Translational Stroke Research (2019) 10:389–401
https://doi.org/10.1007/s12975-018-0652-9

ORIGINAL ARTICLE

MMP10 Promotes Efficient Thrombolysis After Ischemic Stroke in Mice with Induced Diabetes

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Received: 21 February 2018 / Revised: 16 July 2018 / Accepted: 20 July 2018 / Published online: 27 July 2018

Abstract

Diabetes is an important risk factor for ischemic stroke (IS). Tissue-type plasminogen activator (tPA) has been associated with less successful revascularization and poor functional outcome in diabetes. We assessed whether a new thrombolytic strategy based on MMP10 was more effective than tPA in a murine IS model of streptozotocin (STZ)-induced diabetes. Wild-type mice were administered a single dose of streptozotocin (STZ) (180 mg/kg) to develop STZ-induced diabetes mellitus. Two weeks later, IS was induced by thrombin injection into the middle cerebral artery and the effect of recombinant MMP10 (6.5 μg/kg), tPA (10 mg/kg) or tPA/MMP10 on brain damage and functional outcome were analysed. Motor activity was assessed using the open field test. Additionally, we studied plasminogen activator inhibitor-1 (PAI-1) and thrombin-antithrombin complex levels (TAT) by ELISA and oxidative stress and blood-brain barrier (BBB) integrity by immunohistochemistry and western blot. MMP10 treatment was more effective at reducing infarct size and neurodegeneration than tPA 24 h and 3 days after IS in diabetic mice. Locomotor activity was impaired by hyperglycemia and ischemic injury, but not by the thrombolytic treatments. Additionally, TAT, oxidative stress and BBB permeability were reduced by MMP10 treatment, whereas brain bleeding or PAI-1 expression did not differ between treatments. Thrombolytic treatment with MMP10 was more effective than tPA at reducing stroke and neurodegeneration in a diabetic murine model of IS, without increasing haemorrhage. Thus, we propose MMP10 as a potential candidate for the clinical treatment of IS in diabetic patients.

Keywords Fibrinolysis · Thrombosis · Stroke, diabetes mellitus · Matrix metalloproteinases

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12975-018-0652-9) contains supplementary material, which is available to authorized users.

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Introduction

Diabetes mellitus (DM) is a severe health problem associated with micro- and macrovascular complications leading to a 3- to 6-fold higher risk of ischemic stroke (IS) [1]. The prevalence of DM is estimated to be around 15 to 25% among IS patients, and data suggest that it is increasing over time [2, 3]. Moreover, IS patients with diabetes exhibit a worse functional outcome than non-diabetic patients [4, 5].

Tissue plasminogen activator (tPA) is the only current approved pharmacological treatment for acute IS [6]. This thrombolytic treatment in diabetes is associated with increased incidence of intracranial haemorrhage, less successful revascularization and poor functional outcome [7, 8]. Alterations in inflammatory and oxidative stress pathways, together with a hypercoagulable state, may contribute to the lower efficacy and safety [9, 10]. Elevated oxidative stress has been reported in diabetic patients and in animal models of diabetes, while preserved β cell function in streptozotocin (STZ)-induced diabetes was described in Nox2-deficient mice [11]. Moreover, increased thrombotic tendencies and decreased fibrinolytic activity have been associated with diabetes [12, 13]. Overall, these abnormalities could lead to changes in the composition of the extracellular matrix (ECM), vascular dysfunction and brain damage which could impair the thrombolytic response [14, 15].

ECM regulation is partially dependent on matrix metalloproteinases (MMPs), which degrade the ECM and control tissue remodelling [16]. Among other MMPs, MMP10 has been involved in the development of microvascular complications, and diabetic Mmp10−/− mice exhibit better renal function, with lower glomerular mesangial expansion and interstitial macrophage accumulation. Furthermore, Mmp10−/− mice presented partial protection from diabetes-induced retinal injury with normalization of the inner nuclear layer and retina thickness [17]. On the other hand, MMP10 has also shown beneficial thrombolytic effect either alone or in combination with tPA without haemorrhagic complications in an experimental model of IS [18–20]. We have reported that administration of MMP10 is as efficient as tPA to reduce infarct size and demonstrated that a combination of MMP10 with tPA achieves further reduction in brain damage after experimental IS. In vitro studies suggest that MMP10 may exert a protective effect on endothelial barrier properties by modulating tight junction (TJ) proteins and ERK 1/2 phosphorylation [21]. Moreover, the cytoprotective effect of MMP10 on neuron cultures blocking tPA-induced neuronal excitotoxicity further supports its pharmacological potential in IS.

In this study, we investigated the effects of MMP10 and tPA/MMP10 treatments in a murine model of IS in the presence of STZ-induced diabetes, assessing brain damage and functional outcome as main endpoints. Additionally, we explored whether MMP10 treatment influences systemic haemostasis, oxidative stress and blood-brain barrier (BBB) integrity.

Methods

Animals and Experimental Model

Diabetes was induced in 12 weeks old male C57BL/6 J (Envigo, Barcelona, Spain) mice by a single intra-peritoneal dose (180 mg/kg) of STZ (Sigma-Aldrich, St. Louis, MO, USA). Glucose levels were monitored every 2–3 days using the Accu-check system (Roche, Mannheim, Germany). To render animals hyperglycaemic without becoming ketoacidotic, long-acting insulin (Actrapid® and Insulatard® mixed 1:1, Novo Nordisk, Bagsvaerd, Denmark) was subcutaneously given at a dose of 2.8–4.2 U to mice with glucose levels > 400 mg/dL. Two weeks later, experimental ischemic stroke was carried out on diabetic mice randomly assigned to each experimental group as described before [6]. Mice were anesthetized with 2.5% isoflurane and 2 UI of murine α-thrombin (Sigma-Aldrich) was injected into the middle cerebral artery (MCA) bifurcation to induce a clot which was left to stabilize for 10 min. A stable clot was defined when laser Doppler flowmetry displayed a reduction of brain perfusion > 50%. Twenty minutes after the thrombin injection, effective doses of tPA (10 mg/kg) and MMP10 (6.5 μg/kg), previously assayed in this stroke model [6, 19], were administered alone or in combination. As a control, sham-operated animals were subjected to the same procedure without MCA thrombin injection. Experiments were performed in accordance with European Communities Council Directive guidelines (2010/63/EU) for the care and use of laboratory animals and were approved by the University of Navarra Animal Research Review Committee.

