Rapid Differential Endogenous Plasminogen Activator Expression After Acute Middle Cerebral Artery Occlusion

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Background and Purpose—During focal cerebral ischemia, the microvascular matrix (ECM), which participates in microvascular integrity, is degraded and lost when neurons are injured. Loss of microvascular basal lamina antigens coincides with rapid expression of select matrix metalloproteinases (MMPs). Plasminogen activators (PAs) may also play a role in ECM degradation by the generation of plasmin or by MMP activation.

Methods—The endogenous expressions of tissue-type plasminogen activator (tPA), urokinase (uPA), and PA inhibitor-1 (PAI-1) were quantified in 10-μm frozen sections from ischemic and matched nonischemic basal ganglia in the plasma of 34 male healthy nonhuman primates before and after middle cerebral artery occlusion (MCA:O).

Results—Within the ischemic basal ganglia, tissue uPA activity and antigen increased significantly within 1 hour after MCA:O (2P<0.005). tPA activity transiently decreased 2 hours after MCA:O (2P=0.01) in concert with an increase in PAI-1 antigen (2P=0.001) but otherwise did not change. The transient decrease in free tPA antigen was marked by an increase in the tPA–PAI-1 complex (2P<0.001). No significant relations to neuronal injury or intracerebral hemorrhage were discerned.

Conclusions—The rapid increase in endogenous PA activity is mainly due to significant increases in uPA, but not tPA, within the ischemic basal ganglia after MCA:O. This increase and an increase in PAI-1 coincided with latent MMP-2 generation and microvascular ECM degeneration but not neuronal injury. (Stroke. 2001;32:1341-1348.)

Key Words: basal ganglia ■ cerebral ischemia, focal ■ microcirculation ■ plasminogen activators ■ tissue plasminogen activator ■ baboons

Focal cerebral ischemia is responsible for a loss of microvascular integrity, manifested by increased endothelial cell permeability and loss of basal lamina matrix antigens. After experimental middle cerebral artery (MCA) occlusion (MCA:O) and during reperfusion (MCA:O/R), the major basal lamina constituents, laminin, collagen IV, and cellular fibronectin, decrease roughly in parallel, in association with microvascular hemorrhage. Loss of basal lamina integrity coincides with a rapid increase in the expression of latent matrix metalloproteinase (MMP)-2 in the ischemic core (Ic) in a nonhuman primate MCA:O model. Plasminogen activators (PAs) may also play several roles in cerebral ischemic injury to facilitate degradation of basal laminal ligands. Collagen, laminin, fibronectin, elastin, and/or myelin basic protein are degraded either directly by plasmin or by select activated MMPs requiring PA expression. The disruption of normal cell-matrix adhesion has been associated with cell death, edema, loss of cell viability, and hemorrhage in several systems. Latent MMP-9 expression is associated with hemorrhagic transformation in the nonhuman primate, but the nature of its activation remains uncertain. A contribution of plasmin, via PA generation, to pro-MMP-9 activation in the ischemic parenchyma is a potential mechanism. Some or all of these PA-dependent effects could contribute to central nervous system (CNS) injury. However, during focal cerebral ischemia, the tissue-related contributions of PAs to cerebral vascular and neuronal injury and recovery processes have not been detailed.

PA activities have been described in the normal rodent brain. Recent reports have indicated that tissue-type PA (tPA) or urokinase (uPA) may increase in the rodent brain after experimental MCA:O. Increased PA activity was detected within the caudate putamen after MCA:O in mice. But, there has been considerable disagreement about the nature of the PA responses to MCA:O in rodents and their significance. Although uPA activity appears to be increased in several studies, there is no agreement concerning the responses of tPA-like proteinase activity to MCA:O. Wang et al have suggested that increases in tPA-like proteinase activity may contribute to excitotoxic neuronal damage in mice. Therefore, the activation of the PA system during focal cerebral ischemia requires further scrutiny. Furthermore, the tissue content of the PAs and their principal inhibitor must be separated from the contributions of plasma.
We hypothesized that the upregulation of tissue-related tPA and/or uPA must occur rapidly to initiate the loss in microvascular integrity and accompany the changes in microvascular integrin receptor expression and neuronal injury. Increased PA expression would suggest a mechanism for pro-MMP-9 activation, whereas a linear association of increased PA expression with neuronal injury would tie the PA to neurodegeneration. We demonstrate rapid significant increase in PA expression with neuronal injury would tie the PA pro-MMP-9 activation, whereas a linear association of increased PA expression with neuronal injury would tie the PA to neurodegeneration. The sources of these PA-PAI axis components appear distinctly separate.

