

Different therapy options protecting microvasculature after experimental cerebral ischaemia and reperfusion

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Summary

Recombinant tissue plasminogen activator (rt-PA) is successfully used in human stroke, but often shows serious drawbacks. To find an alternative, we hypothesised that the novel thrombolytic microplasmin would have fewer adverse effects on haemoglobin extravasation and microvascular damage compared with the effects of rt-PA and tenecteplase (TNK). A constant period of ischaemia (3 hours) was induced in a rat suture model followed by reperfusion (24 hours). Mikroplasmin (10 mg/kg), TNK (5 mg/kg), rt-PA (9 mg/kg) and saline (control), were administered. The volume of the ischaemic lesion was calculated, the loss of collagen type IV and the extravasation of haemoglobin were quantified by Western blotting. The matrix-metalloproteinases 2 and 9 (MMP-2/-9) were quantified by zymography and their endogenous tissue inhibitors (TIMPs) were analysed by reverse zymography. Microplasmin treatment caused the lowest volume of the ischaemic lesion ($51.0 \pm 22.6 \text{ mm}^3$) compared with control ($167.3 \pm 13.1 \text{ mm}^3$; $p < 0.05$). The content of col-

lagen type IV was significantly increased and haemoglobin extravasation reduced ($154 \pm 24\%$; $p < 0.05$) compared with control ($442 \pm 124\%$); MMP-2/-9 and the corresponding TIMPs remained unchanged. In comparison, TNK did not significantly reduce basal lamina damage and caused the highest extravasation. MMP-2/-9 were severely increased after TNK treatment ($p < 0.05$). Thus, the balance between MMPs and TIMPs was shifted toward the inhibitory side with TNK. Microplasmin had a protective effect on the microvascular basal lamina and blood-brain barrier, whereas TNK was significantly disadvantageous from the viewpoint of ischaemic damage. Microplasmin also appears to be safer than other PAs in terms of damage to the microvasculature associated with thrombolytic therapy of ischaemic stroke.

Keywords

Cerebral ischaemia, microplasmin, MMPs, plasminogen activator, rt-PA, tenecteplase

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Introduction

So far the only pharmacological therapy approved for acute stroke patients is thrombolysis with recombinant tissue-type plasminogen activator (rt-PA, alteplase). While it improves the clinical outcome in patients with acute ischaemic stroke and reduces ischaemic brain damage (1), its beneficial effect is partially offset by the 10-fold increase in the rate of symptomatic intracerebral bleeding (1). Furthermore, a delay in treatment is associated with an increased risk of haemorrhagic transformation and enhanced brain injury (2). Novel agents that achieve higher recanalisation rates, lower haemorrhage rates, or both might improve this delicate trade-off between benefit and risk.

Thrombolytics with novel properties include tenecteplase (TNK), staphylokinase, microplasmin (μ -Pli), PAs originating from the *Desmodus* species, and other preparations in addition to rt-PA, urokinase PA, recombinant single-chain urokinase PA, and streptokinase (3, 4). Surprisingly, no systematic experimental

comparisons of the effect equivalence at equipotent doses of these agents have yet been made.

TNK is a genetically modified form of t-PA with 14-fold greater fibrin specificity, longer half-life (5) and 80-fold greater resistance to inhibition by plasminogen activator inhibitor type 1. Although TNK has been studied in detail in acute myocardial infarction, little is known about its potential usefulness for the treatment of stroke.

μ -Pli, a truncated form of plasmin that lacks the five kringle domains, is an emerging direct fibrinolytic agent (6). Whereas standard plasminogen-activating drugs depend on the local availability of plasminogen to generate active, fibrin-digesting Plasmin, μ -Pli acts directly (7). Nagai and colleagues (8) previously observed that recombinant human μ -Pli reduced lesion size in an ischaemic stroke model in mice when administered within several hours after middle cerebral artery (MCA) occlusion. Furthermore, μ -Pli improved behavioural rating scores after intracerebral embolisation in rabbits, at a dose that did not increase haemorrhage (7).

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Focal cerebral ischaemia causes a loss of microvascular integrity that is manifested by major alterations in vascular permeability (9, 10). The loss of basal lamina integrity has been postulated to be the primary cause of microvascular haemorrhage during focal cerebral ischaemia (11). Cerebral ischaemia triggers a series of pathways that result not only in intracellular proteolytic cascades, but also extracellular proteolytic processes involving the extracellular matrix (ECM). This process begins with the breakdown of the blood-brain-barrier (BBB) and the degradation of the basal lamina, a specialised part of the ECM; it leads to cellular extravasation and a loss of microvascular integrity (12). The plasminogen system and the matrix metalloproteinases (MMPs) are thought to be the terminal enzymes in the ECM-remodelling cascade (13). MMPs, mainly MMP-2 and MMP-9, mediate blood-brain-barrier disruption. Both are up-regulated in the ischaemic brain contributing to the degradation processes of the basal lamina components like collagen IV (14).

In this study, it was our hypothesis to demonstrate a safer alternative with the new thrombolytic microplasmin; further, we wanted to reveal the different effects to the MMP/TIMP (Tissue Inhibitor of Metalloproteinase) system following thrombolysis.

The mechanism of tissue injury after thrombolysis is not well understood, and the effects of plasminogen activators or plasmin on the microvasculature also remain largely unclear. In view of the potentially wide-spread use of thrombolysis in stroke patients, we compared the effects of intravenously administered μ -Pli, t-PA and TNK on basal lamina damage. MMP and the plasminogen system alteration under these conditions were also tested.

Material and methods

All experimental procedures were approved by the government of Upper Bavaria (permission number: 211–2531/76/03). Every effort was made to ensure that the animals were free of pain and discomfort.

Animal experiments

All experiments used male Wistar rats (250–300 g) (Charles River Laboratories, Sulzfeld, Germany). The rats were intubated and anaesthetised with isoflurane inhalation (1.7% isoflurane in a mixture of 70%/30% of N₂O/O₂). They were not allowed to wake up and were kept under anaesthesia until reperfusion started (for details, see [12]). The filament model of transient ischaemia was chosen to accurately measure the effects of the tested thrombolytics without unquantifiable effects deriving from thrombi in an alternative thromboembolic model: the MCA was occluded for 3 hours (h) by advancing an intraluminal thread rostrally into the internal carotid artery up to the origin of the MCA. After ischaemia (I), the onset of reperfusion (R) was induced by withdrawing the thread into the lumen of the internal carotid artery. At the end of the reperfusion period (24 h after reperfusion following 3 h MCA occlusion), rats were sacri-

ficed by transcardial perfusion with 300 ml cold physiological saline containing bovine serum albumin (10 g/l), heparin (10 IU/l), and 2 ml/l nitroprussin solution (1.8 g/l) diluted in 1 l of NaCl isotonic solution (9 g/l) under deep anaesthesia. Sham-operation was done by advancing the thread only 12 mm toward the intracranial part of the internal carotid artery so that it did not occlude the MCA.

