which results in an inactive enzyme, is linked to an increased risk of stroke. In vitro, FSAP cleaves fibrinogen to promote fibrinolysis, activates protease-activated receptors, and decreases the cellular cytotoxicity of histones. Based on these facts, we hypothesized that FSAP can be used as a treatment for ischemic stroke. A combination of tissue plasminogen activator (tPA), a thrombolytic drug, and recombinant serine protease domain of FSAP (FSAP-SPD) improved regional cerebral perfusion and neurological outcome and reduced infarct size in a mouse model of thromboembolic stroke. FSAP-SPD also improved stroke outcomes and diminished the negative consequences of co-treatment with tPA in the transient middle cerebral artery occlusion model of stroke without altering cerebral perfusion. The inactive MI-isoform of FSAP had no impact in either model. FSAP enhanced the lysis of blood clots in vitro, but in the tail transection model of hemostasis, FSAP-SPD treatment provoked a faster clotting time indicating that it also has pro-coagulant actions. Thus, apart from enhancing thrombolysis, FSAP has multiple effects on stroke progression and represents a promising novel therapeutic strategy in the treatment of ischemic stroke.

KEYWORDS

bleeding, brain, FSAP, HABP2, hemorrhage, ischemic stroke, protease, thrombectomy, thrombolysis, tPA

Abbreviations: APC, activated protein C; ABF, arterial blood flow; CBF, cerebral blood flow; FSAP, factor VII activating protease; HMW-HA, high molecular weight Hyaluronic acid; LMW-HA, low molecular weight Hyaluronic acid; FSAP, factor VII activating protease; MI, marburg I; MCA, middle cerebral artery; NETosis, neutrophil extracellular trap formation; PAI, plasminogen activator inhibitor; PAR, protease activated receptor; SNP, single nucleotide polymorphism; SPD, serine protease domain; TES, thromboembolic stroke; TFPI, tissue factor pathway inhibitor; tMCAO, transient middle cerebral artery occlusion; tPA, tissue plasminogen activator; uPA, urokinase type plasminogen actvator.

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1 | INTRODUCTION

An abrupt loss of brain blood supply due to arterial thrombosis is the major cause of ischemic stroke and it afflicts a large number of people worldwide.[1] Along with the surgical removal of clots from larger arteries, the only approved pharmacological therapy is tissue plasminogen activator (tPA).[2] Thrombolysis with tPA is effective in the 0–4.5 h time frame, after which the adverse effects outweigh the positive attributes.[2] Moreover, it is efficacious in only 30% of the patients,[3] and its use is associated with hemorrhagic transformation in about 5%–10% of cases.[4] Thrombectomy is effective over a longer time range, it can be combined with tPA and has a better cost-benefit ratio.[5] Due to their location and composition, some thrombi are irretrievable by thrombectomy and hence there is a need to improve the thrombolytic treatment of ischemic stroke. The pathology of ischemic stroke involves a large number of molecular pathways and cellular interactions and the thrombo-inflammation nexus may provide alternative treatment strategies.[6]

Factor VII activating protease (FSAP) is a circulating serine protease, and individuals with the Marburg-I (MI) single nucleotide polymorphism (SNP), which leads to a protein with low proteolytic activity,[7] have an increased risk of stroke and carotid stenosis.[8,9] In one GWAS study, a locus near this gene was found to increase the risk of stroke in the young.[10] Circulating FSAP levels are increased in patients with ischemic stroke[11,12] and correlate with poor collaterals and worse neurological outcome.[11] Mice have elevated levels of FSAP in the brain and plasma after stroke[11] and FSAP-deficient mice subjected to thromboembolic stroke exhibit a larger infarct volume and increased behavior deficit than wild type (WT) mice.[13]

Although the activation of FVII has been the name-giving property of this protein,[7] a number of other activities have been ascribed to it in relation to thrombosis and hemostasis. It can activate pro-urokinase (uPA),[7] and decrease tissue factor pathway inhibitor (TFPI) activity[14] thereby promoting coagulation, cleave fibrinogen and speed up the lysis of clots.[15] FSAP-deficient mice exhibit delayed thrombosis as well as hemostasis.[16] Reflecting the opposing actions of FSAP on the coagulation and fibrinolysis pathways, the MI-SNP is not decisively linked to thrombosis.[17]

FSAP has a broad substrate specificity[18] and among others, it stimulates protease-activated receptors (PARs) and increases vascular permeability in the lungs,[19] as well as inhibiting apoptosis.[20] FSAP is exceptionally effective in blocking the toxicity of histones[21] and is more potent in this activity than activated protein C (APC).[22,23] Histones are released during necrosis, apoptosis, or during neutrophil extracellular trap formation (NETosis)[24] and are potent activators of the zymogen form of FSAP.[21] Thus, histones trigger a self-destructing cycle that leads to their own neutralization via FSAP. The interaction between FSAP and NETs is further supported by the fact that FSAP is co-localized with NETs[22] in coronary artery thrombi.

Thus, converging lines of evidence from human genetic investigations, mouse models and in vitro studies[8,10,13,15,20] indicate that FSAP may be a candidate for the therapy of ischemic stroke. To address these questions, we tested the effects of recombinant serine protease domain (SPD) of FSAP in ischemic stroke in mouse models.

2 | METHODS

2.1 | Mice

Adult male C57BL6J mice (10–15 weeks old) were obtained from Charles Rivers (Sulzfeld, Germany) and bred in our institution under specific pathogen-free (SPF) conditions. All animal experiments were approved by the Norwegian food safety authority (Application Nos. 7557, 9769, and 9772). The group size was calculated for the effect of treatment on the infarct area 24 h after stroke in WT mice. We assumed a 40% difference in mean, 30% standard deviation, 80% power, and 95% confidence which gave a group size of 7. We expected a premature termination of the experiment in about 20%–25% of the animals due to premature death or abortive death due to excessive pain or moribund state as stipulated by institutional guidelines. To account for the differences in attrition rates between the groups, we operated 8–10 animals per group.

2.2 | Recombinant FSAP-SPD

The C-terminal His-tagged serine protease domain (SPD) of human WT-FSAP, hereafter referred to as WT-SPD, and the MI mutation (MI-SPD) was prepared and characterized as described before.[25]

2.3 | TES model

Studies were performed in accordance with the "ARRIVE Essential 10" guidelines[26] except that the operator was not blinded to the treatments. Mice were randomly assigned to different treatment groups (PBS, tPA, WT-SPD plus tPA, or MI-SPD plus tPA). Mice were anesthetized with isoflurane and the core temperature was maintained at 37°C with a rectal-probe temperature-controlled homeothermic blanket (Panlab, Barcelona, Spain). Cerebral blood flow (CBF) was measured by a laser Doppler flowmeter

(Moor, Axminster, UK) with a flexible fiber-optic probe affixed to the skull over the parietal cortex perfused by the MCA after making an incision in the skin. The neck area was dissected to expose the carotid artery and another fiber-optic probe was attached under the carotid artery bifurcation to measure arterial blood flow (ABF). After exposing the carotid artery a $1 \times 1\,mm^2$ filter paper soaked in 20% $FeCl_3$ (w/v) solution was placed on the central section of the carotid artery for 20 min to induce thrombosis and stroke as described before.[27] Sixty minutes after the onset of thrombosis, the treatment with tPA (Altepase, Boehringer Ingelheim, Ingelheim, Germany), (10 mg/kg body weight) and SPDs (8 mg/kg/body weight), combinations of tPA and FSAP-SPD, as well as, PBS was initiated as an intravenous infusion over 30 min (10% bolus) through the dorsal penial vein. The neck and head wound was sutured and the animals were transferred to temperature-controlled recovery cages, and at 4 and 24 h, a 5-point behavior score was determined.[28] Normal motor function was scored as 0, flexion of the contralateral torso and forelimb on lifting the animal by the tail as 1, circling to the contralateral side but normal posture at rest as 2, leaning to the contralateral side at rest as 3, and no spontaneous motor activity as 4.