Brain Collection and Tissue Preparation

Animals were euthanized 24 h and 3 days after Middle Cerebral Artery Occlusion (MCAO) using a CO_2 chamber and perfused with cold PBS. Brains were removed, frozen in isopentane and stored until use at − 80 °C. Frozen brains were cut into 20-μm-thick coronal sections and stained to analyse lesion volume, neurodegeneration and microhemorrhage.

Effect of Treatments on Body Weight, Temperature and Locomotor Activity

To evaluate the potential biological effects of tPA and MMP10, a separate group of wild-type (WT) 12-week-old male mice ($n = 24$) were injected i.v. with saline, tPA, MMP10 and the combination of both with the same doses as described previously. Open field test was performed, and body

temperature and body weight were measured before and 24 h, 3, 7 and 14 days after the treatment.

Open Field Test

Behavioural assessments were done in all mice before and after the induction of DM and 24 h and 3 days after MCAO. For all behavioural testing, observers were blinded to the treatment. Mice were group-housed in standard cages with bedding and nesting material under a 12-h light-dark cycle. Food and water were provided ad libitum. Mice were subjected to the open field test using a white plexiglass cage (35 × 35 × 30 cm). Mice movements were recorded (c920 HD Pro Webcam, Logitech, Lausanne, Switzerland) for 5 min and analysed by a computer tracking system (Ethovision XT11.5, Noldus Information Technology B.V., The Netherlands) which divided the arena in different quadrants (centre and periphery) and measured the distance and velocity of the animals.

Brain Damage Assessment

Lesion Volume

To assess the lesion volume, brain sections were stained with thionin solution (0.05%). One section out of every 10 was stained and analysed (covering the entire lesion). Stained sections were scanned (3200 Photo Scanner, Epson, Amsterdam, The Netherlands) and the ischemic lesion (non-stained area) was quantified using an image analysis system (ImageJ, National Institutes of Health, Bethesda, MD, USA).

Fluoro-Jade C Staining

To evaluate neuronal degeneration, sections post-fixed with 4% paraformaldehyde were treated with a solution of Fluoro-jade C (FJC) (2×10^{-4}%) (Merck, Darmstadt, Germany) and DAPI (10^{-6}%) in 0.1% acetic acid. For the analysis, one section out of every 10 was stained and the images captured with the automated microscope (Zeiss LSM-510 Meta, Jena, Germany). Stained neurons in lesion area were quantified using ImageJ.

Microhemorrhage

Brain sections were stained with Perl's Prussian blue to identify the presence of microhemorrhage, in the form of iron deposits in cerebral tissue. Air-dried sections were post-fixed with 4% formaldehyde and treated with a mixture of potassium ferrocyanide and hydrochloric acid (Hematognost Fe®, Merck). Then, preparations were counterstained with nuclear red (Hematognost Fe®, Merck) and dehydrated. Stained sections were scanned at 20× (Aperio Scanner, Leica Biosystems, Wetzlar, Germany), and positive blue areas were measured using ImageJ.

Blood-Brain Barrier Evaluation

A different group of animals was euthanized 24 h after MCAO and perfused with cold PBS and 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde for 24 h and then frozen in isopentane and stored until use at − 80 °C. The barrier properties were evaluated analysing the distribution of laminin and endothelial cell marker CD31 by immunohistochemistry. Brain sections were immunostained for laminin (rabbit anti-laminin, Sigma-Aldrich, Munich, Germany) and CD31 (rat anti-CD31/DIA310, Optistain, Hamburg, Germany) followed by incubation with Alexa fluor-labelled secondary IgG goat-anti-rabbit488 (Invitrogen, Darmstadt, Germany) and TSA Cyanine 3 System, (PerkinElmer, Waltham, MA, USA). Slides were mounted with DAPI (Vectashield, Vector, Peterborough, UK) and visualized under fluorescence microscope (Nikon Eclipse 80i, Amsterdam, The Netherlands).

For measurement of IgG leakage, post-fixed brain sections were blocked for endogenous peroxidase and interspecific bindings, followed by incubation with biotinylated anti-mouse IgG antibody (EnVision-HRP labelled System, Dako, Carpentaria, CA, USA). Sections were then incubated with diaminobenzidine (Liquid-DAB Substrate Chromogen System, Dako). One section out of every 10 was stained and analysed. Stained sections were scanned (3200 Photo Scanner), and the stained IgG volume was quantified using an image analysis system (ImageJ).

Western Blot Analysis

Brain sections were carefully scraped from the slides, and the ipsilateral (IL) and contralateral (CL) hemispheres collected separately and homogenized with 50 μL (1:10 weight:volume) of ice cold RIPA buffer (Sigma-Aldrich) using a micropestle (Sigma-Aldrich). Homogenates were vortexed for 20 s, spinned down and quickly frozen in liquid nitrogen and thawed. Finally, samples were incubated at 4 °C while gentle shaking for 1 h and then centrifuged for 10 min at 13,000×*g* (4 °C). Total protein was measured with the Bradford protein assay kit (Bio-Rad, Munich, Germany).

Samples (20 μg) were heated in loading buffer (Invitrogen) at 70 °C for 5 min, loaded and electrophoretically resolved in Bis-Tris 4–12% gels (Novex Life Sciences, Invitrogen). After semi-dry transfer to nitrocellulose membranes (iBlot, Thermo Fisher, Darmstadt, Germany), blots were blocked for 1 h with 5% non-fat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, 0.01% Tween-20, pH = 7.6) at room temperature.

To assess TJ proteins, blots were incubated with rabbit polyclonal anti-claudin-5 antibody (Invitrogen) overnight at 4 °C. For occludin detection, a mouse monoclonal anti-occludin antibody (BD Biosciences, San José, CA, USA) was employed. MAPK signalling pathway and MMP9 were also evaluated by rabbit anti-ERK and anti-phosphorylated-ERK 1/2 (Cell Signalling Technology, Danvers, MA, USA), as well as rabbit anti-MMP9 (Thermo Fisher). Blots were incubated with secondary antibody goat anti-rabbit (Dako) or goat anti-mouse (Santa Cruz, Dallas, TX, USA) as needed. Then, membranes were washed and developed with HRP substrate TMA6 (Lumigen, Southfield, MI, USA) and re-probed with rabbit anti-β-actin antibody (Sigma Aldrich) after removing the primary antibodies with stripping buffer (Invitrogen). Immunoreactive bands were detected by chemiluminescence using an imaging system (Odyssey FC, LI-COR, Lincoln, NE, USA) analysed and quantified using Image Studio software (LI-COR).

