Research report

Plasminogen activation in experimental permanent focal cerebral ischemia

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Abstract

Background: Previous experimental work using in situ zymography has shown very early increased plasminogen activation in ischemic regions after 3 h of ischemia with and without reperfusion. The objective of the present study was to evaluate the time course and extent of plasminogen activation in long-term permanent focal cerebral ischemia. Material and methods: The middle cerebral artery in male Fisher rats was irreversibly occluded by electrocoagulation. Duration of ischemia was 48, 72, and 168 h. Occlusion was controlled in vivo by MRI at day 2. Plasminogen activation was detected by in situ zymography of 10 μm cryosections with an overlay containing plasminogen and the plasmin substrate caseine. Areas of plasminogen activation were compared to structural lesions (immuno-histochemical loss of microtubule-associated protein 2; MAP 2). Results: Compared to controls, increased plasminogen activation was observed in the basal ganglia and the cortex of the ischemic hemisphere after 48, 72, and 168 h (affected area of basal ganglia: 44.5 ± 21.9, 70.1 ± 2.3 and 70.1 ± 2.8%, respectively; affected area of cortex: 63.4 ± 9.8, 67.7 ± 0.7 and 64.0 ± 3.7%, respectively). The duration of ischemia had no significant influence on the extent of plasminogen activation. Areas of increased plasminogen activation significantly overlapped with and exceeded areas of MAP 2 loss (P<0.005). Discussion: Permanent focal cerebral ischemia leads to increased plasminogen activation in ischemic regions. This plasminogen activation remains elevated at persistent levels over days. It may contribute to extracellular matrix (ECM) disruption, secondary hemorrhage, and brain edema in subacute stages of ischemic stroke. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Intravenous thrombolysis with tissue type plasminogen activator (t-PA) was shown to improve outcome in acute ischemic stroke, when therapy was started in the first 3 h after symptom onset [25]. However, the positive effect of early recanalisation may be in part outweighed by an increased rate of intracerebral hemorrhage. In an European study, the rate of symptomatic intracerebral hemorrhage in the t-PA-treated group was 19.8% compared to 6.5% in the placebo group [7].

Possible mechanisms of plasmin-mediated intracranial bleeding include extracellular matrix (ECM) degradation with consecutive vessel leakage, extravasation, and petechial or symptomatic hemorrhage [6]. The ECM components laminin, fibronectin, and collagen IV were shown to be reduced in ischemic regions after focal cerebral ischemia and reperfusion [8]. Using antibodies against these ECM components and hemoglobin, extravasation of blood components could be demonstrated around
leaking vessels by immunofluorescence double staining techniques [9].

Plasmin was shown to directly degrade ECM components such as laminin and fibronectin [14,15]. Besides direct plasmin effects, activation of matrix metalloproteinases (MMPs) by plasmin may play a crucial role in ischemia-related ECM degradation. Pro-MMP 9 was shown to be converted to its active form (MMP 9, gelatinase B) in the presence of plasmin [17]. In accordance with this study, pro-MMP 9 activation was reduced in plasminogen deficient knock-out mice [13]. In a cell line experiment, pro-MMP 2 activation was inhibited by plasmin inhibitors, suggesting a role of plasmin in the conversion of pro-MMP 2 to its active form (MMP 2, gelatinase A) as well [2].

The role of MMPs in focal cerebral ischemia has been addressed in several studies. In experimental focal cerebral ischemia and reperfusion in primates, MMP 2 was increased after 1 h of middle cerebral artery occlusion (MCAO) and remained elevated for at least 7 days [10]. A similar increase was observed for MMP 9 in subjects with hemorrhagic transformation [10]. A clinical study in humans revealed increased MMP 2 and 9 levels in the cerebrospinal fluid of patients with acute stroke. These levels correlated with the extent of peri-infarct edema [3]. In a post-mortem study, MMP 2 and 9 were increased in infarcted brain regions 2 days and several months after stroke [5]. These studies suggest detrimental effects of MMP 2 and 9 in cerebral ischemia such as brain edema and secondary hemorrhage.

Little is known about plasminogen activation in focal cerebral ischemia. Investigations in mice showed increased plasminogen activation in infarcted regions after 2 h of focal cerebral ischemia [27]. In previous experiments in rats, plasminogen activation was increased in regions of structural damage after 3 h of focal ischemia and different reperfusion intervals [19]. The time course of plasminogen activation in subacute stages (days 2–7) of focal permanent cerebral ischemia has not yet been investigated. However, it may play a role in subacute hemorrhage and edema formation after ischemic stroke.

To investigate plasminogen activation in long-term ischemia we used a rat model with permanent MCAO for 2–7 days.

2. Materials and methods

2.1. Animal model

All animal experiments were performed after ethical approval by the responsible government institution (the ‘Kantonales Veterinäramt’, Basel, Switzerland, reference number 1365). In accordance with these standards, every effort was made to reduce the number of animals used and to ensure that they were free of pain and discomfort.