2.4 | tMCAO model

Mice were randomly assigned to different treatment groups; PBS, tPA, WT-SPD plus tPA, MI-SPD plus tPA, WT-SPD, or MI-SPD. All procedures were the same as in the TES model except that after the neck area was dissected to free the carotid artery, a filament (0.22 ~ 0.24 mm, 60-2356 PK10, Doccol corporation, Sharon, MA) was inserted through an incision in the external carotid artery and advanced through the internal carotid artery to the origin of MCA as described before.[29] After confirming occlusion by a 70% drop in CBF, the filament was kept in position for 60 min while the mice were maintained under anesthesia. After a 15 min reperfusion period, the treatment was initiated and the rest of the procedures were as described under the TES model.

2.5 | Analysis of hemorrhage, infarct size, and hemisphere swelling

Animals were sacrificed at the 24 h time point, and the brains were retrieved and cut into 2 mm coronal sections on a brain matrix and photographed to assess the extent of hemorrhage. Foci of hemorrhage, with intense red color in patches, were counted on one face of the brain slices to provide a qualitative estimate of hemorrhagic transformation. Infarct size was determined from sections stained with 2,3,5 triphenyltetrazolium chloride (TTC) (2% w/v) in PBS at 37°C for 30 min. The areas of both the hemispheres and the infarct area (white) were measured using Photoshop and the infarct size was calculated across all sections. In order to reduce any bias due to the selective representation of images, TTC staining was pseudo-colored to combine all images from all mice within a group using Image J. The swelling of the infarcted hemisphere was determined by the formula: ([entire brain-infarct hemisphere × 100]/infarct hemisphere − contralateral hemisphere).

2.6 | Pharmacokinetics

Mice (n = 3 mice) were given WT-SPD at 12 mg/kg body weight intravenously as a bolus and citrate-plasma (0.38% w/v citrate) was collected at 0–3 min or 0–10 min in two independent experiments. Ac-Pro-*D*Tyr-Lys-AMC (amino-methyl-coumarin) was used as a sensitive and specific substrate for human FSAP as described before.[30] Hydrolysis of the fluorogenic substrate was measured using a plate reader with excitation at 320 nm and emission at 460 nm (37°C for 60 min). The maximal velocity was calculated from the linear part of the progress curve. Western blotting and enzyme-linked immunoassay (ELISA) was used to determine the concentration of FSAP-SPD in mouse plasma.[30] The antibody for Western blotting and ELISA was specific for human FSAP with no or little cross-reactivity with mouse FSAP.

2.7 | Tail resection model of hemostasis

The effect of WT-SPD, MI-SPD, Heparin, and PBS on hemostasis was tested. Isoflurane-anesthetized mice (n = 5 mice per group) were given different treatments i.v. through the dorsal penile vein and 15 min later 0.5 cm tip of the tail was resected before the tail was placed in PBS at 37°C. The PBS solution was changed every 5 min for 30 min and the time to stop bleeding was noted. The experiment was terminated after 30 min by sacrificing the animals. Hemoglobin concentration in the PBS solutions was measured using the Hemoglobin Assay kit (Merck, MAK115).

2.8 | Fibrinogen ELISA

Citrate plasma was collected from mice from the TES and tMCAO experiments at sacrifice. Mouse total

fibrinogen ELISA (#IMSFFBGKTT) from Innovative Research (Novi, MI) was used to analyze the samples in duplicates.

2.9 | In vitro clot lysis experiments

Whole blood from C57BL6J mice ($n = 9$ mice) without anticoagulants was directly collected into a polyethylene tube (PE-50, I.D. 0.58 mm) and allowed to clot for 2 h at RT. Thereafter, the tubing was cut into 2 cm sections and the clots ejected into wells of a microtiter plate with PBS (triplicate wells per treatment). Clot lysis was initiated with tPA (20 μg/ml) in 10% plasma from donor mice in the absence or presence of WT-SPD or MI-SPD (10 μg/ml) and the liberation of erythrocytes/hemoglobin from the clots was measured at 37°C at 510 nm in a plate reader (Biotek). Time to a maximal velocity of clot lysis and the end absorbance were quantified across different experiments.

2.10 | Effect of HMW-HA on plasma FSAP activity

FSAP was purified from human plasma as described before.[31] FSAP activity was measured in buffer (20 mM Tris, 150 mM NaCl, 1% BSA (w/v), pH 7.5) by incubating purified FSAP at 10 nM with 200 μM of substrate Ac-Pro-DTyr-Lys-Arg-AMC[32] in the presence of HMW-HA and LMW-HA (R&D Systems, Minneapolis, MN). For experiments involving the activation of pro-FSAP in human citrate plasma, plasma was first mixed with buffer (1:4) and fluorogenic substrate. Histones (25 μg/ml) were then added and fluorescence was recorded every 30 s for a further 75 min. All experiments were performed in black, flat-bottom microtiter plates (ThermoFisher Scientific, Carlsbad, CA, USA) in an Infinite 200 fluorescence reader (Tecan, Crailsheim, Germany) at 360 nm excitation and 450 nm emission wavelength, using the Magellan Software package. From the raw data in relative fluorescence units (RFU) the peak amount of FSAP and total FSAP generated were calculated. These experiments were repeated independently thrice.

2.11 | Statistical analysis

All data are expressed as mean ± SEM. Results from each individual mouse are shown as a point in all panels. Data were analyzed by one-way analysis of variance (ANOVA) with post hoc Bonferroni test for continuous measures; $^{*}p < .05$ and $^{**}p < .001$ $^{***}p < .0001$. For categorical measures, we used the Kruskal–Walis test with Mann–Whitney post-test. SSPS statistical package was used for all analyses (IBM, SPSS 26).

3 | RESULTS

Before testing recombinant human WT-SPD in stroke models, we performed a pilot pharmacokinetic study to gain information about its half-life in mice. WT-SPD was measured in plasma by Western blotting, human FSAP-specific ELISA as well as using a fluorogenic substrate specific for the enzymatic activity of FSAP.[30] ELISA and Western blotting indicated a shorter half-life (2–3 min) compared to the activity-based assay (4–5 min) in mice (Figure S1A–D). The rapid formation of high MW complexes with inhibitors (Figure S1A) most likely accounts for its short half-life. Based on these results we elected a 30-min infusion protocol for the application of WT-SPD so that its levels remained elevated for at least 35 min during the treatment.

We first used a model of thromboembolic stroke (TES) where thrombosis was induced in the carotid artery with a topical application of $FeCl_3$, which causes oxidative damage to the vessel wall.[33] A primary thrombus blocks the carotid artery while microthrombi embolize to the brain microvasculature,[27] representing a model of large vessel TES. WT-SPD was infused together with tPA, the standard thrombolytic treatment, and compared to tPA alone and PBS, 60 min after the onset of thrombosis in the carotid artery (Figure 1A). Since FSAP alone did not exhibit any fibrinolytic activity,[15] WT-SPD was not tested on its own in this experiment. In pilot studies, we observed a superior effect of 8.0, compared to 2.0, mg/kg body weight of WT-SPD and thus the higher concentration was used throughout the study. This is similar to the mode of delivery and dose of tPA used in such experiments.[34] In all experiments, we also tested the enzymatically inactive MI-SPD,[35] which differs by a single amino acid, as a control.