Oxidative Stress

To assess oxidative stress, blots from brain homogenates were incubated with primary antibody mouse monoclonal anti-Nox2 (GP91) (BD Biosciences).

Haemostatic Parameters

Thrombin/antithrombin complexes (TAT, Enzygnost®, Siemens Healthcare, Erlangen, Germany) and plasminogen activator inhibitor-1 (PAI-1, Stachrom®, Stago, Asnières-sur-Seine, France) were measured in citrated plasma samples following the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Behavioural test and treatments biological effect data were analysed by analysis of variance (ANOVA) for repeated measurements followed by Bonferroni or Tamhane post-hoc comparison according to Levene's test of equality of variances. A two-way ANOVA was used to study the effect of tPA and MMP10 treatment and their interaction on studied variables. If an interaction effect was found, one-way ANOVA followed by Tamhane post-hoc test was carried out. Differences in reperfusion frequency data were analysed by the chi-square test. Two independent samples were compared using the Mann-Whitney *U* test, and finally, two related samples were compared using the Wilcoxon signed-rank test. Statistical significance was established as $p \leq 0.05$ (SPSS version 15.0 for Windows).

Results

Impact of STZ-Induced Diabetes and Ischemic Stroke on Functional Outcome

We assessed the influence of STZ-induced diabetes on body weight, glucose levels and behaviour of the animals 2 weeks after STZ injection. As expected, we observed that STZ diabetes impairs the welfare of the animals, reducing the body weight of the animals by 6% ($p \leq 0.01$) and increasing glucose levels from 169 ± 26 to 552 ± 82 mg/dL ($p \leq 0.01$, see Electronic supplementary material, Fig. S1A, B).

Then, we evaluated the impact of stroke on the locomotor activity in STZ-induced diabetic mice. The open field test revealed significant reductions in total distance ($p \leq 0.05$), distance travelled in the central area ($p \leq 0.05$) and velocity ($p \leq 0.05$; Fig. 1a). Finally, as shown in Fig. 1b, stroke further diminished motor activity in diabetic mice, being even lower 24 h after IS ($p \leq 0.05$). These data suggest a strong impairment in locomotor activity induced by diabetes and ischemic injury.

MMP10 Reduces Lesion Volume in the Experimental IS Model in the Presence of STZ-Induced Diabetes

First, we verified that the thrombolytic treatments had no potential side effects in healthy animals, since none of the treatments induced fever, loss of body weight or changes in locomotor activity (adjusted $p > 0.05$; Fig. S2A–D).

Following this, a two-way ANOVA was conducted to examine the effect of MMP10 and tPA treatments on cerebral blood flow in a diabetic setting. Average flow Doppler reduction after thrombin injection was similar in all experimental groups (Table 1); likewise, the number of reperfused animals (≥ 50%) 1 h after tPA and MMP10 treatments was comparable (χ^2, $p > 0.05$; Fig. S3). Regarding the lesion volume, there was a borderline statistically significant interaction between the effects of MMP10 and tPA ($p = 0.055$) 24 h after IS. Simple main effects analysis showed that MMP10 alone or in combination was more effective than tPA in infarct size reduction ($p \leq 0.01$ and $p \leq 0.001$, respectively), but only the combination therapy achieved a pronounced reduction of the ischemic lesion when compared to saline (65%, $p \leq 0.05$; Fig. 2a, b). As anticipated, sham-operated mice did not develop cerebral injury (Fig. 2b).

At day 3, the statistically significant interaction between the effect of MMP10 and tPA treatments ($p = 0.03$) followed by subgroup analysis showed that only MMP10 treatment (alone or in combination with tPA) reduced infarct volume compared to saline ($p \leq 0.05$; Fig. 2c). Furthermore, maximal reduction was obtained with tPA/MMP10 combination (85%, Fig. 2c), although there were no differences when compared to MMP10. Altogether, these results demonstrate that MMP10 treatment is effective in reducing infarct size in STZ-induced

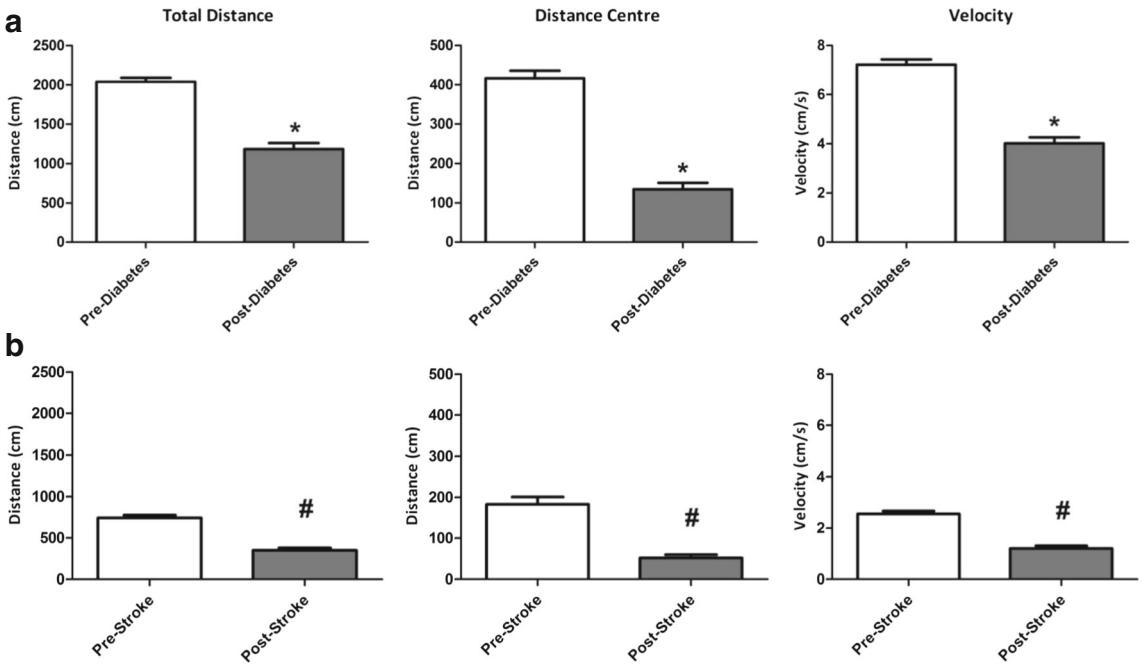

Fig. 1 Impaired locomotor activity after diabetes and IS induction. **a** Locomotor activity; total distance (left), distance in the centre (middle) and velocity (right); before and 2 weeks after STZ-induced diabetes. **b** Impaired motor activity in diabetic animals; total distance (left), distance in the centre (middle) and velocity of the animals (right) before and 24 h after stroke onset. Mean ± SEM, $^{*}p \leq 0.05$ vs. pre-diabetes and $^{\#}p \leq 0.05$ vs. pre-stroke, using Wilcoxon signed-ranked test, $n \geq 44$/group