Administration of the thrombolytic agents

The thrombolytics (Actilyse, Boehringer Ingelheim, Ingelheim, Germany; μ -Pli, Thromb-X, Leuven, Belgium; Metalyse, Boehringer Ingelheim) were intravenously infused in the right external jugular vein by an indwelling catheter of PE 50 tubing at the following doses: rt-PA: 9 mg/kg body weight (bw); TNK: 5 mg/kg bw and μ -Pli: 10 mg/kg bw.

Starting 30 minutes (min) before reperfusion, i.e., after 2.5 h after onset of ischaemia induction, a slow bolus injection of 10% of the rt-PA dose, followed by a continuous infusion of the remaining 90% over 1 h, was administered by an external pump (Precidor 5003 INFORS AG, Basel, Switzerland). μ -Pli was administered in the same schema. The application protocol was identical to the protocol used in stroke therapy (1).

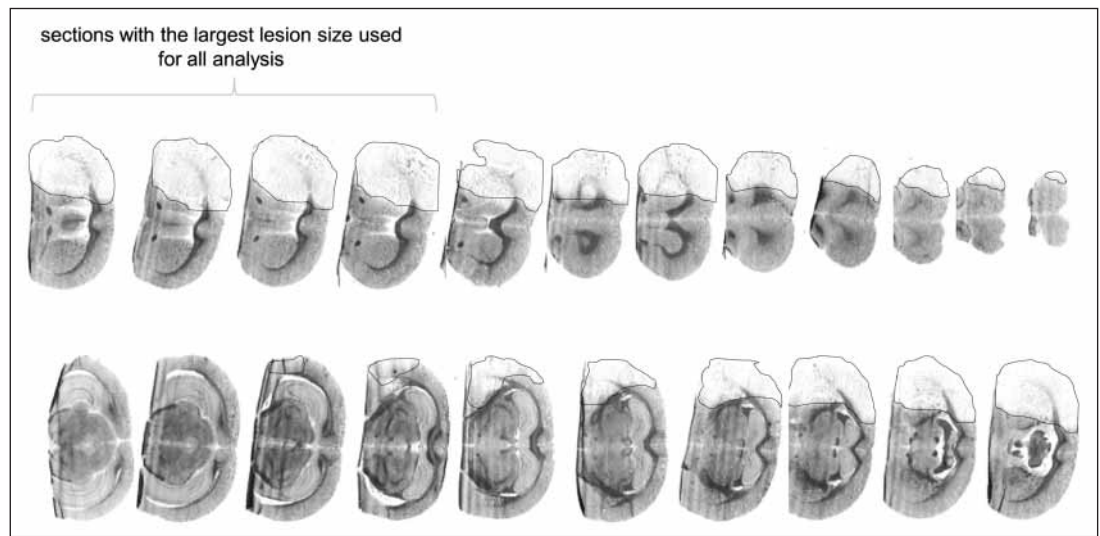
At the same time point before reperfusion, TNK was infused at a dose of 5 mg/kg bw (bolus) over an interval of 5 min using a syringe infusion pump, adapted from treatment of acute myocardial infarction (15). An additional group received saline as control; a group of sham operated animals served as a second control. Each group was assessed with six rats.

Tissue isolation

After perfusion the brain was removed, immediately frozen and cut in a cryostat at -80°C in a volumetric manner. Ten μm thick sections were collected. For volumetry of the lesion size consecutive sections with 500 μm clearance were used (total about 24 sections; ► Fig. 1). Every 50 consecutive sections, one section was used for volumetry (based on MAP-2 immunostaining). The region 0–1 mm behind the bregma (16) appeared to consistently have the largest ischaemic lesion size. One hundred adjacent sections were taken from this region (Fig. 1) and were stored without fixation at -80°C for further use.

For all biochemical analysis brain areas of the cortex and the basal ganglia in the region 0–1 mm behind the bregma were excised with a scalpel. The same size of the ischaemic and non-ischaemic hemispheres was used. For each Western blot or Zymography analysis six consecutive 10 μm -thick cryosections from this region were taken (for details, see [17]). This material was homogenised for zymography and Western blot analysis. Protein concentrations were routinely determined by protein assay (Pierce, Rockford, IL, USA).

Figure 1: All volumetric sections of a representative control rat brain stained with MAP-2 antibody showing ischaemic lesions in the rats. Between each volumetric section there is a clearance of 500 μm . The region 0–1 mm behind bregma reliably showing the largest lesion sizes is indicated.



Gelatine-zymography

For the non-reducing SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), equal amounts of protein (20 μg) were run without β -mercaptoethanol on a 10% polyacrylamide gel containing 0.5% gelatine (Biorad, Munich, Germany). Molecular standards (Biorad) and recombinant human MMP-2 and MMP-9 standards (Sigma, Munich, Germany) were used to calibrate molecular weights and the amount of MMPs between the different gels (17). Active and the inactive proform MMP-9 and pro-MMP-2 were shown and quantified. Reverse zymography was performed in a similar manner with the exception that conditioned medium from HT1080 (human fibrosarcoma) cells, which express MMP-2 and MMP-9, was included in the gel mix with the gelatine (25 μl of conditioned medium was added to 15 ml of the gel mix) (17). Recombinant human TIMP-1 and -2 standards (Chemicon Int., Temecula, CA, USA) were used.

Western blot analysis

Brain lysates (20 μg of protein) were boiled in a reducing SDS sample buffer (containing 25 mM β -mercaptoethanol), then electrophoresed and transferred to a PVDF membrane (Polyvinylidene Fluoride, Sequi-Blot, Biorad). Collagen type IV was detected using goat anti-collagen type IV antibody at a dilution of 1:500 (Southern Biotechnology, Birmingham, AL, USA). Haemoglobin was detected using a polyclonal rabbit anti-haemoglobin antibody at a dilution of 1:200 (DPC Biermann, Eschborn, Germany) (17).