Laser Doppler flowmetry was used to track the consequences of thrombosis and the subsequent fibrinolysis on cerebral blood flow (CBF) in the middle cerebral artery (MCA) territory as well as the carotid artery blood flow (ABF) downstream of the thrombus. The validity of this experimental design was first tested by temporarily ligating the carotid artery with a suture. This reduced CBF, whereas compensatory mechanisms increased ABF via collateral circulation as described before[36] (Figure S2). Thrombosis in the carotid artery reduced CBF, whereas ABF first increased transiently before decreasing (Figure 1B). tPA treatment in all combinations elevated ABF (Figure 1B–E), but this was not statistically significant (Figure 1F). CBF was not influenced by treatment with tPA or tPA plus MI-SPD (Figure 1B–E), but only

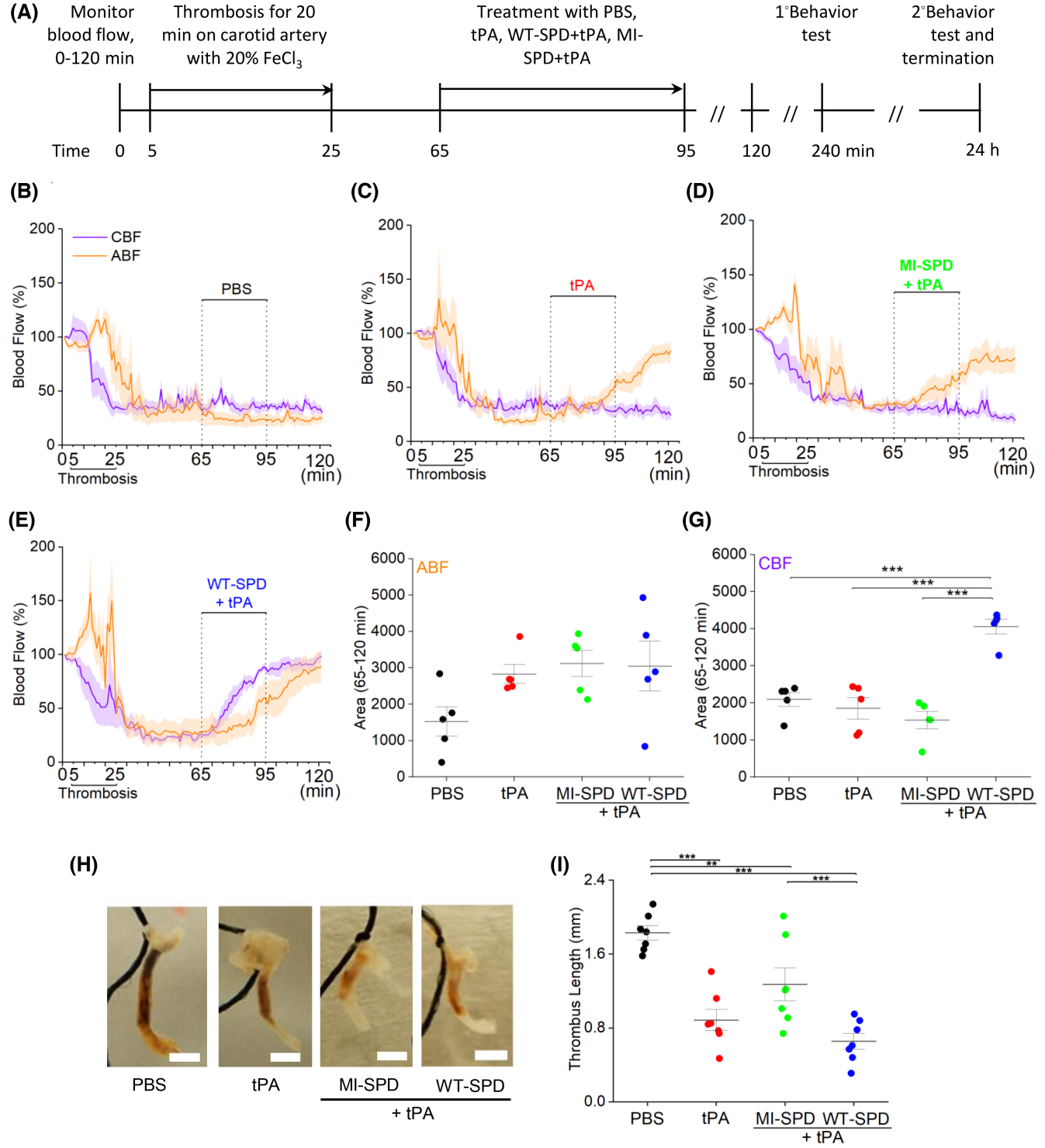

FIGURE 1 Effect of WT-SPD on blood flow and thrombus size in the TES model. (A) Outline of the experiment. (B–E) Cerebral blood flow (CBF) and carotid artery blood flow (ABF) was measured during and after different treatments (0–120 min). (F, G) Cumulative ABF and CBF between 65–120 min for each mouse is shown ($n = 5$ mice per group). (H, I) Images of carotid arteries from one representative mouse in each group and thrombus length (mm) ($n = 7$ mice per group) (Scale bars = 1 mm). The data represent mean ± SEM and each dot represents one mouse. p-Value was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni test; *$p < .05$, **$p < .001$, ***$p < .0001$.

significantly with tPA plus WT-SPD (Figure 1G). We then assessed the size of the primary clot in the carotid artery. The size of the occluding clot was reduced in all groups with tPA treatment, confirming arterial thrombolysis, but the additional presence of either isoform of SPD did not influence the residual clot size significantly (Figure 1H,I).

After 24 h, similar infarct size, involving much of the dorsal and ventral striatum with overlying cortical areas,

was observed in animal groups treated with PBS, tPA, and tPA with MI-SPD, while infarct size in mice treated with tPA plus WT-SPD was strikingly smaller (Figure 2A). Behavioral scoring showed that mice treated with tPA performed worse after 24 h, compared to the PBS, highlighting the adverse effects of tPA that are also observed in patients.[37] These scores were better in mice treated with tPA plus WT-SPD compared to tPA plus MI-SPD (Figure 2B). There were no, edema-related, changes in hemisphere volumes (Figure 2C). The mortality was 2/9 in PBS, 3/10 in tPA, 3/10 in tPA plus MI-SPD, and 1/8 in tPA plus WT-SPD group.

Based on the known effects of FSAP on promoting fibrinolysis in human plasma,[15] we postulated that better clot lysis could account for the above results and therefore studied the lysis of mouse whole-blood clots in vitro. We found that the overall dissolution of clots with tPA plus WT-SPD was significantly better compared to using tPA alone or tPA plus MI-SPD (Figure S3A,B). The time to a maximal velocity of clot lysis was also shorter in the presence of WT-SPD and tPA (Figure S3C,D). WT- or MI-SPD alone, had no direct effect on clot lysis as reported earlier with human plasma-FSAP in human plasma clots.[15] Thus, better clot lysis with WT-SPD and tPA could account for the improved CBF in the TES model.