diabetes, displaying maximal efficacy when combined with tPA. Moreover, no differences in microhemorrhages were observed with any of the treatments since hemosiderin deposition in brain tissue (Fig. 2d) was similar in all treated animals 24 h and 3 days after stroke (data not shown). Furthermore, the locomotor activity diminished by ischemic stroke was not further modified by the different thrombolytic agents (data not shown).

MMP10 Treatment Reduces Neuronal Degeneration and Improves EC Barrier Properties

We previously described that MMP10 prevented neuronal excitotoxicity induced by tPA and protected EC barrier properties in oxygen-glucose deprived endothelial and neuron cultures [21]. To investigate the neuroprotective effect of these treatments in the diabetic model of IS, we examined the number of FJC positive cells in the ischemic area (Fig. 2e). We observed that mice treated with MMP10, regardless of the combination with tPA, presented a significant reduction of neuronal degeneration 24 h and 3 days after IS induction ($p \leq 0.05$; Fig. 2f, g). These data demonstrate that MMP10 reduces neurodegeneration in this diabetic stroke model.

Table 1 Cerebral blood flow in diabetic mice treated with tPA and MMP10 after thrombin injection and the percentage of reperfused animals 1 h later (two-way ANOVA)

tPA	MMP10	Doppler reduction (%)	Reperfusion (%)
No	No	69.4 ± 9.9	42
	Yes	64.7 ± 9.8	58
Yes	No	70.2 ± 13.2	33
	Yes	63.5 ± 11.1	67

To investigate BBB damage, we performed immunostaining for laminin and CD31, an endothelial cell marker, 24 h post-IS. CD31 immunostaining was more intense in the ischemic area as compared with the contralateral area and was higher in MMP10-treated mice, whereas laminin staining was similar in both hemispheres despite the different uneven patterns observed in tPA-treated mice (Fig. 3; Fig. S4). Next, the extravasation of endogenous IgG into the brain parenchyma (Fig. 4a) was analysed. We observed that stroke diabetic mice treated with MMP10, either alone or in combination with tPA, had smaller IgG staining when compared to non-MMP10-treated mice 24 h after ischemia (Fig. 4b), suggesting that MMP10 treatment reduced BBB permeability. We also assessed claudin-5 and occludin expression, sensitive structural proteins of BBB impairment, using western blot. We compared the protein levels of both TJ proteins in the injured (IL) and non-injured (CL) hemispheres 24 h after IS, but we found no significant differences between them regardless of the treatment. Then, we analysed claudin-5 and occludin protein expression only in the injured