Materials and Methods

The experimental procedures used to obtain the tissues for the present study were approved by the institutional Animal Research Committee and were performed according to standards published by the National Research Council (Guide for the Care and Use of Laboratory Animals) and the US Department of Agriculture Animal Welfare Act. Every effort was made to ensure that the animals were free of pain and discomfort. All experiments and procedures were attended by at least 1 institutional veterinarian, members of the primate handling staff, research associates, and the principal investigator.

Experimental Stroke Model

Cerebral tissues from 34 adolescent male baboons (Papio anubis/cynocephalus) were used for the present study. The procedures for development of the MCA:O/R stroke model have been detailed in previous studies. All animals were allowed a 7-day procedure-free interval after transorbital implantation of an eccentric balloon compression device around the proximal MCA. Twenty baboons underwent MCA:O for 1 hour (n = 5), 3 hours (n = 5), or 24 hours (n = 5) pro-MMP-9 activation, whereas a linear association of increased PA expression with neuronal injury would tie the PA to neurodegeneration. We demonstrate rapid significant increases in uPA and PA inhibitor (PAI)-1, but not tPA, in the parenchyma of the ischemic basal ganglia very early after MCA:O. The sources of these PA-PAI axis components appear distinctly separate.

Blood Sampling

Blood samples were taken from the peripheral vein into tubes containing heparin or EDTA at various times and centrifuged at 3000 rpm for 20 minutes, and the plasmas were stored at −80°C. Preliminary experiments determined the optimal conditions for homogenization and activity extraction.

Plasminogen-Gelatin Zymography

Plasminogen-gelatin zymography was performed by a modification of published methods. All tissue samples (10 μg protein) were resolved under nonreducing conditions on 8% SDS-polycrylamide gels containing 0.01 U/mL plasminogen and 1.5 mg/mL gelatin (Sigma). For standardization, 10 pg of human melanoma tPA (Sigma) and 10 pg of recombinant human uPA (a kind gift of Drs Andrew P. Mazar, Attenuon, San Diego, Calif, and Don Eisenhauer, Abbott Laboratories, Chicago, Ill) were loaded onto each gel. After electrophoresis, the gels were washed 3 times in 2.5% Triton X-100 solution and then incubated in 100 mmol/L Tris-HCl (pH 8.2) buffer for 18 hours at 37°C. The gels were stained with 0.1% amido black solution. On destaining, gelatinolytic activity was demonstrated as clear zones (Figure 2).

Tissue Processing and Sample Extracts

Experiments were terminated under thiopental Na anesthesia by left ventricular transcardiac perfusion at 180 to 200 mm Hg with chilled perfusate containing heparin (200 IU/L), nitroprusside (1 mg/L), and BSA (50 g/L) (Sigma Chemical Co). The perfused brains were subdivided into aliquots and stored at −80°C. Preliminary experiments determined the optimal conditions for homogenization and activity extraction.

Immunoblotting

Samples (100 μg protein per lane) were separated under nonreducing conditions on 8% SDS-polycrylamide gels and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell Inc) by the semidry blotting method. Blocking with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) was followed by incubation with the primary antibody for 1 hour at 37°C and with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG antibody (Santa Cruz Biotechnology Inc or Vector Laboratories, Inc) at 20°C for 1 hour. After each incubation, the blots were washed in PBST to remove unbound antibody. Bound antibody was detected by enhanced chemiluminescence (NEN Life Science Products Inc).

Figure 1. Time course of protein content changes in ischemic and nonischemic basal ganglia after MCA:O.
Quantification of Immunoblotting and Zymography

Immunoblot films and zymography gels were scanned by using a Personal Densitometer SI (Molecular Dynamics Inc) under the mode of 12 bits per pixel digital resolution and 50-μm pixel size and quantified by NIH Image 1.61 on a Macintosh platform. Each measurement was calibrated with a step tablet (Kodak Scanner Step Tablet, ST-34). The area under the densitometry curve is expressed as the integrated density (IDA).