Considering the number of opposing actions FSAP has with respect to coagulation and fibrinolysis,[7,14,15] we examined the effects of WT-SPD on hemostasis in a tail-transection model (Figure 3A). WT-SPD decreased the bleeding time (Figure 3B) and the amount of blood loss (Figure 3C) compared to MI-SPD or PBS treatment, which is in agreement with our earlier results.[16] Mice treated with heparin bled during the entire experiment (Figure 3B,C). Since FSAP cleaves fibrinogen and promotes fibrinolysis, we also assessed the effect of the different treatments in the

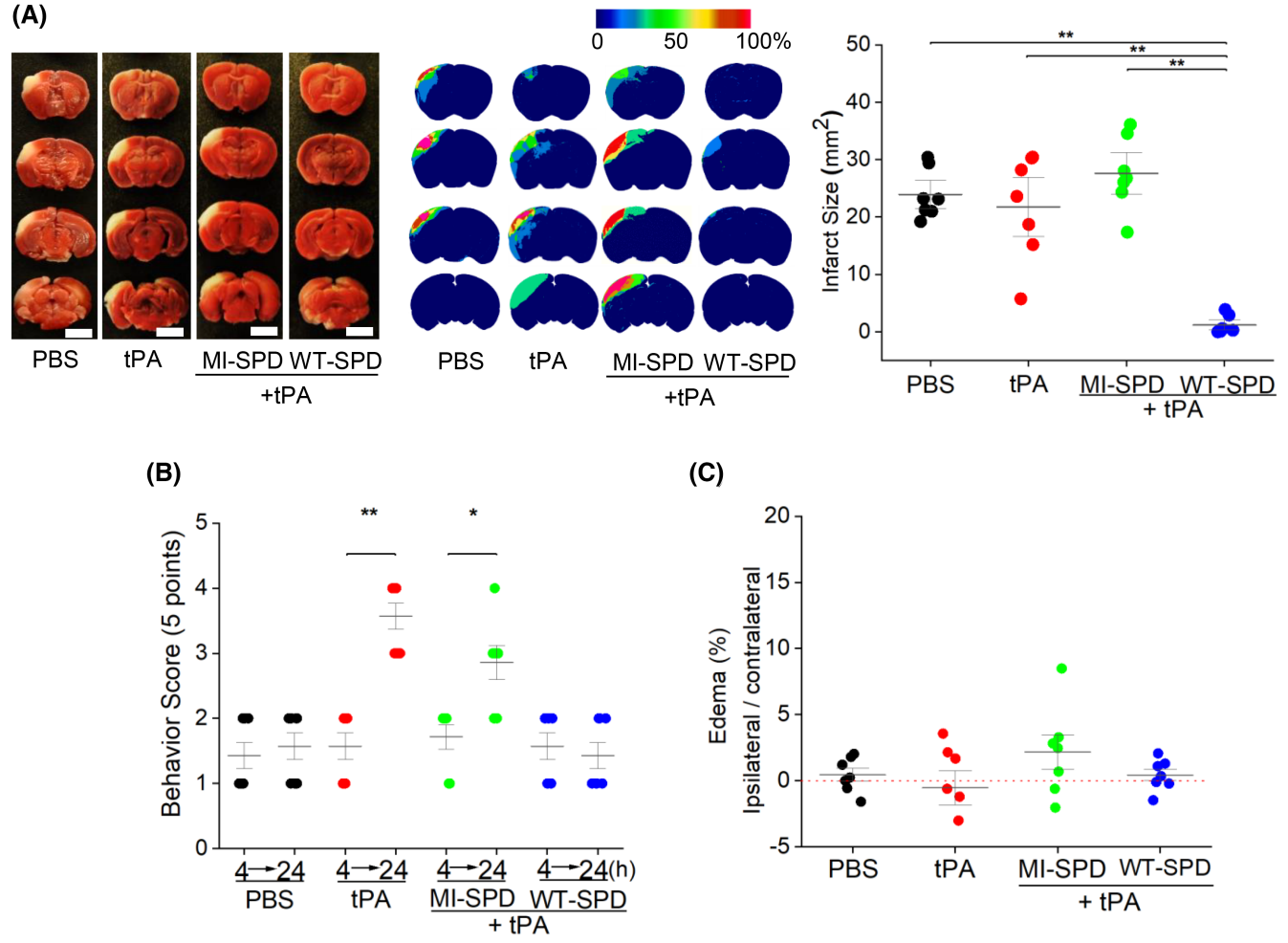

FIGURE 2 Effect of WT-SPD on stroke outcomes in the TES model. (A), TTC staining of brain sections from one representative mouse in each group and pseudo color representation of infarct size from all mice in the treatment group and quantification of infarct area ($n = 7$ mice per group) (Scale bars, 5 mm). (B) Behavior score was measured with a 5-point scale (0–4) ($n = 7$ mice per group). (C) Edema-mediated change in the size of ipsilateral over the contralateral hemisphere ($n = 7$ mice per group). The data represents mean ± SEM and each dot represents one mouse. p-Value was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni test except in panel B where Kruskal–Wallis test with Mann–Whitney post-test was used; *$p < .05$, **$p < .001$, ***$p < .0001$.

