Deleterious Effects of Plasminogen Activators in Neonatal Cerebral Hypoxia-Ischemia

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The immature brains of newborns often respond differently from the brains of adults when exposed to similar insults. Previous studies have indicated that although hypoxia-ischemia (HI) induces persistent thrombosis in adult brains, it only modestly impairs blood perfusion in newborn brains. Here, we used the Vannucci model of HI encephalopathy to study age-related responses to cerebral HI in rat pups. We found that HI triggered fibrin deposition and impaired blood perfusion in both neonatal and adult brains. However, these effects were only transient in neonatal brains (<4 hours) and were accompanied by acute induction of both tissue-type and urinary-type plasminogen activators (tPA and uPA), which was not observed in adult brains subjected to the same insult. Interestingly, activation of the plasminogen system persisted up to 24 hours in neonatal brains, long after the clearance of fibrin-rich thrombi. Furthermore, astrocytes and macrophages outside blood vessels expressed tPA after HI, suggesting the possibility of tPA/plasmin-mediated cytotoxicity. Consistent with this hypothesis, injection of α -2-antiplasmin into cerebral ventricles markedly ameliorated HI-induced damage to neurofilaments and white matter oligodendrocytes, providing a dose-response reduction of brain injury after 7 days of recovery. Conversely, ventricular injection of tPA increased HI-induced brain damage. Together, these results suggest that tPA/plasmin induction, which may contribute to acute fibrinolysis, is a critical component of extravascular proteolytic damage in immature brains, representing a new therapeutic target for the treatment of HI encephalopathy. (*Am J Pathol* 2008, 172:1704–1716; DOI: 10.2353/ajpath.2008.070979)

Hypoxic-ischemic encephalopathy (HIE) is a leading cause of mortality and long-term neurological morbidity in premature infants.¹ Previous studies have indicated the immature brain of newborns has a higher susceptibility to hypoxia-ischemia (HI) than adult brains.² The increased vulnerability to HI in newborn brains can be attributed to several reasons, including a greater risk of energy failure, the immaturity of blood-brain-barrier (BBB) and white matter, a high susceptibility to glutamate excitotoxicity,³ low expression of the glutamate transporters,⁴ as well as other unknown mechanisms. These neonate-specific responses are important considerations in designing effective therapies of HIE in infants.⁵

Accumulating evidence demonstrates the tissue-type plasminogen activator (tPA) has complex functions in the nervous system.⁶ By converting plasminogen to active fibrinolytic plasmin, tPA is currently the only Food and Drug Administration-approved specific therapy for acute ischemic stroke. However, tPA can also induce metalloproteases to damage the BBB, leading to hemorrhagic complications.⁷ Moreover, the brain parenchyma has a low level of tPA and plasminogen expression that is markedly increased by seizures and excitotoxins.^{8,9} Because plasmin is a broad-spectrum protease, the excitotoxin-induced endogenous tPA/plasmin can degrade the extracellular matrix, activate protease-activated receptors, and cause neuronal degeneration.^{10,11} Further, tPA has plasmin-independent mechanisms to stimulate microglia¹² and enhance *N*-methyl-D-aspartate receptor-mediated excitotoxicity.¹³ Hence, tPA may have both protective (thrombolytic) and deleterious (neurotoxic) effects in ischemic brain.

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Although the risk of tPA neurotoxicity associated with thrombolytic therapy is well-recognized, the roles of endogenous tPA and plasmin in ischemic brain injury are unclear. This uncertainty is partly because, except in animal models of permanent cerebral ischemia, transient cerebral ischemia does not significantly induce the expression nor activity of endogenous tPA and plasmin.^{14–16} Moreover, although initial studies suggested *tPA-null* mice had smaller infarct volumes than wild-type mice after the suture model of transient focal ischemia, a later study using the genetic background-matched wild-type and *tPA-null* mice revealed opposite results in similar experiments.^{17–19} Even with a photochemically induced thrombosis model of stroke, studies using wild-type and mutant mice indicated that tPA has paradoxical functions in protecting against large thrombi, but increasing the damage by small thrombi.²⁰ Hence, the roles of endogenous tPA and plasmin(ogen) are uncertain in adult ischemic brain injury, and almost unexplored in neonatal HIE.

The present study was stimulated by our recent publication showing while HI only transiently impairs cerebral perfusion in newborns,²¹ it produces persistent occlusive fibrin deposition in adult brains.²² In light of the contrasting effects of HI in newborn versus adult brains, we hypothesized that neonatal brains have an age-specific response to HI through activation of the endogenous plasminogen system. To test this hypothesis, we examined the thrombosis-fibrinolysis responses and the tPA/plasmin activity in rat pups subjected to the Vannucci model of cerebral HI.² Results of these experiments supported our hypothesis, but also indicated persistent extravascular induction of the tPA/plasmin system is a critical factor of brain injury in this paradigm. These results shed new insight into the mechanism of brain injury in neonatal HIE in infants.