Intragel and Intergel Reproducibility

Preliminary plasminogen zymography experiments with human melanoma tPA and recombinant human uPA (from 1 to 1000 pg) demonstrated that the technique could detect as little as 1 pg of each

Figure 2. Representative plasminogen-gelatin zymograms of the ischemic (R) and nonischemic (L) basal ganglia display clear evidence of uPA (54 kDa), tPA (61 kDa), and tPA–PAI-1 complex (105 kDa) activity. Sample incubation with respective antibodies (Abs) deleted the corresponding gelatinolytic band(s) (see text) (A). Also shown are time-course studies of PA activities from 3 animals each: no ischemic challenge (B), 2 hours after MCA:O (C), and 24-hour reperfusion after 3 hours of MCA:O (D). The uPA activity band, but not the tPA band, increased 2 hours after MCA:O.

Figure 3. Time course of changes in uPA activity (A) and antigen (B), tPA activity (C) and antigen (D), and PAI-1 antigen (E) in ischemic and nonischemic basal ganglia and plasma. At each time point, uPA activity and antigen and PAI-1 antigen increased within 1 hour after MCA:O in the ischemic basal ganglia. In contrast, tPA activity, but not antigen, decreased transiently at 2 hours after MCA:O.
PA. The log (integrated density) for each sample was linear with respect to the log (PA content) \((r=0.992\) and \(2P<0.0001\) for tPA, \(r=0.988\) and \(2P<0.0001\) for uPA). The PA activities from both brain and plasma samples were all within that range. The intragel reproducibility for tPA and uPA were quite acceptable, with coefficients of variability of 2.3% \((n=8)\) and 5.4% \((n=8)\), respectively, by use of 10 pg of each PA. The intergel reproducibility of tPA and uPA was satisfactory, providing coefficients of variability of 11.7% and 11.0%, respectively \((n=6\) gels each).

**dUTP Incorporation and Region of Cellular Injury**

Evidence of nuclear DNA scission/repair was taken as an indication of significant cellular injury, as previously described.\(^{19}\) Incorporation of digoxigenin-dUTP on 10-μm cryosections was detected by the DNA polymerase I-based procedure. The region containing cells with nuclear dUTP incorporation \((dUTP^*)\) was defined as the Ic. All activities were normalized for the Ic region. The validity of this approach was confirmed by immunohistochemistry of adjacent sections \((\text{D.-I. Chang, G.J. del Zoppo, unpublished data, 2001})\). The absolute numbers and densities of total dUTP cells and dUTP neurons were determined within a 1.5-mm\(^2\) region of interest centered within the Ic by computer-assisted quantitative video-imaging microscopy.

**Enzyme-Linked Immunosorbent Assays**

tPA, uPA, and PAI-1 antigen contents in brain tissue extract and plasma were determined by a commercially available ELISA kit \((\text{American Diagnostica Inc})\). Assays were performed according to the manufacturer’s instructions. Antigen concentrations were calculated in nanograms per milligram protein.

**Analysis of Data**

All data are presented as mean±SD. Differences in the time courses of molecules between ischemic and matched nonischemic brain samples were assessed by using 2-way ANOVA, with Bonferroni corrections for multiple comparisons. One-way ANOVA was used for comparison between ischemic and nonischemic animals or between hemorrhagic and nonhemorrhagic animals. Associations between PA (activity and antigen) and ischemic injury were performed by means of linear regression. Jackknifed standard errors \((\text{SEs})\) of reported correlation coefficients are provided for assessment of the precision of the estimated correlations. Significance was set at \(2P<0.05\).

**Results**

**Zymographic Characterization of uPA, tPA, and PAI-1 Expression**

Plasminogen-gelatin zymographic studies displayed clear evidence of tPA and uPA activity within 10-μm frozen sections from both ischemic and nonischemic basal ganglia \((\text{Figure 2})\). Three bands \((105, 61, \text{and } 54\text{ kDa})\) were identified in all plasma samples, which were inhibited by phenylmethylsulfonyl fluoride but not the cysteine protease inhibitor E-64. No proteolytic bands were seen by 2 hours after MCA:O in the ischemic basal ganglia compared with the nonischemic regions \((\text{Figure 3C and 3E})\). A transient increase in tPA and PAI-1 antigen and activity, and PAI-1 antigen between animals with and without ischemic injury. tPA activity and antigen and PAI-1 antigen were significantly greater in the Ic region of the animals undergoing MCA:O than in those without ischemic injury. Symbols represent individual animals from each time point. \(^{*2P<0.01}\) vs nonischemic basal ganglia.