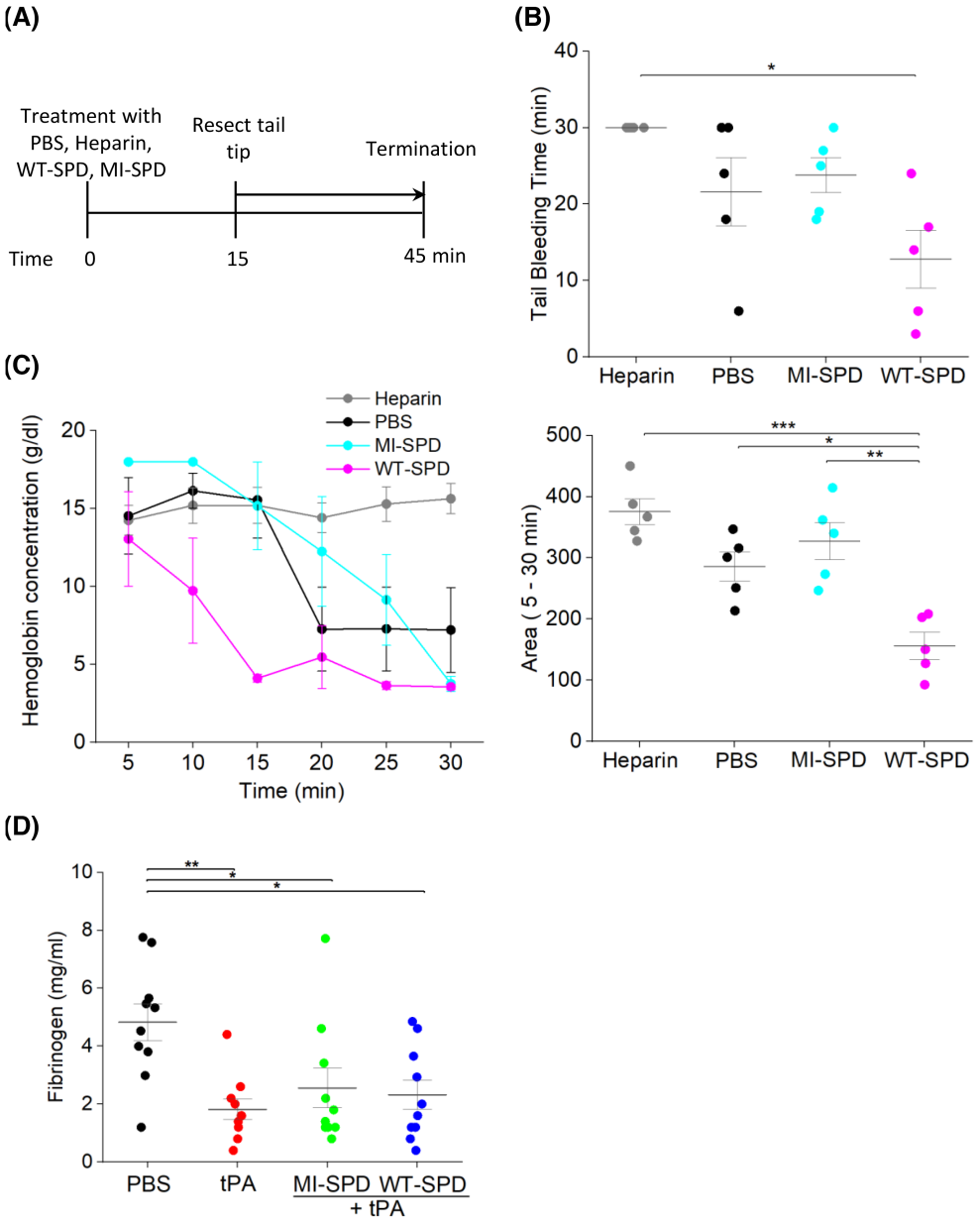

FIGURE 3 Effect of WT-SPD on hemostasis. (A) Outline of the experiment. (B) Mice were treated with PBS, MI-SPD, or WT-SPD (both, 8 mg/ kg body weight) and heparin (10 IU/kg body weight) ($n = 5$ mice per group) and the time to stop bleeding from the resected tail were determined. (C) Blood loss was measured in 5 min intervals and cumulative blood loss over the 30 min for each mouse was calculated. (D) Plasma fibrinogen (mg/ml) was measured in citrate plasma 24-h after the induction of TES in the treatment groups described in Figure 1A ($n = 10$ mice per group). Each dot represents one mouse and the data represents mean ± SEM. *p*-Value was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni test; *$p < .05$, **$p < .001$, ***$p < .0001$.

TES model on circulating levels of fibrinogen. Fibrinogen levels were reduced in the tPA-treatment group compared to mice given PBS, but this reduction was not affected by the co-administration of WT-SPD (Figure 3D).

We then tested the FSAP-SPD in the tMCAO model of stroke which features ischemia–reperfusion injury and microvascular obstruction, rather than overt large vessel thrombosis.[38] Treatments were initiated 75 min after the onset of ischemia (Figure 4A). CBF remained depressed in the reperfusion phase in all groups (Figure S4A,B), most likely due to non-thrombotic obstruction of the microvessels. No differences in plasma-fibrinogen were observed (Figure S4C) indicating a lack of involvement of overt coagulation/fibrinolysis in this model.

After 24 h, all stroke parameters were significantly improved in the WT-SPD plus tPA groups, compared to the other groups. The number of hemorrhagic foci was increased in tPA-treated mice, while only to a lesser extent in mice also receiving WT-SPD. For unknown reasons, the number of foci was significantly elevated in the MI-SPD

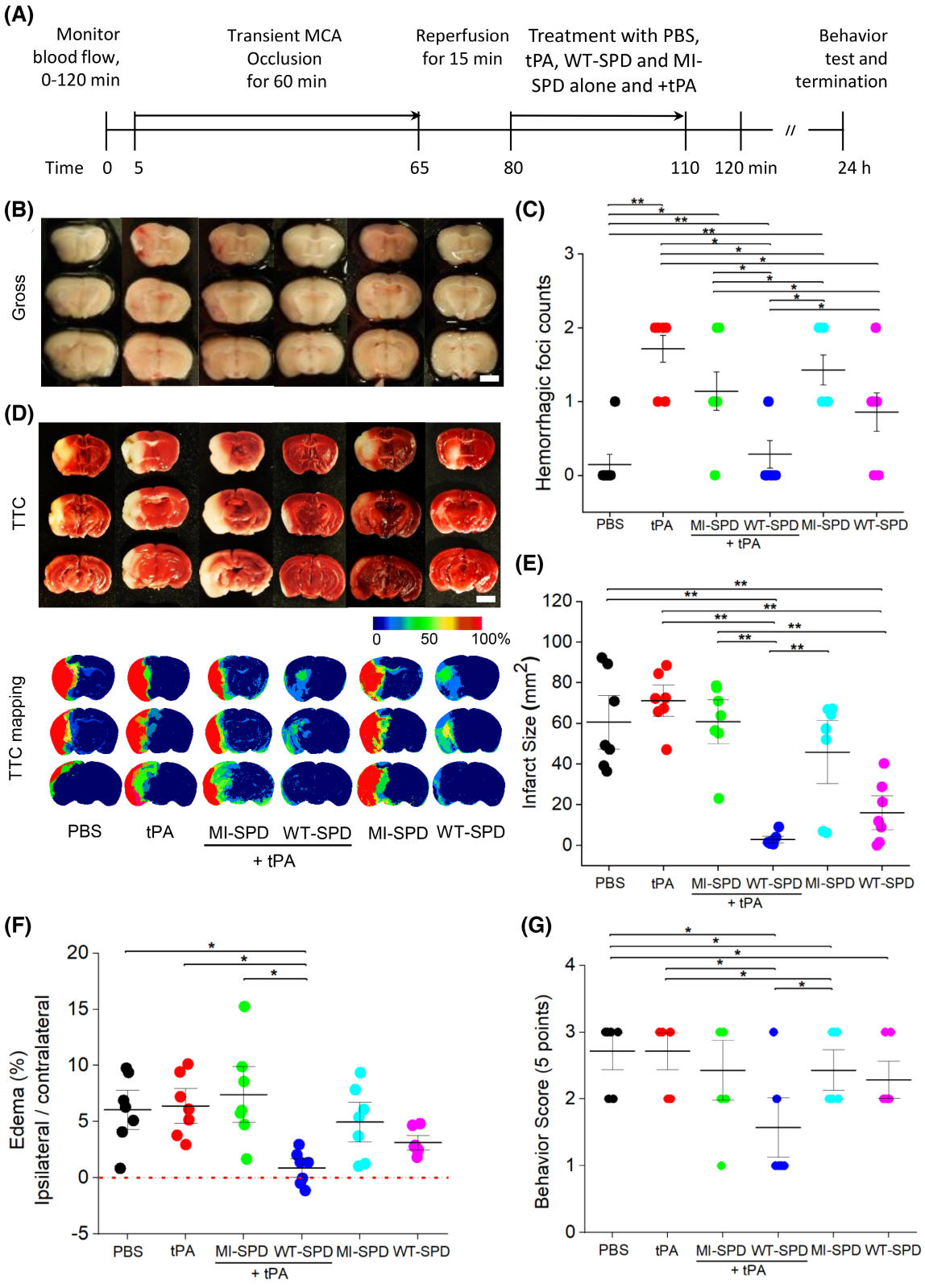

FIGURE 4 Effect of WT-SPD in the tMCAO model. (A) Outline of the experiment ($n = 7$ mice per group in all panels). (B) Hemorrhagic foci from the brain sections of one mouse in each group. (C) Quantification of hemorrhagic foci. (D) TTC staining of brain sections from one mouse in each group and pseudo color representation of infarct size from all mice in the treatment groups E, Quantification of infarct area. (F) The percentage change in the size of the infarct hemisphere over the contralateral hemisphere due to edema. (G) Neurological outcome of a 5-point scale (0–4). Each dot represents one mouse and data are mean ± SEM ($n = 7$). p-Value was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni test except in panels C and G where Kruskal–Wallis test with Mann–Whitney post-test was used; *$p < .05$, **$p < .001$, ***$p < .0001$. In B and D, scale bar, 5 mm.

group (Figure 4B,C). The infarct size was also larger in groups treated with tPA, MI-SPD, or their combination while being significantly smaller in mice that received WT-SPD alone or together with tPA (Figure 4D,E). WT-SPD plus tPA treatment decreased the edema-related expansion of the stroke hemisphere (Figure 4F) and improved the behavior score (Figure 4G). The mortality was 2/9 in PBS, 2/9 in tPA, 2/9 in tPA plus MI-SPD, 2/9 in tPA plus WT-SPD, 3/10 in MI-SPD, and 1/8 in the WT-SPD group (Figure 4A–G). Hence, in a stroke model with lesser involvement of coagulation, FSAP-SPD had a beneficial effect on its own as well as reduced the deleterious effects of tPA.