Experiments were performed in male Fisher 344 rats (Ifa Credo, France) weighing 220–300 g. Animals were housed under standard conditions with free access to rat chow and tap water before and after surgery. Irreversible occlusion of the right middle cerebral artery was performed as described previously [22,24]. Briefly, animals were anesthetized with 2% isoflurane in a 70/30 (v/v) nitrous oxide/oxygen mixture and, using an operating microscope (Wild), the right MCA was exposed by a subtemporal craniectomy. The artery and its lenticulostriate branches were then occluded by bipolar electrocoagulation. Afterwards, retracted soft tissues were replaced, wounds were sutured, anesthesia was discontinued, and the rats were put back into their cages. Body temperature was maintained at 37°C by means of a rectal probe connected to a heating pad (CMA 150, Carnegie Medicine) during surgery and until animals regained consciousness. Thereafter, rectal temperature was checked frequently (every 10–15 min) during the following 2 h and, if necessary, it was corrected to 37°C using a heating pad which was placed under the cage. After this, animals were returned to their home cages and allowed free access to food and water.

In order to verify successful MCAO and to visualize the lesioned territory all brains were investigated by NMR imaging after 48 h of permanent ischemia. MRI investigations were performed on a Bruker DBX 47/30 (4.7 T) equipped with an actively shielded gradient insert (max. 2.2. Materials and methods

2.2. Preparation of cryostat sections

Cryostat sections of 10 μm thickness were taken from regions 0–1 mm behind the bregma [18], were prepared at −20°C, and stored at −80°C. To ensure comparability,
adjacent sections were examined for MAP 2 immunohistochemistry and zymographic plasminogen activation.

2.3. Zymographic detection of plasminogen activation

The method was described previously [21]. After thawing and drying at room temperature, sections were incubated with an overlay consisting of commercial non-fat milk powder (containing the plasmin substrate casein), agarose, and human plasminogen. The overlay was prepared in several steps. Three different solutions were prepared. Solutions 1 (100 ml PBS + 24.6 mg MgSO\textsubscript{4} + 13.2 mg CaCl\textsubscript{2}) and 2 (10 ml dH\textsubscript{2}O + 1.6 g commercial non-fat milk powder) were separately heated to 50°C, solution 3 (10 ml dH\textsubscript{2}O + 250 mg agarose) to 70°C. The solutions were mixed (0.75 ml of solution 1, 0.70 ml of solution 2, 0.50 ml of solution 3), and the mixture cooled to 37°C. Then 100 µl of plasminogen (1.5 mg/ml) were added. This mixture was evenly spread over the heated sections (37°C) and immediately covered by a coverslip. After incubation at 37°C for 22 h the sections were immediately inspected for zones of overlay lysis and digitally scanned for later computer analysis. The incubation period of 22 h was chosen, since plasmin-mediated lysis had then reached a maximum. Longer incubation periods up to 36 h showed no further expansion of lytic regions. To exclude unspecific overlay lysis, control sections of all groups were incubated with an overlay lacking plasminogen.

2.4. Immunohistochemistry

For MAP 2 detection, a monoclonal mouse antibody (Boehringer Mannheim, dilution 1:800, incubation for 2 h at 37°C, followed by 24 h at 4°C) was used. Before incubation, sections were fixed in a 1:1 mixture of acetone and chloroform, washed in phosphate buffer solution (PBS), and incubated with blotto (50 g non-fat dry milk, 10 ml horse serum, 0.3 mmol/l sodium azide diluted in Tris–saline stock (38.5 mmol/l Tris, 150 mmol/l NaCl)) to reduce unspecific binding. After repeated washing in PBS, the sections were incubated with the secondary biotinylated antibody (horse, anti-mouse, Vector Laboratories, dilution 1:200, incubation period 2 h at 37°C). This was followed by blocking endogenous peroxidase with H\textsubscript{2}O\textsubscript{2} for 20 min. Thereafter sections were incubated with the ABC-Complex (Vectastain-Elite-Kit, Vector Laboratories, incubation 30 min at 37°C). Peroxidase signal was developed with AEC (AEC-Kit, Biomedica). Nuclei were counterstained with Mayer’s hematoxylin (Sigma), and tissue was blued in saturated sodium bicarbonate.

2.5. Morphometric analysis

To evaluate areas of visible plasminogen activation and decreased MAP 2 antigenicity, a morphometric program (Optimas 6.5, Media Cybernetics, MD) was used. Areas of plasmin-mediated lysis and MAP 2 loss were measured and expressed as percent of the total ischemic hemisphere. Affected areas were also evaluated separately for the basal ganglia and the cortex (Fig. 1). To minimize the error of spatial resolution, adjacent brain sections were evaluated for zymography and MAP 2 immunohistochemistry. Corresponding MRI images were selected from the individual MRI series to illustrate overlapping of MRI changes with overlay lysis and MAP 2 loss. This selection was guided by a rat brain atlas [18].