**uPA Expression During MCA:O**

Parenchymal uPA activity and antigen increased significantly within 1 to 2 hours after MCA:O in the ischemic basal ganglia \((2P<0.005)\) \((\text{Figures 2, 3A, and 3B})\). Both uPA activity and antigen were significantly greater in the Ic regions than in identical nonischemic tissues \((2P<0.001)\) \((\text{Figure 4})\). No difference in uPA activity or antigen was observed between the sham-operated and control animals.

In the plasma, uPA activity, but not antigen, demonstrated a small but significant increase early after MCA:O \((2P<0.05)\) \((\text{Figure 3A and 3B})\). Plasma uPA antigen content did not change appreciably over the time from MCA:O.

**tPA and PAI-1 Expression During MCA:O**

A significant increase in PAI-1 antigen \((2P=0.001)\) and a decrease in free tPA activity \((2P=0.01)\) were seen by 2 hours after MCA:O in the ischemic basal ganglia compared with the nonischemic regions \((\text{Figure 3C and 3E})\). A transient increase in the tPA–PAI-1 complex and a coordinate decrease in unbound tPA at 2 hours after MCA:O \((2P<0.001)\) explained these changes in the ischemic tissue \((\text{Figure 5})\). Total paren-
chymal tPA antigen content remained unchanged throughout MCA:O/R (Figure 3D), and both tPA antigen and activity in the Ic region were not different in control and sham-operated animals ($P > 0.55$) (Figure 4). But PAI-1 antigen in the Ic region in all ischemic animals increased significantly ($P < 0.003$).

In plasma, only the gelatinolytic activity of the tPA–PAI-1 complex was detected. Plasma PAI-1 antigen increased significantly ($P < 0.05$) after MCA:O, in parallel with the increase in PAI-1 antigen in the ischemic basal ganglia (Figure 3E).

PA Expression and Neuronal Injury

The relations of uPA activity/antigen, tPA activity/antigen, and PAI-I antigen content to the density of dUTP$^+$ cells and neurons at 2-hour MCA:O were examined. $^{3,10}$ No significant linear relationship between the contents of each PA or PAI and the density (number per unit area) of dUTP$^+$ neurons was observed: uPA activity/antigen ($r=0.739$, SE=0.257, $2P=0.093$); tPA activity/antigen ($r=0.218$, SE=0.539, $2P=0.678$); and PAI-1 antigen ($r=0.687$, SE=0.232, $2P=0.132$). However, a weak association with uPA content might exist. This is because of the large SE in the uPA relationships.

Association of PAs With Hemorrhagic Transformation

At least 1 animal at each time point, 15 in total (44.1%), demonstrated visible evidence of hemorrhagic transformation within the Ic region after MCA:O. No significant differences in tPA, uPA, and PAI-1 contents between the animals that displayed hemorrhagic transformation and those without hemorrhagic transformation after MCA:O were detected (Figure 6).

Discussion

Plasmin, in addition to dissolving fibrin-containing thrombi and fibrinogen, is capable of degrading selected matrix proteins of the basal lamina, elastin, and myelin basic protein either directly or through the activation of select latent MMPs. $^{4-8}$ The ability of PAs to alter microvascular matrix integrity in response to focal cerebral ischemia has so far not been clearly defined. $^{13,14,23}$ The initial step is to examine the effect of MCA:O on the PA/PAI axis within the brain parenchyma. From these experiments and those of others, $^{12}$
there appears to be no zymographic evidence of free plasmin in normal brain tissue. We demonstrate an immediate significant increase in gelatin-proteolytic activity after MCA:O that was mainly due to significant increases in uPA within the ischemic basal ganglia. Parallel increases in PAI-1 were reflected by an increase in tPA–PAI-1 complex formation and a transient decrease in tPA activity in the same territory. uPA antigen and activity and tPA-associated PAI-1 antigen (assigned to the lc region) increased significantly during focal cerebral ischemia compared with no ischemic injury. However, no relation to neuronal injury was evident. Furthermore, there was no significant difference among the animals that displayed hemorrhagic transformation and those without hemorrhagic transformation with respect to uPA and tPA/PAI-1.