Immunohistochemistry and volumetry

Before immunohistochemistry the frozen sections were air-dried for 10 min and fixed with cold acetone and chloroform (1:1) for 5

min at room temperature. The loss of MAP-2 (microtubule-associated protein) coincides with neuronal loss and parallels ischaemic injury (18). MAP-2 immunostaining (MAP-2 antibody: Chemicon Int.) was used to delineate the borders of the ischaemic lesion. The staining protocol was published earlier (17). This staining was also used to calculate the lesion size. All volumetric sections were stained with the MAP-2 antibody, scanned and the files imported into Optimas 6.2 imaging software (Media Cybernetics, L.P., Silver Spring, MD, USA). The lesioned areas were delineated (Fig. 1A) and the partial volume between two adjacent sections was computed using the formula for a conic section. Then all partial volumes were added to find the total lesion volume (19).

Quantification of vessel count by video-imaging microscopy

To quantify the microvessels, their number per area unit (vascular density) was calculated in the ischaemic and non-ischaemic cortex and basal ganglia region: First two sequential sections from each rat brain were used: 1) one was stained with an antibody against MAP-2 (► Fig. 2A); 2) the adjacent section was stained with an antibody against collagen IV. At first the MAP-2-stained section was digitised using a conventional flatbed scanner and the respective region of interest (ROI) was delineated (basal ganglia and cortical areas) using the OPTIMAS image analysis system. Basal ganglia and cortex were defined according to the anatomic landmarks (16) as described by Hamann et al. (12). In addition, a contralateral area that mirrored the ROI was delineated as control (Fig. 2A). Next the complete collagen-stained section was digitised with a Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped with a five-fold objective using a Sony Power HAD 3CCD color video camera (Sony, Tokyo, Japan) resulting in 96 fields of view per section. The composite image from these fields was imported into OPTIMAS (Fig. 2B).

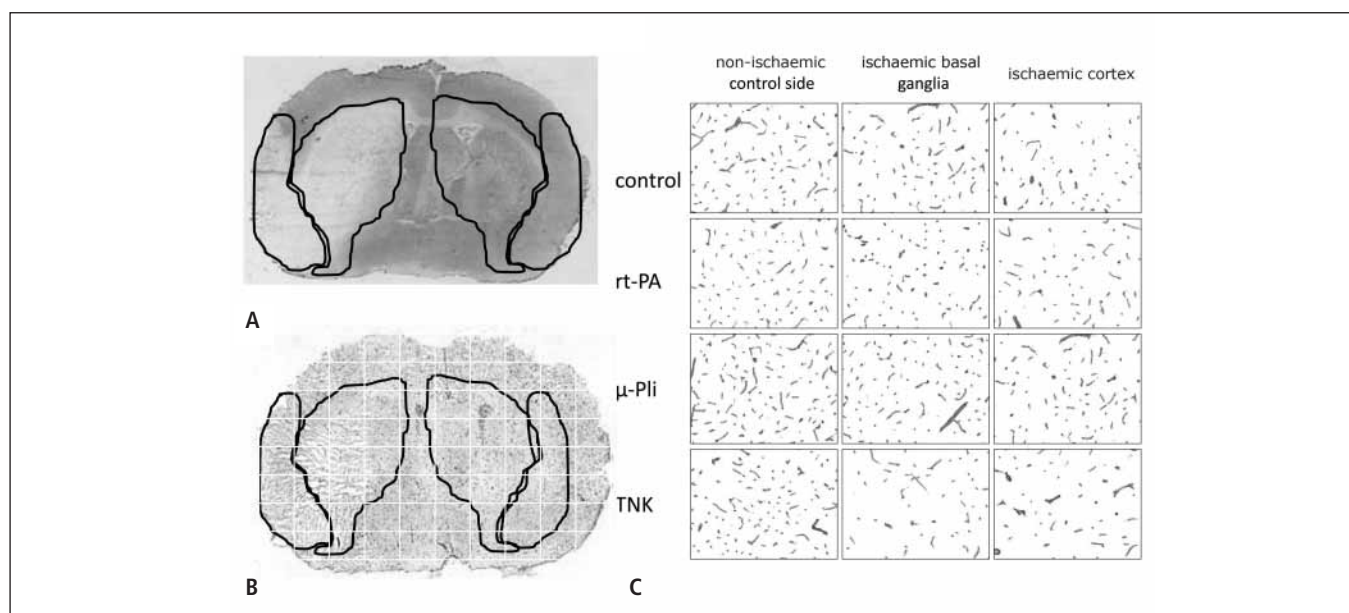


Figure 2: Immunostaining of representative cryosections of ischaemic rat brain. A) Representative brain section +1 mm bregma according to (16) stained by a MAP-2 antibody (dilution 1:800; Chemicon, USA). The ischaemic lesion area of the cortex and the basal ganglia region and the contralateral regions are indicated. B) Collagen type IV immunostaining of a representative section of a saline-treated control animal. The complete collagen-stained section was digitised with a Zeiss microscope

equipped with a five-fold objective using a CCD video camera (Sony, Japan) resulting in 96 fields of view per section. The composite image from these fields is shown. The masks of the region of interest, based on the MAP-2 staining (A) were transferred to this composite. C) Representative fields of view from each experimental group are shown; separated into the non-ischaemic control side (basal ganglia, cortex not shown here) and the ischaemic cortical and basal ganglia region.

Subsequently, the ROI masks were transferred to the collagen-stained section and the number of vessels in each ROI was calculated with OPTIMAS and EXCEL (Microsoft, Redmond, WA, USA).

The values of the vessel count are mean values of $n=6$ sections per animal.

Data analysis

The signals from the zymography and Western blots were scanned and analysed with an optical analysis program (TINA, Version 2.08, Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany) by optical densitometry. Results were displayed on an arbitrary optical density scale. The data were expressed as relative lysis zone (pixels) per microgram protein and per mm lane width. The ratio of the data on the ischaemic to the non-ischaemic area was determined to avoid the influence of inter- and intra-gel irregularities. Data were expressed as mean values \pm standard error of the mean (SEM). For each brain region, the ipsilateral area was compared with the contralateral one using the Mann Whitney-U Test with a level of significance of 5%. Percent values are calculated; 100% was the non-ischaemic control region (contralateral) in each animal. Statistical comparisons of the drug-treated groups to the control were performed by unpaired Student t-test and one-way

ANOVA followed by post-hoc Bonferroni analysis (SPSS version 15.0 for windows, SPSS GmbH, München, Germany).