During the completion of this study, it was reported that inhibition of FSAP improves stroke outcomes in the tMCAO model.[11] FSAP was inhibited with HMW-HA based on an earlier report that it inhibits FSAP activity in vitro.[19] To resolve this apparent contradiction to our conclusions with recombinant FSAP-SPD, we investigated the inhibition of FSAP with HMW-HA in more detail. HMW-HA, and for comparison LMW-HA, did not in any way modulate the activity of purified full-length FSAP (Figure S5A). FSAP in plasma is in a zymogen form with no activity and can be activated with histones.[21] Using a very selective and sensitive assay to detect FSAP activity in plasma we found no effect of HMW-HA and LMW-HA on the histone-mediated increase in the activity of FSAP (Figure S5B). Therefore, HMW-HA does not inhibit the enzymatic activity of FSAP.

4 | DISCUSSION

A pharmacological treatment for ischemic stroke that can promote the actions of tPA, as well as block its negative effects, will improve the management of ischemic stroke patients considerably.[39] This study is the first reported use of recombinant FSAP-SPD as a therapeutic agent in ischemic stroke in mice in combination with tPA and demonstrates improvement in stroke outcome in two mouse models. In the TES model, tPA plus FSAP-SPD improved cerebral blood flow, decreased infarct size, and reversed tPA-mediated motor impairment. In the tMCAO model, FSAP-SPD decreased infarct size, and reversed tPA-mediated motor impairment, hemorrhage as well as edema without influencing cerebral blood flow. On its own, FSAP-SPD reduced infarct size but did not significantly affect other outcomes. These results are congruent with earlier observations in relation to FSAP gene-polymorphisms and stroke,[8,9] and changes in plasma levels of FSAP in human stroke patients.[11,12] An advantage of using FSAP is that it promotes fibrinolysis without altering tPA or plasmin activity since it directly cleaves fibrin/ogen rendering clots more susceptible to fibrinolysis.[15] This avoids the typical problems of pro-thrombolytic drugs related to increased hemorrhage and/or bleeding associated with increased tPA and/or plasmin activity.[37] In line with this hypothesis, we expected FSAP-SPD treatment to influence plasma fibrinogen levels. There was a tPA-related decrease in plasma fibrinogen in the TES model, but not in the tMCAO model, indicating consumption of fibrinogen, but FSAP-SPD did not influence this further. This needs to be investigated in detailed time course studies in the future.

The TES model mimics the often encountered clinical situation where tPA recanalizes the primary blocked artery, but fails to establish distal cerebral reperfusion.[40] The likely reasons for this resistance to thrombolysis are vascular dysfunction, cellular aggregation, biomechanical factors, ischemia–reperfusion injury, and clot composition.[41] Apart from fibrinogen, other components such as the von Willebrand factor, NETs and DNA may contribute significantly to clot stability.[39,41] FSAP-SPD promotes fibrinolysis by cleavage of the α- and β-chain of fibrinogen,[15] but it may also influence proteolysis of some of the other factors. The observed pattern of blood flow in the brain and carotid artery confirmed the successful use of FSAP-SPD and tPA to re-establish cerebral blood flow in the brain and was confirmed by the in vitro clot lysis experiments. Cerebral blood flow is indirectly related to the thrombus load in the brain, but the latter was not quantified in the current study.

Application of FSAP-SPD in vivo leads to the formation of FSAP inhibitor complexes, amongst others, with plasminogen activator inhibitor-1[42] and alpha2-antiplasmin.[43] Hence, the faster lysis of clots in the presence of FSAP-SPD seen in our experiments could also be due to the scavenging of these inhibitors of fibrinolysis. Cellular uptake of FSAP-SPD-inhibitor complexes probably accounts for its short half-life in vivo similar to that for tPA.[44] This necessitated the use of relatively high concentrations of FSAP-SPD (200 μg/25 g mouse). Although this is similar to the concentration of tPA used in mouse experiments, it exceeds the normal circulating levels of FSAP (12 μg/ml).

We studied the effects of FSAP-SPD on hemostasis because of the known effects of FSAP on coagulation and fibrinolysis pathways. FSAP-SPD shorted bleeding time in the tail transection model, as observed in a previous study with full-length FSAP in FSAP-deficient mice.[16] This pro-coagulant effect is most likely due to the inactivation of TFPIα.[14,25] In mice, the main source of TFPIα that is responsible for its hemostatic effects are platelets[45] but we did not investigate this point further in the current study. The seemingly incongruent actions seen with the pro-hemostatic effect and, at the same time, improved stroke outcome, can be reconciled with the hypothesis that in the stroke model the pro-fibrinolytic effect of FSAP predominates over the pro-hemostatic effect, leading to an overall positive outcome. Counterintuitively, the pro-hemostatic effect might negate the hemorrhagic transformation due to tPA. Through detailed structure–function studies it may be possible to mutate away the

pro-coagulant effects in FSAP, as has been successfully done with other coagulation factors such as APC.[46]

The positive outcome of FSAP treatment in the tMCAO model, which is largely devoid of thrombosis, indicates that FSAP is likely to have additional mechanisms of action independently of its effects on coagulation and fibrinolysis. A number of FSAP substrates have been identified in-vitro that are relevant in stroke. Extracellular histones are released during necrosis, apoptosis or during NETosis[24] are found in stroke-thrombi.[47] They are key mediators in stroke either through their cytotoxic effects or through toll-like receptors.[48] FSAP is a potent neutralizing factor of histones through their proteolytic cleavage[21-23] and this could improve stroke outcome. For instance, reversal of the histone-mediated increase in endothelial permeability[49] by FSAP-SPD could reduce edema, as observed in FSAP-SPD-treated mice.

Activation of PARs is another mechanism relevant for stroke. APC cleaves PARs at non-canonical sites and generates cellular responses that improve stroke outcome,[50] In contrast, FSAP cleaves PARs at their canonical cleavage sites,[20] so the signaling initiated by FSAP will be different from that by APC. In lung cancer cells the activation of PAR-1 by FSAP inhibits apopotosis[20] but, it remains to be tested if this is also the case for neurovascular cells. FSAP also regulates endothelial permeability in the lungs via PARs[19] and this mechanism may also operate in brain endothelium in the context of stroke. The different properties of FSAP that are relevant for stroke are summarized in Figure 5.

In a recent study, it was reported that inhibition of FSAP improves stroke outcome in the tMCAO model[11] which is the opposite of our conclusion. HMW-HA, an acidic polymer of hyaluronic acid that is usually found in the extracellular matrix, was used to inhibit FSAP in these experiments. HMW-HA has pleiotropic effects on many cell types and causes extensive changes in the gene expression profiles of cells via various cell surface receptors including CD44, LYVE-1, RHAMM, and TLR4.[51] In view of these very broad actions of HMW-HA, it is not possible to ascribe its effect on stroke to a single molecular interaction with FSAP. Furthermore, we did not observe any inhibitory effect of HMW-HA on FSAP activity. Thus, the effects of HMW-HA treatment on stroke outcomes are unlikely to be related to its ability to inhibit FSAP.