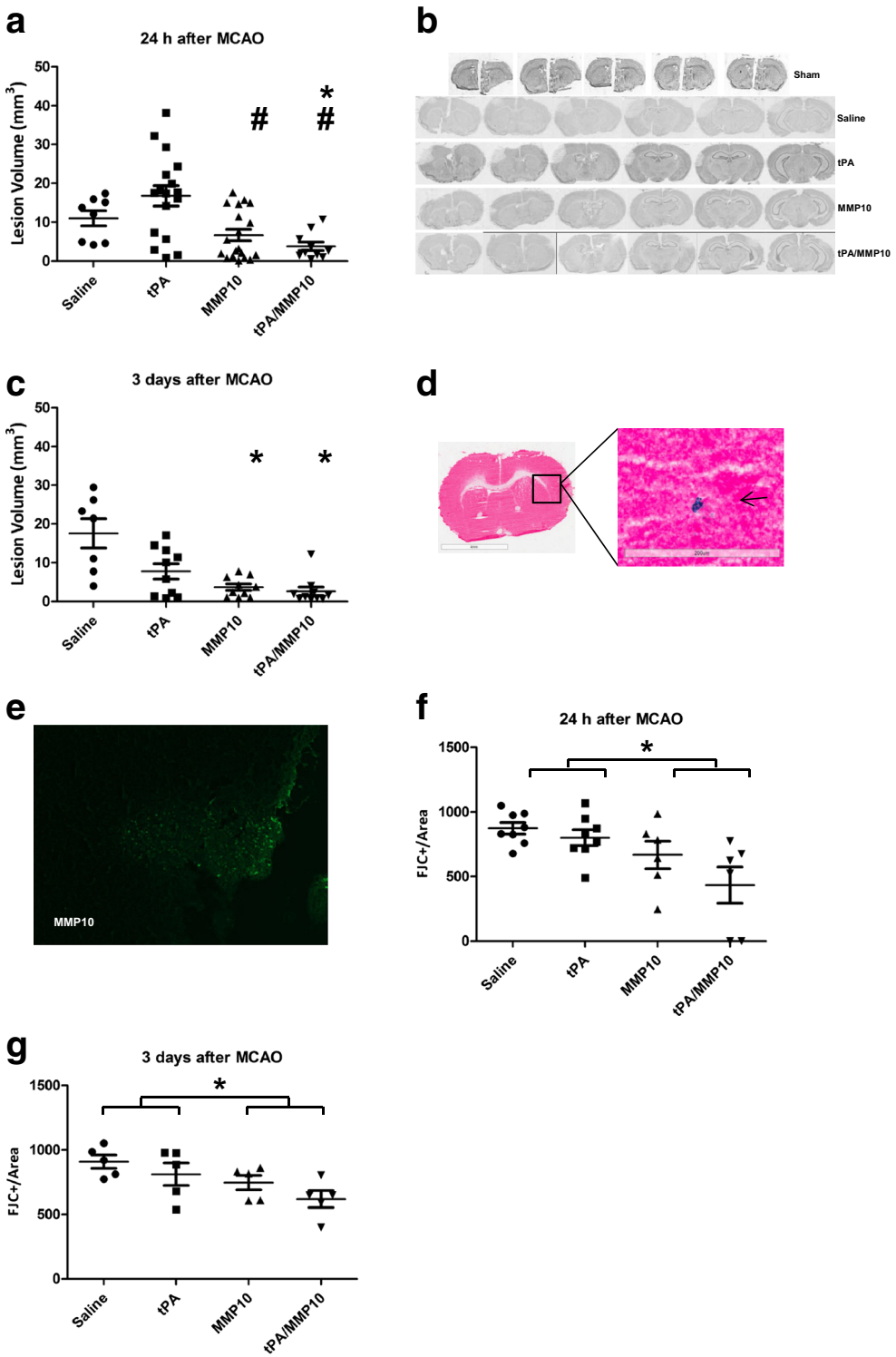

Fig. 2 MMP10 reduces brain injury in diabetic mice. **a** MMP10 treatment either alone or in combination with tPA leads to a larger reduction of lesion volume (mm^3) in experimental model of IS in the presence of diabetes at 24 h after MCAO. **b** Representative images of infarcted brains stained with thionin from one brain of each experimental group 24 h after MCAO. **c** Lesion volume 3 days after MCAO. **d** Representative image of a tPA/MMP10-treated brain 24 h after MCAO stained with Perl's (scale bar = 4 mm). The higher magnification view of the squared area shows the presence of microhemorrhages detected as hemosiderin deposits (arrows, scale bar = 200 μm). **e** Representative image of FJC staining in the lesion area of an MMP10-treated animal 24 h after MCAO. **f** MMP10 alone or in combination with tPA reduces neuronal degeneration (FJC + area) 24 h and **g** 3 days after MCAO. Mean ± SEM; two-way ANOVA ($*p < 0.05$ vs. non-MMP10 treatment) followed by one-way ANOVA with Tamhane correction for multiple comparisons ($*p \leq 0.05$ vs. saline, and $^{\#}p \leq 0.05$ vs. tPA) when p value for interaction was significant; $n \geq 7$ per group

hemisphere (IL) and compared their expression among treatments finding no differences 24 h after IS ($p > 0.05$; Fig. S5). These results show no effect of the treatments in TJ protein expression after IS in diabetic mice.

Since we had previously shown that MMP10 could decrease oxygen-glucose deprivation- induced ERK 1/2 activation in endothelial cells (EC) [21], we evaluated ERK 1/2 phosphorylation in IS tissues. We compared the IL and CL brain hemispheres 24 h after MCAO, but we found no differences in ERK 1/2 phosphorylation. However, our results showed that MMP10 diminished total ERK 1/2 phosphorylation in mice 24 h after MCAO when compared to non-

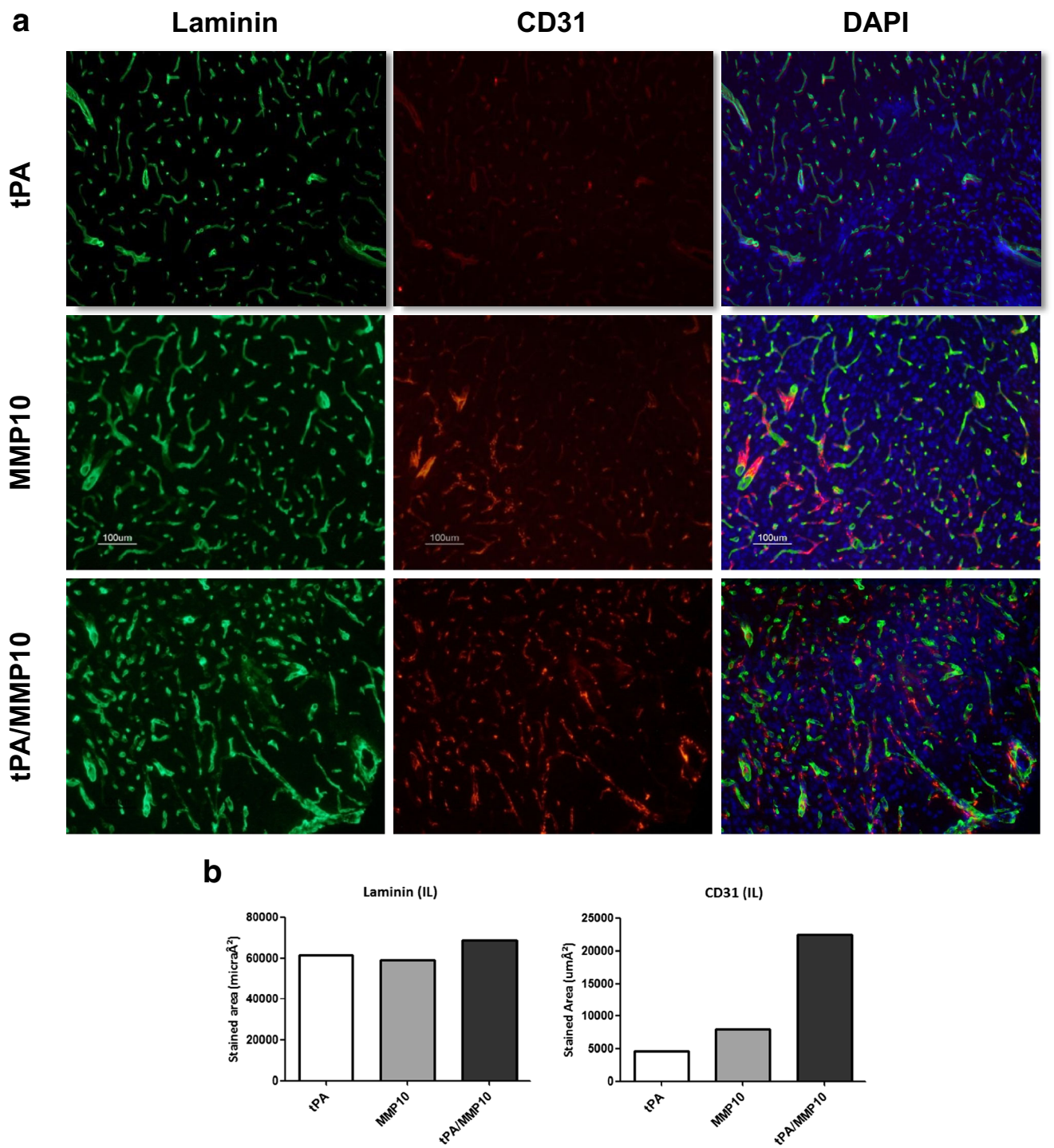

Fig. 3 CD31 expression was preserved in MMP10-treated diabetic mice. **a** Representative immunofluorescence images of laminin, CD31 and DAPI (merge) staining in the injured hemisphere (IL) of mice treated with tPA, MMP10 and tPA/MMP10 24 h following stroke. Brain sections were stained with anti-laminin (green), anti-CD31 (red) and DAPI (blue). Scale bar = 100 μm. **b** Quantification of laminin and CD31 stained areas in the injured hemisphere (IL), $n = 1$ per group

MMP10-treated mice ($p \leq 0.05$; Fig. 4c), suggesting that MMP10 treatment might diminish the signalling response associated with ERK 1/2 activation during the acute phase after IS.