Results

Twenty-four rats with documented cerebral ischaemic lesions underwent further analysis. Six rats in each of the four groups (control, rt-PA, TNK, and μ -Pli group) were assessed. Sham operated animals did not show any lesion and in all tests no differences between the ipsi- and contralateral hemisphere are seen. Data not shown here.

Effects of the different thrombolytics on cerebral lesion size in rats after I3R24

In Figure 1 a representative example of the procedure to delineate the ischaemic lesion throughout a whole rat brain is shown. The distance between two volumetric sections was always 500 μ m. Occlusion of the MCA induced an ischaemic lesion with a volume of 165 ± 21 mm³ ($N=6$) in saline-treated control rats. Injection of 5 mg/kg TNK had no significant effect on the lesion size (222 ± 13 mm³, t-test $p>0.05$ vs. control group), whereas both 9 mg/kg rt-PA and 10 mg/kg μ -Pli significantly reduced the lesion size to 101 ± 17 mm³ (t-test; $p=0.048$ vs.

control group) and $69 \pm 22 \text{ mm}^3$ (t-test: $p=0.017$), respectively (Fig. 4A); Anova: $p=0.012$. The difference of the lesion size in the TNK group vs. the μ -Pli groups was significant (Bonferroni).

Effects of different thrombolytics on microvascular damage, microvessel count and haemoglobin extravasation in rats after I3R24

To determine whether the reduced lesion size correlated with an increase of collagen type IV and a protection of the extracellular basal lamina, the effects of all three drugs on collagen type IV, the main component of the basal lamina, were determined. Quantification of collagen type IV was done by Western blotting. The content of collagen IV in the control group (in the ischaemic side compared with the non-ischaemic side) was $64 \pm 7\%$ (cortex) and $43 \pm 9\%$ (basal ganglia) following ischaemia 3 h, reperfusion 24 h (I3R24). The results showed a statistically significant increase of collagen after administering rt-PA and μ -Pli (Anova: $p<0.05$ cortex and basal ganglia each). This severe loss of collagen IV was significantly and most effectively reduced with μ -Pli: the loss amounted to $94 \pm 3\%$ (Bonferroni and t-test: $p<0.05$) and $96 \pm 7\%$ (Bonferroni and t-test: $p<0.05$ vs. control group) in the ischaemic cortex and basal ganglia respectively, compared with the non- ischaemic side. This effect was also observed in the rt-PA group (cortex: $85 \pm 11\%$, basal ganglia: $75 \pm 8\%$, significant for both regions [t-test: $p<0.01$ vs. control group]), but it was, however, only observed to a minor degree in the TNK group (cortex: $57 \pm 16\%$, $p=0.05$, basal ganglia: $55 \pm 9\%$, not significant) (Fig. 4B). The number of microvessels was severely reduced after treatment with TNK ($p<0.05$, Bonferroni). This decrease in microvessel number was significant compared with the group treated with rt-PA and μ -Pli in the cortical and in the basal ganglia regions. The loss of microvessels after I3R24 was similar for both rt-PA and μ -Pli treatment groups (control: cortex: $88 \pm 7\%$, basal ganglia: $83 \pm 3\%$; rt-PA: cortex: $87 \pm 4\%$, basal ganglia $79 \pm 4\%$; μ -Pli: cortex: $94 \pm 5\%$, basal ganglia: $88 \pm 6\%$; Anova: cortex $p=0.003$, basal ganglia $p=0.025$) (Fig. 2C; 4C).

After I3R24, there was severe extravasation of haemoglobin in the ischaemic brain (► Fig. 3). The extravasated haemoglobin was quantified by Western blotting in the brain tissue (Anova: $p<0.05$). These results showed an enhanced haemoglobin amount of $442 \pm 28\%$ in the basal ganglia and $191 \pm 23\%$ in the cortical region in saline-treated control rats (t-test: $p<0.05$). This haemorrhage was further enhanced by treatment with TNK and tPA. Both TNK and rt-PA significantly increased the amount of haemoglobin in the brain tissue. No difference was observed between these two t-PAs (TNK: cortex: $474 \pm 16\%$, basal ganglia: $653 \pm 20\%$; rt-PA: cortex: $420 \pm 21\%$, basal ganglia: $694 \pm 19\%$), but the difference was significant vs. the untreated control group (Bonferroni post-hoc analysis: TNK and rt-PA each $p<0.05$). Only the plasmin derivative μ -Pli diminished the haemoglobin amount in the cortex ($125 \pm 4\%$) and in the basal ganglia ($134 \pm 12\%$) (t-test: $p<0.05$ vs. control) (► Fig. 4D).

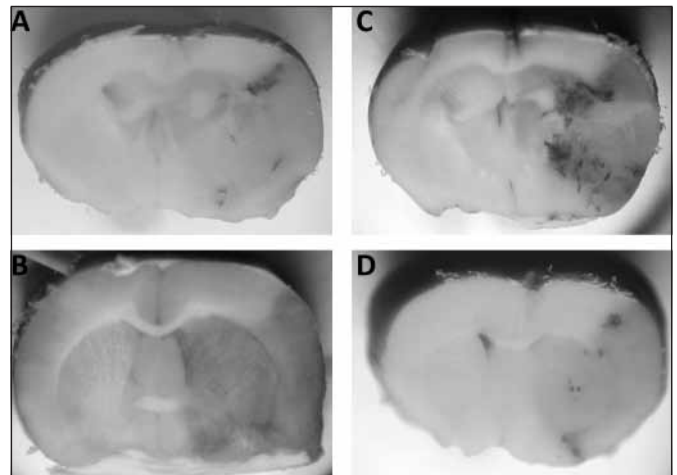


Figure 3: Representative picture during the cutting procedure of the frozen, native brain from the four experimental groups. Haemoglobin extravasation into brain tissue is demonstrated. A) control brain, saline-treated; B) rt-PA; C) TNK; and D) μ -Pli-treated animal.

Rt-PA had different effects on the brain tissue and microvessels. Rt-PA treatment protected BBB integrity (collagen type IV) as microplasmin does, but did not protect BBB leakage (haemoglobin).