2.6. Statistical analysis

For statistical evaluation, data were presented as means.

![Fig. 1](image)
and standard errors of mean (S.E.M.). To analyse the time course of overlay lysis and MAP 2 loss non-parametric testing (Kruskal–Wallis, Mann–Whitney) was performed. To compare areas of overlay lysis with areas of MAP 2 loss in individual sections the Wilcoxon matched-pairs signed-ranks test was used.

3. Results

3.1. Lesions detected by MRI

In all animals, MRI showed clearly delineated lesions in the ischemic hemisphere after 48 h of MCAO (Fig. 2). While the cortex was consistently affected in all animals, the basal ganglia were spared in one animal of group 1 (48 h of ischemia).

3.2. Plasminogen activation in controls

Sections without plasminogen in the overlay showed no zones of lysis (Fig. 1). Using overlays containing plasminogen, limited areas of ‘background’ overlay lysis were seen in the non-ischemic (control) hemisphere. This lysis uniformly occurred in two different patterns: first, small, pin-point-shaped regions with no preference for distinct anatomical structures; secondly, a circular rim of lysis following pial structures around the section (Fig. 2). In all individual animals, this ‘background’ lysis affected less than 20% of the cortex and less than 10% of the basal ganglia of the control hemisphere.

3.3. Plasminogen activation in ischemic regions

Additional extensive plasminogen activation beyond ‘background’ lysis was observed in the basal ganglia and cortical regions of the ischemic hemisphere as large coherent zones of overlay lysis (Fig. 2). After 48, 72, and 168 h of ischemia, the affected area in the basal ganglia was $44.5\pm21.9$, $70.1\pm2.3$ and $66.6\pm2.8\%$, respectively (Fig. 3). The affected area in the cortex measured $63.4\pm9.8$, $67.7\pm0.7$ and $64.0\pm3.7\%$, respectively (Fig. 3). No significant differences in the time course of permanent ischemia from 48 to 168 h were observed. The relatively

![Image](https://example.com/fig2.png)

Fig. 2. MRI hyperintensity, plasminogen activation, and MAP 2 loss at different time points of permanent middle cerebral artery occlusion (MCAO): ‘Background overlay lysis’ is presenting as small pin-point shaped zones of lysis (single arrow) and a circular rim of overlay lysis surrounding the sections. Note the overlapping of MRI lesion, plasmin-mediated overlay lysis, and MAP 2 loss in the ischemic hemisphere. Also note areas of plasminogen activation exceeding areas of MAP 2 loss (double arrows).
Fig. 3. Areas of MAP 2 loss (left column) and plasminogen activation (right column), presented as percent of total basal ganglia and cortex area of the ischemic hemisphere ± standard error of mean. The area of overlap between MAP 2 loss and plasminogen activation is indicated by hatched lines. In the area of MAP 2 loss this overlap is almost complete and significantly higher than expected if areas were randomly distributed (Wilcoxon; P < 0.005). The area of plasminogen activation exceeds the area of structural injury (Wilcoxon; * indicates significance at P < 0.05 for single groups; lack of significance in the other groups may be due to low animal number; for all groups combined: P < 0.005). Duration of ischemia (h) has no influence on the size of affected areas.

Areas showing MAP 2 loss were almost completely affected by increased plasminogen activation (Figs. 2 and 3). Comparing individual sections, this colocalisation was significantly higher than expected if areas were randomly distributed (Wilcoxon; P < 0.005). On the other hand, increased plasminogen activation was not merely restricted to zones with detectable structural injury (Fig. 2). In the basal ganglia and the cortex of the ischemic hemisphere, the area of increased plasminogen activation exceeded the area of MAP 2 loss (Wilcoxon; P < 0.005). Separated for different time points, this excess was significant for the basal ganglia at 72 h and for the cortex after 72 and 168 h (Wilcoxon; P < 0.05; Fig. 3).

4. Discussion

Our results demonstrate increased plasminogen activation in the basal ganglia and the cortex following permanent focal cerebral ischemia. This increase was limited to the ischemic hemisphere. Comparing different time intervals of permanent ischemia (48, 72, 168 h), no significant differences in the extent of plasminogen activation could be detected.

In a previous study, we found increased plasminogen activation as early as 3 h after onset of ischemia in ischemic regions [19]. In that study, the extent of plasminogen activation beyond background lysis after 3 h of ischemia was restricted to about 5 and 20% of total basal ganglia and cortex area, respectively. In the present study with ischemic intervals between 2 and 7 days, up to 70% of the respective areas showed increased plasminogen activation, which may suggest an expansion of the areas of plasminogen activation between 3 and 48 h. However, this point remains speculative, since different rat strains (Wistar versus Fisher rats) and occlusion techniques (intraluminal thread versus electrocoagulation) were used in the two studies.