Limitations of this, proof-of-concept, study is that the experiments were not carried out in aged mice or female mice, or mice with co-morbidities, which are important factors in determining drug efficacy in stroke treatment as described in the STAIRS guidelines.[52] The hemorrhagic transformation related to tPA treatment becomes more prominent with the passage of time and we did not test the effect of FSAP in a delayed treatment setting. Furthermore, long-term studies with extensive sensory-motor tests are needed to judge the overall translational relevance of these results. Blinding

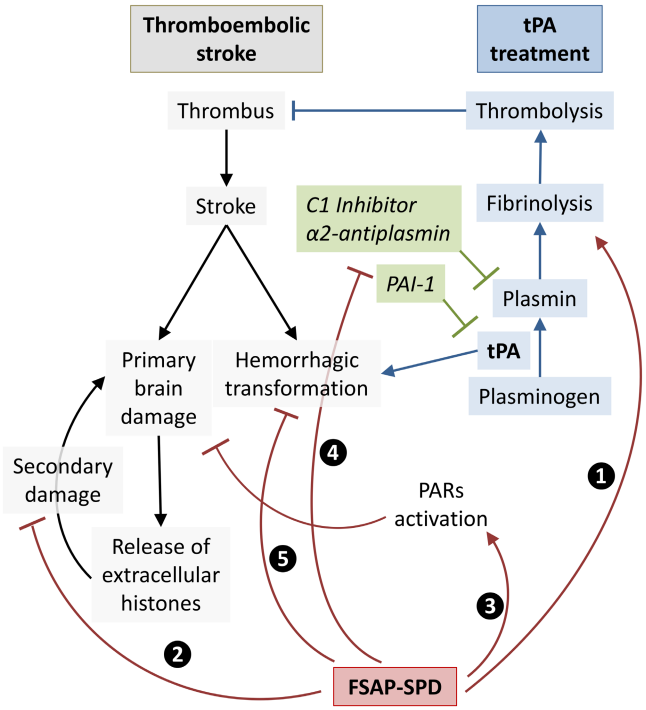

FIGURE 5 Schematic representation of the effects of FSAP-SPD on thromboembolic stroke: Primary brain damage caused by stroke is exasperated by the release of mediators, e.g., histones that cause secondary damage (gray boxes and lines). tPA mediates lysis of the primary thrombus but re-establishment of the distal perfusion is not always successful and rates of hemorrhagic events are high (blue boxes and lines). FSAP-SPD enables better distal perfusion as well as reverses some of the negative effects of tPA treatment (red lines). The effects of FSAP-SPD which contribute to a better stroke outcome are numbered (1–5) in the figure. (1) Fibrin(ogen) cleavage and improved fibrinolysis,[15] (2) histone cleavage to reduce its cytotoxicity,[21-23] (3) activation of PARs[19,20] to mediate favorable regulation of endothelial permeability, (4) scavenging of serine protease inhibitors[42,43] to promote the activity of plasmin, and (5) pro-coagulant effect, through inactivation of TFPI[14,16] which may negate the hemorrhagic tendency. The latter four properties of FSAP-SPD will also operate in occlusive models of stroke in the absence of a thrombus.

was not used during treatments, due to practical reasons, and this may have influenced the outcome of the study. Although the group size was judged appropriately during the design of the stroke experiments, 7 animals per group constitute a small group size. A disadvantage of using the serine protease domain of FSAP, and not the full-length protein, is that the full complement of regulatory interactions is lost with the use of the SPD domain thus limiting the extrapolation of these results to a physiological context. However, we confirmed that SPD recapitulates all, the tested, activities of the endogenous full-length protein.[20,25,30] The use of recombinant human protein in the mouse may lead to aberrant conclusions that need to be replicated using recombinant mouse FSAP. However, the same expression system, used for producing human FSAP-SPD, was not suitable for

mouse FSAP-SPD expression. One possible solution to the problems of expressing recombinant FSAP is to identify a selective and potent activator of the endogenous pro-FSAP zymogen, as we have done using phage display,[53] which then may achieve the same end-effect.

In our studies, FSAP-SPD showed promising results in a pharmacological context by significantly improving stroke outcomes in mice. FSAP has the advantage of being an endogenous blood protein with a plausible mechanism of action that is readily amenable to further development.

AUTHOR CONTRIBUTIONS

Sandip M. Kanse designed the study, obtained funding, supervised experiments, and drafted the manuscript. Jeong Yeon Kim performed all of the animal experiments, the imaging studies, analyzed the data, performed the statistics, and prepared the figures. Dipankar Manna prepared and characterized the recombinant proteins used in the study. Michael Etscheid performed all the enzyme activity assays. Trygve B. Leergaard contributed to the morphological analyses. All authors added intellectual content to the manuscript as well as read, edited, and approved the final version of the manuscript.

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DISCLOSURES

The authors declare that they have no financial or any other conflicts of interest in relation to the contents of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Feigin VL, Nguyen G, Cercy K, et al. Global, regional, and country-specific lifetime risks of stroke, 1990 and 2016. *N Engl J Med*. 2018;379(25):2429-2437.
2. Campbell BCV, Khatri P. Stroke. *Lancet*. 2020;396(10244):129-142.
3. Bhatia R, Hill MD, Shobha N, et al. Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: real-world experience and a call for action. *Stroke*. 2010;41(10):2254-2258.
4. Zhang J, Yang Y, Sun H, Xing Y. Hemorrhagic transformation after cerebral infarction: current concepts and challenges. *Ann Transl Med*. 2014;2(8):81.
5. Waqas M, Gong AD, Levy BR, et al. Is endovascular therapy for stroke cost-effective globally? A systematic review of the literature. *J Stroke Cerebrovasc Dis*. 2021;30(4):105557.
6. Stoll G, Nieswandt B. Thrombo-inflammation in acute ischaemic stroke—implications for treatment. *Nat Rev Neurol*. 2019;15(8):473-481.
7. Romisch J. Factor VII activating protease (FSAP): a novel protease in hemostasis. *Biol Chem*. 2002;383(7–8):1119-1124.
8. Trompet S, Pons D, Kanse SM, et al. Factor VII activating protease polymorphism (G534E) is associated with increased risk for stroke and mortality. *Stroke Res Treat*. 2011;2011:424759.
9. Willeit J, Kiechl S, Weimer T, et al. Marburg I polymorphism of factor VII-activating protease: a prominent risk predictor of carotid stenosis. *Circulation*. 2003;107(5):667-670.
10. Cheng YC, Stanne TM, Giese AK, et al. Genome-wide association analysis of young-onset stroke identifies a locus on chromosome 10q25 near HABP2. *Stroke*. 2016;47(2):307-316.
11. Tian DS, Qin C, Zhou LQ, et al. FSAP aggravated endothelial dysfunction and neurological deficits in acute ischemic stroke due to large vessel occlusion. *Signal Transduct Target Ther*. 2022;7(1):6.
12. Hanson E, Kanse SM, Joshi A, et al. Plasma factor VII-activating protease antigen levels and activity are increased in ischemic stroke. *J Thromb Haemost*. 2012;10(5):848-856.
13. Joshi AU, Orset C, Engelhardt B, et al. Deficiency of factor VII activating protease alters the outcome of ischemic stroke in mice. *Eur J Neurosci*. 2015;41(7):965-975.
14. Kanse SM, Declerck PJ, Ruf W, Broze G, Etscheid M. Factor VII-activating protease promotes the proteolysis and inhibition of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2012;32(2):427-433.
15. Etscheid M, Subramaniam S, Lochnit G, et al. Altered structure and function of fibrinogen after cleavage by factor VII activating protease (FSAP). *Biochim Biophys Acta Mol Basis Dis*. 2018;1864(10):3397-3406.
16. Subramaniam S, Thielmann I, Morowski M, et al. Defective thrombus formation in mice lacking endogenous factor VII activating protease (FSAP). *Thromb Haemost*. 2015;113(4):870-880.
17. Da L, Jiahui Z, Xiaoqiang L. Association between FSAP 1601G>A polymorphism and venous thromboembolism risk: a meta-analysis. *Phlebology*. 2020;35(5):345-353.
18. Kara E, Manna D, Loset GA, Schneider EL, Craik CS, Kanse S. Analysis of the substrate specificity of factor VII activating protease (FSAP) and design of specific and sensitive peptide substrates. *Thromb Haemost*. 2017;117(9):1750-1760.
19. Mambetsariev N, Mirzapoiazova T, Mambetsariev B, et al. Hyaluronic acid binding protein 2 is a novel regulator of vascular integrity. *Arterioscler Thromb Vasc Biol*. 2010;30(3):483-490.
20. Byskov K, Le Gall SM, Thiede B, Camerer E, Kanse SM. Protease activated receptors (PAR)-1 and -2 mediate cellular effects of factor VII activating protease (FSAP). *FASEB J*. 2020;34(1):1079-1090.
21. Yamamichi S, Fujiwara Y, Kikuchi T, Nishitani M, Matsushita Y, Hasumi K. Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation in vivo. *Biochem Biophys Res Commun*. 2011;409(3):483-488.
22. Grasso S, Neumann A, Lang IM, Etscheid M, von Kockritz-Blickwede M, Kanse SM. Interaction of factor VII activating protease (FSAP) with neutrophil extracellular traps (NETs). *Thromb Res*. 2018;161:36-42.
23. Marsman G, von Richthofen H, Bulder I, et al. DNA and factor VII-activating protease protect against the cytotoxicity of histones. *Blood Adv*. 2017;1(26):2491-2502.
24. Murao A, Aziz M, Wang H, Brenner M, Wang P. Release mechanisms of major DAMPs. *Apoptosis*. 2021;26(3–4):152-162.