Effects on the equilibrium between MMP and TIMP after treatment with different thrombolytics after I3R24

Gelatin zymographic studies of ischaemic and non-ischaemic basal ganglia and cortex were used to quantify the amounts of MMP-2 and MMP-9 (► Fig. 5A). One-way Anova analysis showed significant effects after thrombolytic treatment for MMP-9 in both brain regions and for MMP-2 in basal ganglia only. Both MMP-2 and MMP-9 were significantly increased after injection of TNK compared with saline and μ -Pli-treated animals (Bonferroni post-hoc analysis: $p<0.05$). There was also a significant increase of both MMPs after rt-PA treatment compared with control animals (Bonferroni post-hoc analysis: $p<0.05$); the μ -Pli group did not show a different MMP amount compared with the control group. The amount of MMP-2 was significantly lower compared with both t-PA groups. However, μ -Pli treatment caused no changes of MMP-9 compared with rt-PA treatment (Fig. 5B and C).

The endogenous inhibitors TIMP-1 and TIMP-2 were quantified by reverse zymography. A significant increase of TIMP-1 and TIMP-2 (t-test: $p<0.05$) was shown after focal ischaemia compared with sham operated animals (Fig. 5D). This effect was attenuated by treatment with all the thrombolytic drugs (Anova: $p>0.05$; Fig. 5E and F).

Prior studies suggested specific interactions between MMP-9 and TIMP-1 and MMP-2 and TIMP-2 (20). In order to evaluate the TIMP/MMP balance, the ratio of the enzymatic activity of the inhibitors to their preferred protease TIMP-1/MMP-9 and TIMP-2/MMP-2 was calculated. An effective evaluation of the impact of

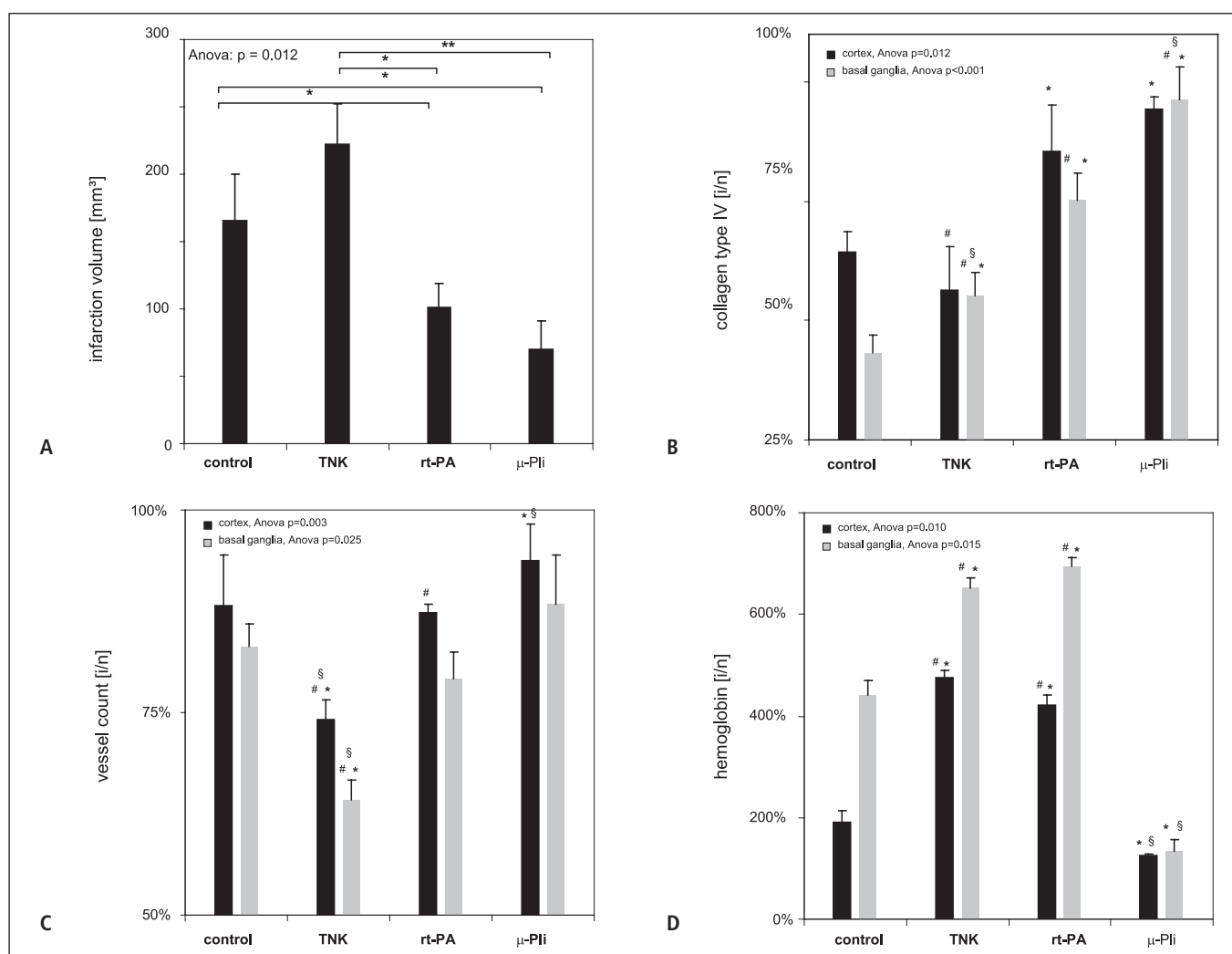


Figure 4: Quantification of microvascular and neurological damage after I/R. A) Effect of administration of TNK, rt-PA and μ -Pli on lesion size following 3 h of ischaemia and 24 h reperfusion in rats. Ischaemic lesion size is expressed in mm³. B) Effect on collagen type IV amount following ischaemia and reperfusion in the cortical and basal ganglia regions. Densitometric analysis of the Western blot analysis. The ratio of the ischaemic to the contralateral area was calculated and expressed as percentage. C) Number of cerebral microvessels

measured by quantification of the results of the immunohistochemistry of the brain sections. D) Haemoglobin extravasation measured by quantification of Western blot results. The ratio between the ischaemic and the non-ischaemic side was calculated separately for the cortical and the basal ganglia regions. Data are expressed as mean value of N=6 experiments (mean \pm SEM). P is calculated by the Students t-test. *: $p < 0.05$ vs. control, **: $p < 0.001$ vs. control, §: $p < 0.05$ vs. rt-PA-treated rats; #: $p < 0.05$ vs. μ -Pli-treated rats; all Anova $p < 0.05$.

the different thrombolytics on the MMP equilibrium could be given with this ratio.