In the present study, one animal of group 1 (48 h of ischemia) showed no major overlay lysis in the basal ganglia. This lack of plasminogen activation corresponded to basically normal MAP 2 immunohistochemistry and lack of major MRI changes in the basal ganglia. It is known from the used rat model, that, regarding MRI alterations due to ischemia, the basal ganglia can be spared in rare cases [24]. The lack of basal ganglia affection in one animal explains the low mean and high variability for area quantification of plasminogen activation and MAP 2 loss in group 1 (Fig. 3).

In all groups, areas with structural injury (loss of MAP 2 antigenicity) constantly showed increased plasminogen activation. Beyond that, the area of increased plasminogen activation exceeded the area of structural injury. This exceeding plasminogen activation was mainly located around the border zone of the structural injury and may therefore simply represent diffusion of proteolytic agents (plasmin, plasminogen activators) into the casein overlay. In some cases, however, areas of increased plasminogen activation in the ischemic hemisphere had no contact to and were remote from areas of structural injury (as demonstrated in Fig. 2). This suggests that plasminogen activation in focal ischemia does not necessarily depend on structural injury. Plasminogen activation and structural damage may both be independent consequences of focal ischemia. Alternatively, a cause-and-effect relationship with increased plasminogen activation leading to the
aggravation of structural injury after focal ischemia may be considered. One previous study supports this interpretation: Wang and co-workers found a decrease in infarct size after experimental focal ischemia in t-PA-deficient mice, suggesting negative effects of plasminogen activation on structural integrity [27]. However, these findings could not be confirmed by intervention experiments with exogenous t-PA [11,12,16]. Supposing a negative effect of plasminogen activation on structural integrity, an expansion of the structural injury into areas with excess plasminogen activation over time should be expected. In our experiments, however, the area of increased plasminogen activation compared to MAP 2 loss remained stable over the time course of permanent ischemia.

The areas of increased plasminogen activation were detected by histological in situ zymography. Using an overlay lacking plasminogen, no lysis was seen. Overlay lysis in the presence of plasminogen depended on the presence of brain tissue. Therefore, overlay lysis unquestionably represented endogenous plasminogen activator-mediated proteolysis. Two limitations of the method have to be mentioned: first, in situ zymography does not reveal information about the degree of activation. Second, in situ zymography does not allow to differentiate between different PAs. From other studies, limited data regarding the roles of different PAs in plasminogen activation following focal cerebral ischemia are available. A possible role of urokinase type plasminogen activator (uPA) in plasminogen activation following focal cerebral ischemia was suggested by a study of Rosenberg et al., that found an increase of uPA in the ischemic hemisphere of spontaneously hypertensive rats after 12 and 24 h and 5 days of permanent MCAO but a decrease of t-PA after 12 and 24 h [20]. These results have not yet been confirmed in normotensive rats.

As stated in Section 1, increased plasminogen activation to plasin may effect the ECM in two ways. First, plasin directly degrades ECM components. Secondly, it is involved in the activation of pro-MMP 2 and 9 to their active forms, the collagen IV degrading gelatinases A and B. Besides its ECM disrupting properties with consecutive microvascular injury, the PPS may also be involved in neuronal damage. This was demonstrated in experiments with knock-out mice, where t-PA deficiency protected against neuronal damage provoked by excitatory amino acids [26] and focal ischemia [27]. As in PPS-mediated microvascular injury, this neuronal damage also seems to depend on ECM disruption. In experiments with t-PA deficient mice, the blocking of the ECM component laminin with specific antibodies restored excitotoxic neuronal sensitivity. This suggests that the disruption of the neuron ECM interaction (by PPS-mediated proteolysis or blocking of laminin) substantially contributes to t-PA-mediated neuronal vulnerability [4].

The PPS may not only be affected by t-PA or other PAs. It may also be indirectly influenced by plasminogen activator inhibitors (plasminogen activator inhibitors 1 and 2; PAI 1 and 2). Supposing stable PA activity, decreased PAI activity should lead to increased overall plasminogen activation. Shatos et al. found decreased levels of PAI 1 after exogenous exposure to t-PA in human cell line experiments, suggesting synergistic effects of increased PA and decreased PAI activity [23]. Regardless of the exact mechanisms and the enzymes involved, a shift towards increased plasmin activity is likely to affect ECM integrity. Our experiments demonstrate this ongoing and stable imbalance at subacute stages of permanent ischemia. Potential beneficial effects (maintenance of microvascular flow, capillary remodeling) may be outweighed by brain edema and cerebral hemorrhage due to PPS-mediated ECM disruption.

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