The extracellular matrix provides a substrate for the endothelial cell and astrocyte components of the cerebral microvasculature, contributing a second element of the blood-brain barrier. Intact microvascular basal lamina and integrin-mediated matrix adhesion are required for cell survival. Focal cerebral ischemia degrades microvascular integrity through major alterations in endothelial cell permeability and loss of the vascular matrix. The major basal lamina constituents, including laminin-1, laminin-5, collagen IV, and cellular fibronectin, decrease roughly in parallel during MCA:O/R. That loss is associated with microvascular hemorrhage. From parallel studies, loss of basal laminal integrity and neuronal injury are accompanied by a rapid simultaneous increase in tissue expression of uPA and latent MMP-2, but not tPA, in this model. PAIs may facilitate degradation of basal lamina via several pathways. Plasmin and uPA, but not tPA, can activate latent MMP-1, MMP-3, and MMP-9, or in the case of pro-MMP-2, activation is through the proteolytic activation of membrane type-1–MMP. Some or all of these PA-dependent effects can contribute to postischemic CNS injury.

With a modification of zymography with the use of gelatin (collagen) as the plasmin substrate, tPA and uPA activities from 10-μm frozen sections were readily detectable and reproducible. Within nons ischemic basal ganglia, tPA activity (calculated from the normalized integrated densities) was about 67% of uPA activity in control animals, although tPA antigen content was 33 times that of uPA antigen. When casein was used as the substrate for zymography, tPA activity was about 156% of uPA activity in control animals. These differences may reflect the separate requirements for plasmin formation by tPA and uPA activity with gelatin as the substrate, the known contributions of fibrin to optimal in vivo plasminogen activation by tPA, or other aspects of the activation of plasminogen by tPA. Nonetheless, the simple extraction procedure used in the present study released nearly all uPA and tPA antigen/activity into the soluble phase (96.8%/96.5% and 97.3%/97.4%, respectively). The specific activities of uPA or tPA were similar in the soluble and the nonsoluble phases (data not shown). The low tPA specific activity, compared with uPA specific activity, is not readily explained by inhibition with PAI-1, because the tPA–PAI-1 complex levels in nons ischemic tissue were low. Importantly, although the activity assays attempt to detect matrix-relevant PA activities, the true condition within cerebral tissue is unknown.

Rosenberg et al first described increased uPA-like and decreased tPA-like proteinase activities by 12 to 24 hours after permanent MCA:O in Wistar-Kyoto rats and SHR, whereas Ahn et al noted an increase in uPA-like proteinase activity and no change in tPA-like proteinase activity in C57BL/6J mice. Wang et al suggested that increases in tPA-like proteinase activity after MCA:O contribute to neurodegeneration. tPA, but not uPA, was assigned a role in neuronal injury within the murine hippocampus. The appearance of both PAIs in neuronal cells has been reviewed recently. Pfefferkorn et al also observed increased PA activity within the caudate putamen by 9 hours after MCA:O in Wistar rats, although the PA was not defined. PAI-1 antigen was apparently increased 4 hours after MCA:O in Wistar rats, but no relation of PAI-1 to uPA and tPA activity has been reported. The very rapid (1-hour) increase in uPA, the increase in the tPA–PAI-1 complex, and the transient decrease in free tPA are consistent findings matching the rapid appearance of other gene products in the microvasculature after MCA:O in the nonhuman primate. Differences in the species and models used, ancillary effects of the anesthetics in the rodent studies, retained plasma, the use of the more sensitive zymographic assays in the present experiments, and other technical differences may also contribute to the discrepant findings. In recent studies, we have shown that residual plasma significantly decreases the activity of tPA and uPA in rodent brain tissue samples (G.J. del Zoppo, E. Lo, M. Asahi, S.E. Baer, unpublished data, 2000). uPA synthesis has been attributed to a number of CNS cell types, including endothelial cells, neurons, astrocytes, and microglia in vivo or in vitro. Given the rapid and persistent elevation of uPA in the ischemic basal ganglia, it is unlikely that uPA is derived from the plasma compartment. If only a contribution of edema to the tissue content of plasma and stable plasma uPA content are assumed, a mechanism to concentrate uPA 40-fold from plasma would be required to explain the early tissue increase in uPA content.