Given that MMP9 has been implicated in tPA-enhanced BBB disruption in the ischemic area surrounding blood vessels [22], we analysed MMP9 expression in diabetic brains after stroke. As shown in Fig. 4d, we found no differences

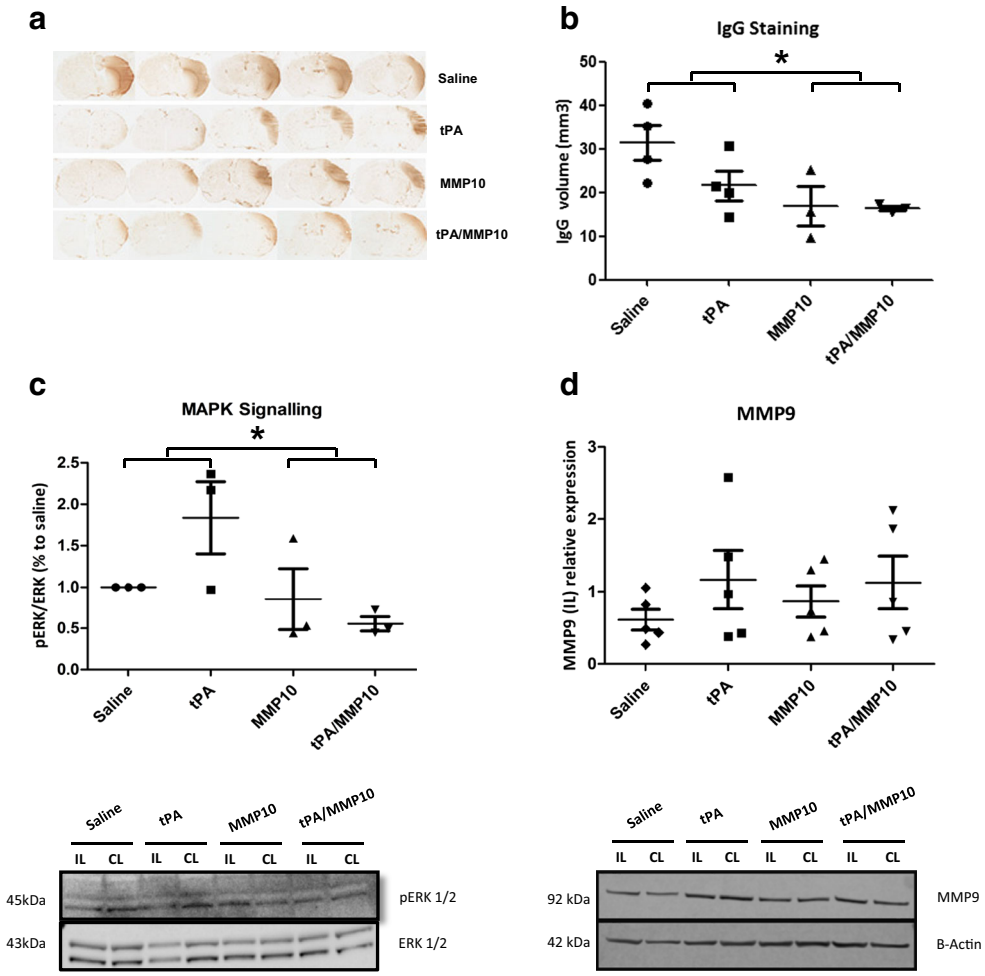

Fig. 4 Effect of MMP10 treatment on BBB properties after IS in diabetic mice. **a** Representative IgG-stained brain sections 24 h after MCAO from one brain of each experimental group. **b** Reduced IgG stained volume in MMP10-treated animals 24 h after ischemia. **c** Representative western blot of brain ERK1/2 protein 24 h after IS. Quantification of total ERK 1/2 protein relative expression 24 h after IS. Reduced ERK 1/2 phosphorylation was found in mice treated with MMP10 regardless of the combination with tPA. **d** Western blot of brain MMP9 protein 24 h after IS. No changes in MMP9 protein relative expression in the injured hemisphere (IL) were detected. Non-injured hemisphere (CL). Mean ± SEM, $p > 0.05$ for Interaction, $^*p \leq 0.05$ vs. non-MMP10 (saline and tPA), using two-way ANOVA, $n \geq 3$/group

between the IL and CL hemispheres. We also found that neither tPA nor MMP10 treatments significantly changed MMP9 expression in the IL hemisphere in diabetic mice 24 h after MCAO. Additionally, we showed no differences in MMP9 (IL) protein expression between saline-treated and sham-operated animals 24 h after MCAO (Fig. S6). These result indicate that none of the treatments modified MMP9 protein expression after IS in diabetic mice.

MMP10 Treatment Decreases Nox2

Oxidative stress is especially relevant during reperfusion after ischemia and contributes to lesion progression [23]. Therefore, we studied brain expression of Nox2 as a marker of oxidative stress. NOX2 levels were similar in the CL hemisphere when comparing different treatments and lower than in the IL (Fig. 5). The IL hemisphere showed differential expression of NOX2 depending on the treatment, being significantly higher in saline and tPA-treated mice compared to MMP10 administered animals ($p \leq 0.05$; Fig. 5). Our results suggest that MMP10 might reduce neurovascular oxidative stress early after stroke in diabetic mice.