The ratio TIMP-1/MMP-9 was significantly elevated in both the cortex and the basal ganglia after ischaemia (TIMP-1/MMP-9: cortex 1.0 ± 0.1 [sham] vs. 1.2 ± 0.1 [ischaemia]; basal ganglia: 1.1 ± 0.1 vs. 1.4 ± 0.1 ; $p < 0.05$). Thus the TIMP/MMP balance shifted toward inhibition after focal ischaemia. This ratio was significantly changed by the treatment with thrombolytics (Anova: $p < 0.05$). After treatment with TNK, the balance shifted to a value smaller than 1 (cortex: 0.52 ± 0.07 ; basal ganglia: 0.46 ± 0.06); thus, the influence of MMP-9 was greater than its inhibitor TIMP-1 ($p < 0.05$, Bonferroni post-hoc analysis for both cortex and basal ganglia). This effect was also observed with rt-PA, but to a minor though sig-

nificant degree (t-test). As shown in ► Figure 6A, μ -Pli treatment caused a balance between MMP-9 and TIMP-1 (cortex: 1.00 ± 0.2 ; basal ganglia: 1.05 ± 0.05 ; t-test: $p < 0.05$).

The ratio TIMP-2/MMP-2 was also shifted toward the inhibitor side after ischaemia and reperfusion (cortex: 1.2 ± 0.1 [sham] vs. 1.25 ± 0.3 [ischaemia]; basal ganglia: 1.0 ± 0.1 vs. 1.5 ± 0.1 , $p < 0.05$). Both TNK and t-PA caused a shift towards the proteolytic side of the equilibrium protease and inhibitor (TNK: cortex: 0.5 ± 0.2 , basal ganglia: 0.6 ± 0.1 ; rt-PA: cortex: 0.6 ± 0.1 ; basal ganglia: 0.6 ± 0.0). The ratio TIMP-2/MMP-2 after μ -Pli treatment did not significantly differ from that in control animals (cortex: 1.0 ± 0.1 ; basal ganglia: 1.1 ± 0.1 ; $p > 0.05$ post-hoc Bonferroni) (Fig. 6B).

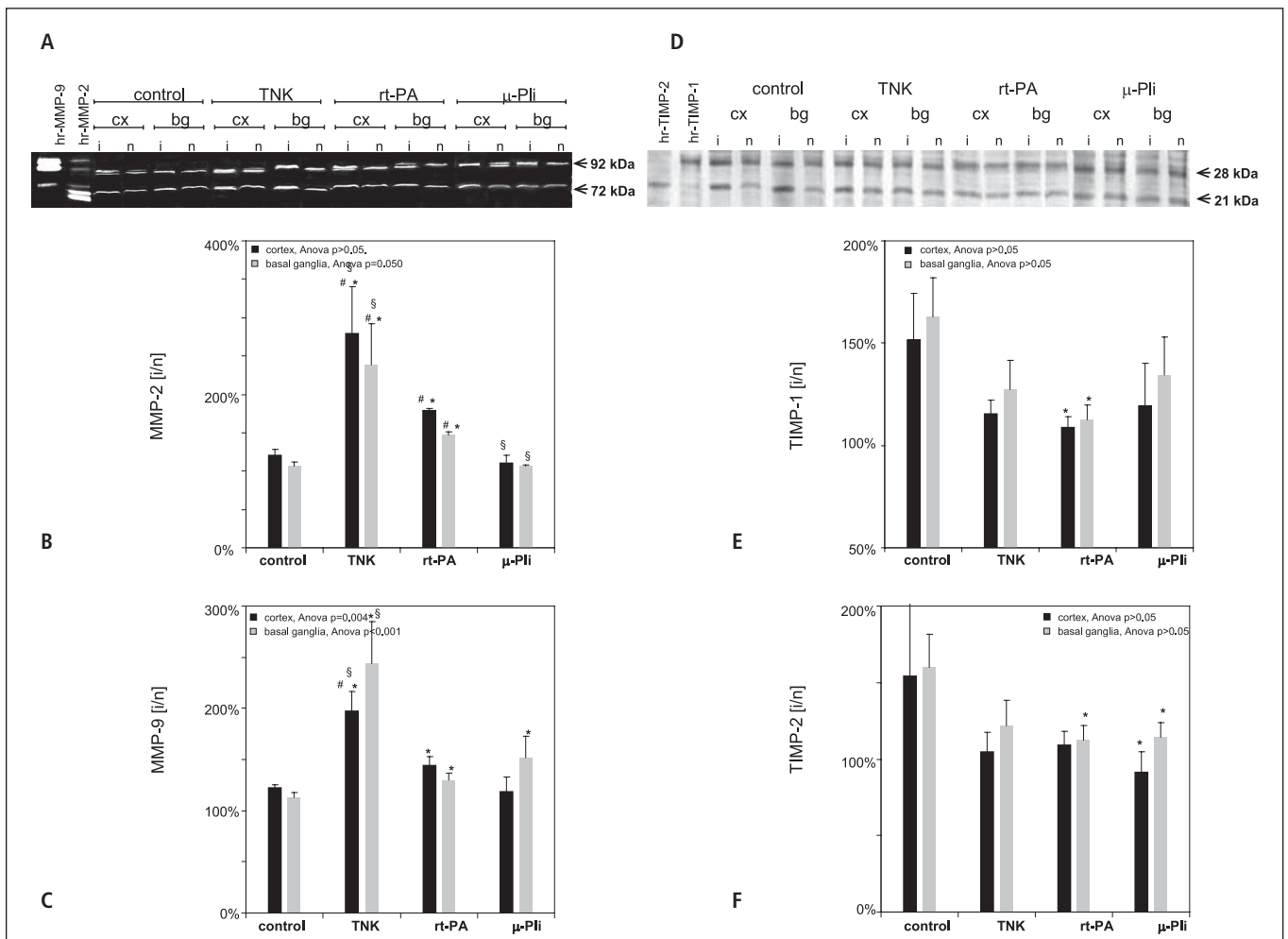


Figure 5: Analysis of components of the MMP-system (MMP-2, -9, TIMP-1, -2) after I/R. A) Gelatine-dependent zymography of protein extracts from ischaemic (i) and non-ischaemic (n) cortex (cx) and basal ganglia (bg) in the control group, the TNK, rt-PA and μ -Pli-treated group after I3R24. MMP-9 and MMP-2 and recombinant human MMP-2 and -9 standards (Sigma, Germany) are shown; B) MMP-2; C) MMP-9; D) Representative reverse zymograms of protein extracts in the control group after I3R24 and the sham operated group without ischaemia are shown as representative

example. Recombinant human TIMP-1 and -2 standards (Chemicon) are shown. Optical density was measured and the ratio between the ischaemic and the non-ischaemic side was calculated separately for the cortical and the basal ganglia region. Data are expressed as mean value of N=6 experiments (mean \pm SEM). P is calculated by the Students t-test. *: $p < 0.05$ vs. control, \S : $p < 0.05$ vs. rt-PA-treated rats; #: $p < 0.05$ vs. μ -Pli-treated rats. Anova is indicated. The calculated ratio ischaemic to non-ischaemic side was the basis for the statistical analysis; E) TIMP-1; F) TIMP-2.