In contrast, the PAI-1 content of ischemic tissue and plasma increased in parallel, suggesting that plasma PAI-1 entered the edema fluid directly as early as 1 to 2 hours after MCA:O. Although PAI-1 synthesis has been attributed to endothelial cells, neurons, and astrocytes, and microglia in vivo or in vitro, any in situ production by normal brain tissue might be overwhelmed by plasma PAI-1. Given the parallel increases in tissue and plasma PAI-1, it is likely that a common source of the inhibitor is the endothelium. Increased PAI-1 mRNA and PAI-1 immunoreactive microvessels have been detected 4 hours after MCA:O in Wistar rats. Docagne et al observed that PAI-1, but not tPA, mRNA expression was increased between 24 hours and 3 days after MCA:O in mice.

In the ischemic basal ganglia, total tPA antigen did not change, although free tPA activity decreased transiently. The early decrease in tPA activity is explained by formation of the tPA–PAI-1 complex. tPA, uPA, and PAI-1 secretion have been attributed variously to stimulated endothelial cells, neurons, astrocytes, or microglia in vivo or in vitro, but tPA antigen and activity did not accumulate in the ischemic
tissue in the present study. In the nonischemic brain, tPA antigen is localized to selected capillary microvessels.39 The differential expression of tPA relative to uPA and PAI-1 might be explained by early stimulation of uPA and PAI-1 generation by tumor necrosis factor-α and -β, transforming growth factor-β, and interleukin-1β (all inflammatory cytokines), which do not stimulate endothelial cell tPA synthesis and secretion.17,32,33 Expression of these cytokines increases early within the ischemic zone in rodents after MCA:O.40,41 With human microvascular endothelial cells, epidermal growth factor increases the synthesis and secretion of tPA, which is inhibited by tumor necrosis factor-α.42 Therefore, microvascular endothelium may be responsible for the increased uPA and PAI-1 antigen, but not tPA antigen.

Recent reports have suggested that tPA might promote neurodegeneration within the rat hippocampus by alteration in laminin integrity.13,43 However, there was no firm relation of uPA, tPA, and PAI-1 content to neuronal injury in the ischemic basal ganglia by 2 hours of MCA:O in the present study. This differs from the significant linear relationship of pro-MMP-2 content to neuronal injury in the same setting.3 Recent reports comparing uPA (−/−), tPA (−/−), PAI-1 (−/−), plasminogen (−/−), and wild-type mice suggest that the absence of tPA, but not uPA, decreased the apparent volume of ischemic injury.23 Furthermore, plasminogen (−/−) constructs had increased regions of ischemic injury. Those experiments do not address the acute condition and are subject to other concerns noted for selected well-characterized knockout and transgenic mouse preparations subject to MCA:O.23,44 – 46 Despite the lack of change in total tPA antigen, it cannot be excluded that tPA increases in microscopically localized areas and contributes to neuronal injury.

Although no direct association of uPA with hemorrhagic transformation was observed in the present study (Figure 6), increased uPA in the ischemic zones of all animals could promote the activation of pro-MMP-9, which is associated with hemorrhagic transformation in this model. At the clinical level, it seems likely that interventional treatment with exogenous PAs could further increase the risk of hemorrhagic transformation if endogenous uPA is already increased. This argument assumes that the extravasation of blood is not itself the source of the increased pro-MMP-9.

The significant increase in uPA within the ischemic basal ganglia coincides with pro-MMP-2 generation and microvascular matrix degeneration, but not neuronal injury, after MCA:O. Despite the rapid and persistent increase in gelatin-proteolytic activity mainly due to significantly increased uPA and the decreased tPA activity 2 hours after MCA:O, the actual compartmentalization of the endogenous PAs in the CNS and their individual effects on microvascular matrix, neuronal integrity, and clinical outcome remain to be defined.

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