MMP10 Reduces Systemic Thrombin Activation

To assess whether the treatments modulate haemostatic balance in diabetes associated IS, we analysed circulating levels of two haemostatic parameters. We found significantly decreased TAT levels in MMP10-treated mice 24 h after MCAO ($p \leq 0.05$), when compared to non-MMP10 administration (Fig. 6a). PAI-1 levels were similar in all experimental conditions 24 h after IS ($p > 0.05$; Fig. 6b). These results suggest a lower pro-thrombotic status in MMP10-treated diabetic mice 24 h after IS.

Discussion

Thrombolytic therapy with tPA in diabetic patients with IS remains a major clinical concern [24]. Here, we show for the first time the effectiveness of MMP10 and a synergistic effect

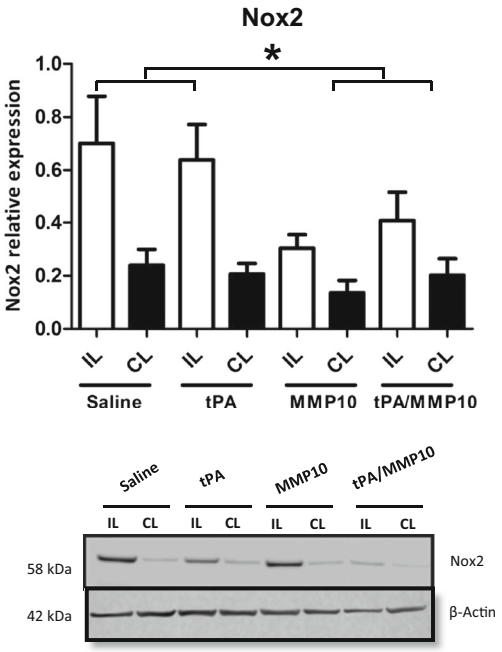

Fig. 5 MMP10 reduces oxidative stress in brain tissue. Quantitative analysis of Nox2 protein relative expression compares the injured (IL) and non-injured (CL) hemispheres 24 h following stroke in diabetic mice and representative western blot. Mean ± SEM, $^{\#}p \leq 0.05$ vs. IL using Wilcoxon signed-ranked test, $p > 0.05$ for Interaction, $^{*}p \leq 0.05$ vs. non-MMP10 (saline and tPA), using two-way ANOVA, $n = 5$/group

with tPA in reducing stroke lesion in STZ-induced diabetic mice. This model recapitulates partially the lower efficacy and safety of tPA in diabetic patients. Our results indicate that from the major mechanisms implicated in this damage, MMP10 treatment reduces neuronal degeneration, BBB permeability, ERK 1/2 phosphorylation, oxidative stress and thrombin generation.

tPA is the only FDA and EMA approved therapy for acute IS patients. Yet, its use in the clinical setting is limited due to its narrow therapeutic window and increased risk of cerebral bleeding, which is even higher under hyperglycaemic conditions [25]. Hyperglycemia is considered a poor diagnostic factor in stroke patients [26] that potentially decreases the beneficial effect of tPA. Therefore, diminished rates of tPA administration are observed in diabetic subjects, although studies did not find an overall reduced benefit of administering tPA in these patients [25]. Moreover, only a handful of studies have involved tPA in experimental hyperglycaemic/diabetic models. Our group has already shown that MMP10 and tPA effectively reduce lesion volume in an experimental model of thromboembolic IS in healthy mice [19], which is further improved when both are administered together [21]. Here, we challenge the thrombolytic properties MMP10 in IS using diabetes as co-morbidity. MMP10 administration reduced infarct size better than tPA, which was not able to display such effects unless administered in combination with MMP10 24 h after MCAO in STZ-induced diabetic mice. Furthermore, 3 days after IS, only MMP10-treated mice presented reduced infarct size, arguing previous experimental data showing delayed brain protection by tPA in hyperglycaemic rats [27]. These data not only support the concern about the benefit of tPA during the therapeutic window in diabetic patients [28] but also confirm that MMP10 treatment is more effective than tPA in terms of infarct size, neuronal degeneration and BBB permeability to IgG in this stroke model. Altogether, our results suggest a possible use of MMP10 as a novel therapeutic alternative in diabetic patients, by quickening the beneficial effects of the thrombolytic therapy compared to tPA. The mechanisms by which hyperglycemia increases ischemic brain injury are not fully understood and several pathways are thought to play a role in the detrimental effect [25].

Diabetes has been associated with increased vascular permeability [29] and also with angiogenesis impairment [30]. In our diabetes model, immunohistochemical analysis showed higher endothelium immunostaining in the ischemic hemisphere when compared to the contralateral non-ischemic. Moreover, specific analysis of the ischemic hemisphere indicates stronger CD31 staining in MMP10-treated animals, whereas no differences were observed for laminin despite

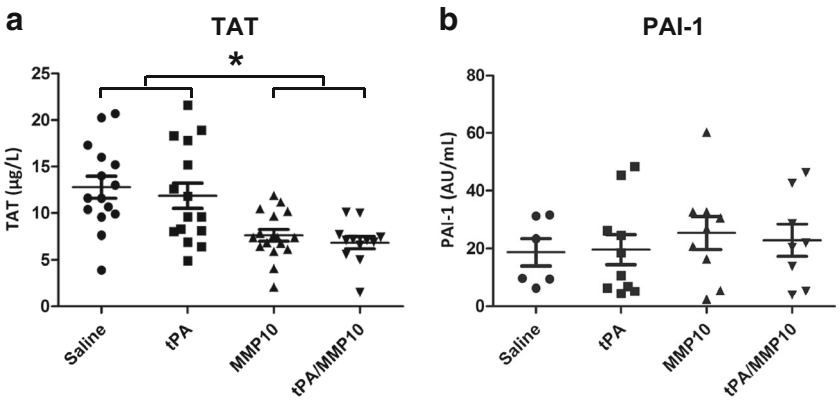

Fig. 6 MMP10 reduces thrombotic status early after IS. **a** Circulating levels of TAT complexes and **b** PAI-1 levels 24 h after IS. Mean ± SEM, $p > 0.05$ for interaction, $^{*}p \leq 0.05$ vs. non-MMP10 (saline and tPA), using two-way ANOVA, $n \geq 8$/group

its fragmented profile in tPA-treated mice. These results suggest that MMP10 might help to preserve endothelium consistency after IS although further research may be performed.