25. Nielsen NV, Roedel E, Manna D, Etscheid M, Morth JP, Kanse SM. Characterization of the enzymatic activity of the serine protease domain of factor VII activating protease (FSAP). *Sci Rep*. 2019;9(1):18990.
26. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol*. 2020;18(7):e3000410.
27. Martinez de Lizarrondo S, Gakuba C, Herbig BA, et al. Potent thrombolytic effect of N-acetylcysteine on arterial thrombi. *Circulation*. 2017;136(7):646-660.
28. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science*. 1994;265(5180):1883-1885.
29. Park JY, Lee SK, Kim JY, Je KH, Schellingerhout D, Kim DE. A new micro-computed tomography-based high-resolution blood-brain barrier imaging technique to study ischemic stroke. *Stroke*. 2014;45(8):2480-2484.
30. Artunc F, Bohnert BN, Schneider JC, et al. Proteolytic activation of the epithelial sodium channel (ENaC) by factor VII activating protease (FSAP) and its relevance for sodium retention in nephrotic mice. *Pflugers Arch*. 2022;474(2):217-229.
31. Etscheid M, Hunfeld A, Konig H, Seitz R, Dodt J. Activation of proPHBSP, the zymogen of a plasma hyaluronan binding serine protease, by an intermolecular autocatalytic mechanism. *Biol Chem*. 2000;381(12):1223-1231.
32. Rut W, Nielsen NV, Czarna J, Poreba M, Kanse SM, Drag M. Fluorescent activity-based probe for the selective detection of factor VII activating protease (FSAP) in human plasma. *Thromb Res*. 2019;182:124-132.
33. Hechler B, Nonne C, Eckly A, et al. Arterial thrombosis: relevance of a model with two levels of severity assessed by histologic, ultrastructural and functional characterization. *J Thromb Haemost*. 2010;8(1):173-184.
34. Korninger C, Collen D. Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro. *Thromb Haemost*. 1981;46(2):561-565.
35. Etscheid M, Muhl L, Pons D, Jukema JW, Koenig H, Kanse SM. The Marburg I polymorphism of factor VII activating protease is associated with low proteolytic and low pro-coagulant activity. *Thromb Res*. 2012;130(6):935-941.
36. Wang J, Zheng C, Hou B, Huang A, Zhang X, Du B. Four collateral circulation pathways were observed after common carotid artery occlusion. *BMC Neurol*. 2019;19(1):201.
37. Thiebaut AM, Gauberti M, Ali C, et al. The role of plasminogen activators in stroke treatment: fibrinolysis and beyond. *Lancet Neurol*. 2018;17(12):1121-1132.
38. Desilles JP, Loyau S, Syvannarath V, et al. Alteplase reduces downstream microvascular thrombosis and improves the benefit of large artery recanalization in stroke. *Stroke*. 2015;46(11):3241-3248.
39. Vivien D. Can the benefits of rtPA treatment for stroke be improved? *Rev Neurol (Paris)*. 2017;173(9):566-571.
40. Espinosa de Rueda M, Parrilla G, Manzano-Fernández S, et al. Combined multimodal computed tomography score correlates with futile recanalization after thrombectomy in patients with acute stroke. *Stroke*. 2015;46(9):2517-2522.
41. Staessens S, De Meyer SF. Thrombus heterogeneity in ischemic stroke. *Platelets*. 2020;32:1-9.
42. Wygrecka M, Morty RE, Markart P, et al. Plasminogen activator inhibitor-1 is an inhibitor of factor VII-activating protease in patients with acute respiratory distress syndrome. *J Biol Chem*. 2007;282(30):21671-21682.
43. Stephan F, Hazelzet JA, Bulder I, et al. Activation of factor VII-activating protease in human inflammation: a sensor for cell death. *Crit Care*. 2011;15(2):R110.
44. Mei T, Kim A, Vong LB, et al. Encapsulation of tissue plasminogen activator in pH-sensitive self-assembled antioxidant nanoparticles for ischemic stroke treatment—synergistic effect of thrombolysis and antioxidant. *Biomaterials*. 2019;215:119209.
45. Maroney SA, Cooley BC, Ferrel JP, Bonesho CE, Mast AE. Murine hematopoietic cell tissue factor pathway inhibitor limits thrombus growth. *Arterioscler Thromb Vasc Biol*. 2011;31(4):821-826.
46. Griffin JH, Mosnier LO, Fernandez JA, Zlokovic BV. 2016 scientific sessions sol Sherry distinguished lecturer in thrombosis: thrombotic stroke: neuroprotective therapy by recombinant-activated protein C. *Arterioscler Thromb Vasc Biol*. 2016;36(11):2143-2151.
47. Laridan E, Denorme F, Desender L, et al. Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol*. 2017;82(2):223-232.
48. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. *Cell Death Dis*. 2014;5(8):e1370.
49. Ramasubramanian B, Kim J, Ke Y, et al. Mechanisms of pulmonary endothelial permeability and inflammation caused by extracellular histone subunits H3 and H4. *FASEB J*. 2022;36(9):e22470.
50. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C, protease activated receptor 1, and neuroprotection. *Blood*. 2018;132(2):159-169.
51. Garantziotis S, Savani RC. Hyaluronan biology: a complex balancing act of structure, function, location and context. *Matrix Biol*. 2019;78-79:1-10.
52. Kahle MP, Bix GJ. Successfully climbing the "STAIRs": surmounting failed translation of experimental ischemic stroke treatments. *Stroke Res Treat*. 2012;2012:374098.
53. Berge-Seidl S, Nielsen NV, Rodriguez Alfonso AA, et al. Identification of a phage display-derived peptide interacting with the N-terminal region of factor VII activating protease (FSAP) enables characterization of zymogen activation. *ACS Chem Biol*. 2022;17(9):2631-2642.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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