Discussion

This study examined the effects of different thrombolytics, alteplase (rt-PA), tenecteplase (TNK) and microplasmin (μ -Pli) on ischaemic brain damage after transient MCAO in rats. We report here that these agents differ with respect to ischaemic lesion size and microvascular injury.

The main finding was that treatment with μ -Pli had the strongest protective effect on both lesion size and microvascular integrity. Furthermore, ischaemia leads to increased haemoglobin extravasation, and both rt-PA and TNK further increased haemoglobin extravasation significantly compared with control. Microplasmin, however, demonstrated significantly decreased haemoglobin

extravasation compared with both PAs and control. We found no improvement of microvascular parameters such as haemoglobin extravasation, collagen content or vessel number in the ischaemic areas compared with the two other thrombolytics.

The use of rt-PA is currently the only beneficial pharmacological approach to limit injury in stroke patients in the acute treatment phase. The time-dependent benefit of rt-PA treatment has been clearly demonstrated by a meta-analysis of 2,775 patients in the ATLANTIS, ECASS and NINDS trials (21). On the basis of multiple studies of the effects of rt-PA on the neurovascular unit (22, 23), we included the results of the rt-PA group as the basis of comparison in our analysis. Treatment with rt-PA after onset of ischaemia significantly reduced ischaemic lesion and concomitantly

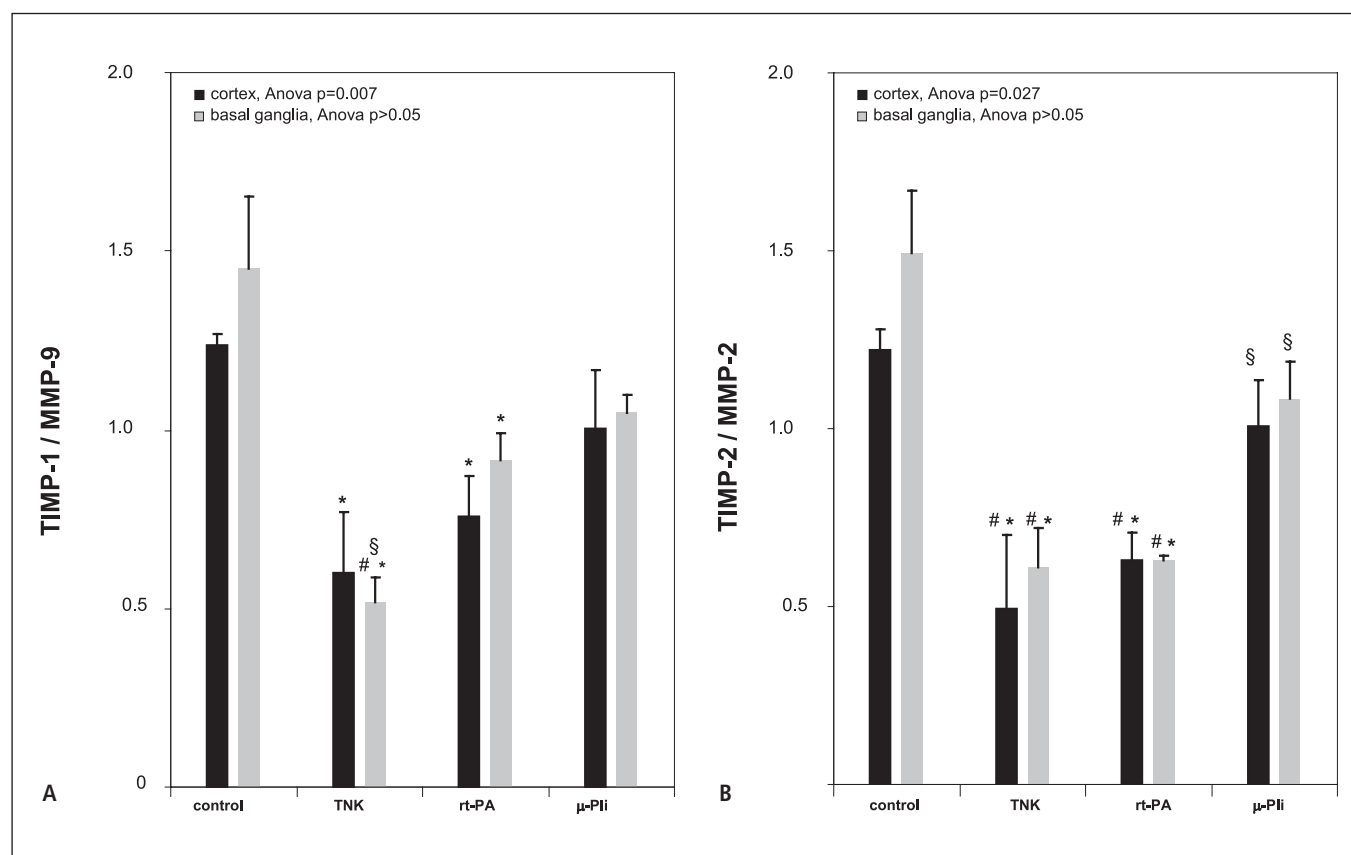


Figure 6: The ratio of TIMP to the preferred MM-Protease is plotted for all groups separately for cortex and basal ganglia. A) Ratio of TIMP-2 to MMP-2; B) of TIMP-1 to MMP-9. Data are expressed as mean value

of N=6 experiments (mean \pm SEM). P is calculated by the Students t-test. *: $p < 0.05$ vs. control, §: $p < 0.05$ vs. rt-PA-treated rats; #: $p < 0.05$ vs. μ -PlI-treated rats; Anova is indicated.

increased microvascular integrity, which is expressed by a conservation of the content of collagen IV. The data concerning the reduction of the ischaemic lesion is in accordance with several groups (24, 25), which also showed the protective effect of rt-PA to the ischaemic lesion size. Controversial data have been obtained concerning these effects (26, 27). The effects of rt-PA remain largely unclear. It has been shown that intravenous injection of tPA in mechanical models of stroke has a beneficial component through degradation of endogenous deposits of fibrin (28, 29). However, rt-PA was shown to have deleterious effects related in part to matrix proteolysis within the neurovascular unit (30). Rt-PA seems to have different effects on the brain tissue and the microvessels. The difference between the observed protection of collagen type IV and the haemoglobin leakage can eventually be attributed to small punctual basal lamina disruptions in the ischaemic region. These disruptions lead to haemoglobin extravasation, which can be quantified by biochemical analysis, even if it is much localised (31). The mechanisms can include different reasons: exogenous rt-PA interfering with the endogenous haemostatic system of the capillary endothelium may contribute to improvement of the microcirculation causing a higher patency rate; further, it may be presumed that secondary clot formation can be prevented.