Next, we explored the effect of tPA and MMP10 treatment on BBB integrity as an essential mechanism for the maintenance of brain homeostasis that prevents brain oedema and intracranial haemorrhage [31]. Based on previous studies, MMPs and tPA have been implicated in basement membrane degradation and haemorrhagic complications. It has been shown that tPA administration 1–2 h after stroke increased haemorrhagic transformation in experimental models of hyperglycaemia [32]. In contrast, our data showed that IS in diabetic mice resulted in small microhemorrhages regardless of the treatment. Differences in the duration of the ischemia/reperfusion time (1–2 h vs. 20 min) could explain this apparent divergence, because late tPA treatment (3 h) has also shown a deleterious effect and haemorrhagic transformation in this thromboembolic stroke model [33].

Increased MMP9 levels have been linked to poor outcome and haemorrhagic transformation in clinical studies of IS [34, 35] and to the development of other diabetic complications such as microangiopathy among others [36]. Desilles et al. described increased MMP9 levels in the brains of hyperglycaemic rats 24 h following stroke [37], and Ning et al. reported elevated MMP9 expression after tPA treatment 48 h after MCAO in STZ-induced diabetic rats [32]. In contrast, we did not find differences in MMP9 brain expression 24 h after stroke, either after tPA or MMP10 treatment. This discrepancy might be due to the different experimental conditions employed, although these findings are in line with our previous results on brain microvascular EC showing that stimulation of these cells with MMP10 did not modify MMP9 expression [21].

TJ proteins, such as claudin-5 and occludin, are important to preserve the BBB integrity and neurovascular unit [38]. Hyperglycemia has been related to increased BBB impairment by decreasing early claudin-5 and occludin brain expression (2–4 h) after ischemia in an experimental model of stroke [37]. However, Liu et al. [39] and Jiao et al. [40] described that ischemia leads to an alternated distribution of claudin-5 and occludin proteins after MCAO in rats rather than to a reduction in their expression. In our study, quantification of TJ proteins by western blot showed no differences in expression of claudin-5 and occludin 24 h after IS in STZ-induced diabetes, although as suggested by other authors [39, 40], it cannot be excluded their redistribution in brain tissue post-IS. Thus, further investigations are required to assess the role of TJ proteins in experimental models of stroke associated with diabetes.

Analysis of the MAPK pathway showed that MMP10 diminished the activation of ERK 1/2 in brain tissue after the ischemic challenge which has been associated with BBB alterations and damage in stroke models [41, 42]. These results would agree with our previous data in brain microvascular EC showing that MMP10 decreased ERK 1/2 phosphorylation and suggest that MMP10 might limit the hyperglycaemic-ischemic damage by interfering with ERK activation and its related brain injury mechanisms. However, further research should be performed about this issue in order to confirm these observations in vivo.

Increase in ROS generation and inflammatory cytokines are among the main mechanisms of damage in diabetes. Hyperglycemia and diabetes have a detrimental effect in brain injury due to enhanced production of free radicals [30, 43] and the activation of NADPH oxidase [44] which can lead to apoptosis and neuronal death [45]. In accordance with the results reported by Liu et al. in a rat model of IS [46], we have observed an increase in Nox2 expression, a subunit of NADPH oxidase, in the ischemic hemisphere 24 h after stroke, which is reduced by MMP10. This is also in line with Miller et al., that described an increased Nox2 activity in the cerebral arteries of the penumbra for 3 days [47]. Together, these data suggest that MMP10 might diminish neuronal death in the ischemic hemisphere by reducing Nox2 expression and ROS production although this hypothesis should be confirmed in future studies.

Increased activation of blood coagulation and decreased fibrinolytic activity has been also found in hyperglycemia. Thus, we analysed the effect of MMP10 and tPA treatments on thrombin generation by measuring TAT and the inhibitor of fibrinolysis PAI-1. PAI-1 has been shown to influence physiological responses to tPA recanalization [48] and its levels were increased in patients with type-1 diabetes, being an independent risk factor for vascular complications [49]. Regarding the coagulation process, lower levels of TAT have been associated with higher recanalization rates with tPA in IS patients [13]. Moreover, elevated TAT levels were described in hyperglycaemic rats as an indicator of coagulation activation after experimental stroke [37]. We found lower levels of circulating TAT in diabetic animals treated with MMP10 regardless of the combination with tPA 24 h after stroke, while no changes in PAI-1 levels were found. This suggests that MMP10 treatment might promote a less pro-thrombotic status after IS in diabetes by reducing TAT complexes.

In conclusion, we present MMP10 as a more effective thrombolytic treatment than tPA reducing stroke lesion and neuronal degeneration in a murine model of IS associated with STZ-induced diabetes. Additionally, our results suggest that MMP10 might play a role in the reduction of BBB permeability, ERK 1/2 phosphorylation, oxidative stress and thrombin generation. Therefore, we propose MMP10 alone or in combination with tPA as a potential candidate for the clinical treatment of IS in diabetic patients.

Acknowledgements We thank Lara Montori for the help with the experimental work and Rosa Tordera and Mikel Aleixo from Pharmacology and Toxicology Department, University of Navarra for their technical assistance with behavioural tests.

Author's Contribution MNO participated in the design of the project, experimental work, statistical analysis, and wrote, reviewed, and edited the manuscript; CR participated in the design of the project and reviewed the manuscript. AS was in charge of the animal experiments; MB performed histological studies; OR participated in the design of the project and reviewed the manuscript; ET participated in the statistical analysis and reviewed the manuscript; BZ participated in the design of the project and reviewed the manuscript; JAR participated in the design of the project and reviewed the manuscript; JAP participated in the design of the project, supervised the work, and edited and reviewed the manuscript; RM participated in the design of the project and edited and reviewed the manuscript; JO was in charge of the whole project design, supervised the work, and wrote, edited, and reviewed this manuscript.

Funding This work was supported by the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III [FIS PI15/01807] and Federal Ministry of Education and Research (FEDER) funds; grants from the Spanish Society of Thrombosis and Haemostasis (SETH), Navarra Government (02/2015), Patrimonio Praga (Mexico) and Virto Group (Navarra, Spain); and PhD scholarship from the Asociación de Amigos de la Universidad de Navarra (ADA).

Compliance with Ethical Standards

Experiments were performed in accordance with European Communities Council Directive guidelines (2010/63/EU) for the care and use of laboratory animals and were approved by the University of Navarra Animal Research Review Committee.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All animal experiments were performed in accordance with European Communities Council Directive guidelines (2010/63/EU) for the care and use of laboratory animals and were approved by the University of Navarra Animal Research Review Committee.

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