Rt-PA increases ischaemic brain matrix metalloproteinase levels (23), and there is a close association between MMP-9 up-regulation and haemorrhagic transformation after ischaemia (32). In this study, TIMP-1 and TIMP-2 expression in ischaemic brain tissue was induced after focal ischaemia. This may represent an attempt of an endogenous protection from severe matrix degrading processes by attenuating MMP activation. The increase of MMPs caused by thrombolytics counteracts these effects. Thus the equilibrium TIMP/MMP is shifting towards a value below or approximately 1, as a function of the applied thrombolytic. Rt-PA-treated animals showed significantly lower TIMP levels compared with control ischaemic rats. The equilibrium between the MMP and its corresponding endogenous inhibitor TIMP revealed a shift toward the inhibitory side of this imbalance with rt-PA treatment. This could in part be responsible for the protective effect on the basal lamina component collagen type IV. Microplasin seems to have the best effect in regard to the strengthening of the inhibitory side of the degrading MMP system.

Well-characterised PAs with novel properties include TNK, a modified form of rt-PA with a longer half-life and greater fibrin specificity (33, 34). In our rat model, TNK produced a larger lesion volume compared with rt-PA, and caused more severe collagen type IV degradation and the loss of microvessels. The haemoglo-

bin extravasation was not further enhanced by TNK, but was already increased by factors of 5 and 7 (in the cortex and the basal ganglia, respectively) compared with untreated control animals. In a rabbit, large clot embolism model haemorrhage was also associated with TNK treatment, but was not statistically different compared with controls or the rt-PA group (35, 7). In a pilot study, TNK doses of 0.1, 0.2 and 0.4 mg/kg are safe in regard to intracerebral haemorrhage in stroke patients (36). Its failure to reduce lesion volume of TNK at the dose of 0.5 mg/kg (human dose corresponds to 5mg/kg bw in rats) was also observed by Zhang *et al.* (37). Our experiments showed that after TNK treatment, the proteases of the MMP system were significantly increased compared with controls and animals receiving rt-PA.

The present study included μ -Pli, a truncated form of plasmin, which in addition to its non-lytic-dependent neuroprotective properties also improves behavioural rating scores (38). We explored whether it is a potential microvascular protective agent and additionally we investigated its effect on MMP level.

Our findings indicate that μ -Pli significantly improved the size of the ischaemic lesion in the suture model of ischaemic stroke in rats, confirming the reduced lesion volumes after μ -Pli treatment in a rat embolic stroke model (39) or a photothrombotic model (40). For the first time, we quantified the consequences of μ -Pli on the microvasculature. The degradation of collagen IV after μ -Pli and the vessel count were less diminished than in control and rt-PA-treated animals. Furthermore, μ -Pli treatment caused a lower risk of haemorrhage than both tPAs in our rat model. This is consistent with earlier findings showing that μ -Pli decreased intracerebral bleeding in an embolic MCAO model in mice (39).

To date no studies have been performed on the effects of μ -Pli on the MMP system. We have shown here that μ -Pli has no effect on MMP-2 compared with control animals, neither in the contralateral brain region nor in the ischaemic area. However, MMP-9 was significantly up-regulated in the ischaemic basal ganglia. This up-regulation following ischaemia and reperfusion was not significantly different from the observed effect to MMP-9 in the basal ganglia after rt-PA treatment.

Looking for explanations concerning the increased damage parameter after treatment with TNK, our study suggests that the increase of both gelatinases may be responsible for these effects. The balance of protease to inhibitor, which expresses the net proteolytic activity, gives a better explanation for the observed matrix-degrading reactions. After I3R24 and treatment with TNK, both endogenous inhibitors TIMP-1 and TIMP-2 were decreased. The balance, MMP/TIMP was shifted significantly toward values lower than 1. The equilibrium, in particular TIMP-1/MMP-9, was significantly lower after TNK than after rt-PA treatment.

The prolonged half-time of TNK in the microvasculature (33) could be responsible for an enhanced accumulation of polymorphonuclear leukocytes. As previously shown by others, an accumulation of polymorphonuclear leukocytes occurs during reperfusion injury (41) which may exacerbate tissue damage via various cytotoxic products, e.g. proteases and oxygen-free radicals. Another reason could be the different efficacy of both TNK and t-PA on microvascular obstruction and hypoperfusion. Furthermore,

TNK and rt-PA differ in terms of their platelet-related properties (42).

In the present study, it was apparent that μ -Pli had the best effect on microvascular parameters after ischaemia and reperfusion in this particular rat model. No increased MMPs levels were found after treatment with μ -Pli. As there is a close association with MMP-9 up-regulation and haemorrhagic transformation after acute stroke (32), our MMP data after μ -Pli treatment could explain the improved results concerning haemorrhage and basal lamina damage. Additionally, the ratio between MMP protease and inhibitors is balanced, so that lower amounts degrading proteases spent less time in abundance in the ischaemic microvessels and, therefore, caused less deleterious effects on vascular and parenchymal matrix integrity.

It should be noted, however, that these results are restricted to male animals, thus avoiding gender-specific effects.

In conclusion, at a dose that is bio-equivalent to rt-PA (43), TNK does not seem to be any safer than rt-PA. It is possible that lower doses of TNK may have similar efficacy and may cause less cerebral bleeding than rt-PA. However μ -Pli showed lower haemorrhage rates and better effect on the microvasculature. Future investigations are planned to clarify the dose dependency and mechanisms of MMP activation following μ -Pli treatment. In view of its properties, combined with a better understanding of damage reactions, μ -Pli is a promising candidate for stroke therapy.

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