Materials and Methods

Animal Surgery

Wistar rat pups were used for the Vannucci model of neonatal HI as previously described.² Briefly, 7-day-old rat pups were anesthetized with 3% isoflurane mixed with ambient air under spontaneous inhalation, and the right common carotid artery was ligated. After a 1-hour recovery period, the pups were placed in glass chambers containing a humidified atmosphere of 10% oxygen and 90% nitrogen and submerged in a 37°C water bath. After 1.5 hours of hypoxia, the pups were returned to their dam for the indicated time. Wistar rats, 8 to 12 weeks of age, were used for adult HI model as previously described.²² Intracerebroventricular injections were done immediately after, 2 hours, or 4 hours after hypoxia with 10 μ l volumes of α 2-antiplasmin (BioDesign International, Saco, ME), saline, tPA [50 μ g recombinant tPA (Activase; Genentech, South San Francisco, CA) and 3.85 mg L-Arginine in 10 μ l], and tPA-vehicle (3.85 mg L-arginine in 10 μ l) with a Hamilton syringe at 2.0 mm rostral and 1.5 mm lateral to the right from the Lambda suture, and at a depth of 2.0 mm from the surface of the brain, as previously described.²³ These animal procedures were approved by the Institu-

tional Animal Care and Use Committee and conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

tPA-Null Mice

Homozygous *tPA-null* mice backcrossed to the C57BL/6 strain were used in the present study. These mice were generated as previously described,²⁴ and obtained from The Jackson Laboratory, Bar Harbor, ME (stock no. 002508).

Assessment of Brain Damage

After 7 days of recovery from HI, pups were sacrificed under deep anesthesia by transcardiac perfusion with saline and then 4% paraformaldehyde. After postfixation, brains were taken through sucrose gradients and frozen in tissue-freezing medium, then cut in coronal sections 50 μ m in thickness. Eight evenly spaced sections were taken to represent the whole brain and stained with cresyl violet. Every section was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD) for area of surviving cortex, striatum, and hippocampus on lesion and contralateral sides as indicated by Nissl stain. The percentage of tissue loss was calculated at every relevant section for each structure as a ratio of lesion to contralateral areas, and this was averaged over eight sections for each animal.

Histology

Brain sections were obtained as above for lesion analysis, but with 30- μ m-thick sections. Immunohistochemistry was performed using rat polyclonal antibodies against P-selectin (BD Pharmingen, Franklin Lakes, NJ), Glycoprotein IIb (BD Pharmingen), mouse monoclonal antibodies against CD11b (OX42 antibody; Serotec, Oxford, UK), myelin basic protein (Chemicon, Temecula, CA) and neurofilament (clone 2H3; Hybridoma Bank, University of Iowa, Ames, IA), as well as, rabbit polyclonal antibodies against tPA (Molecular Innovations, Novi, MI) and fibrin(ogen).²² Biotinylated secondary antibodies and streptavidin conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR) were used to amplify the immuno signals. For mouse primary antibodies, a highly cross-absorbed anti-mouse secondary antibody (Molecular Probes) was used because of cross reactivity with rat IgG. Goat biotinylated anti-rat (H+L; Vector Laboratories, Burlingame, CA) was used to label IgG leakage. The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed as described.²²

Fluorescein Isothiocyanate (FITC)-Dextran Perfusion Analysis

Cerebral perfusion analysis was performed as previously published.²² Briefly, FITC-dextran [5 mg in 100 μ l of phosphate-buffered saline (PBS) of 2×10^6 MW; Sigma,

St. Louis, MO] was injected with a small-bore needle into the left ventricle of pups 1 or 4 hours after ischemia/hypoxia. After 2 minutes, the animal was rapidly decapitated and the brain was removed and postfixed for 48 hours. Sections were cut as above and analyzed with fluorescence microscopy. For quantification, images obtained were analyzed using ImageJ software. An index of perfusion was measured in each hemisphere in an area excluding the midline (matching the maximal possible infarction) in two caudal coronal sections covering the hippocampus in each animal. The number of FITC pixels per number of total pixels in maximal infarct area of ipsilateral hemisphere was first determined. The data from the lesion sides were then normalized to the average of data from the contralateral side at the corresponding time point. Presented are the lesion and the contralateral data at 1 hour and 4 hours after HI, with *P* values reported relative to each time point's contralateral data set.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA of the hippocampus was isolated from the lesion, contralateral, and untreated hemispheres using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen) and oligo dT₁₈ primer were used for cDNA synthesis. For quantification, optical intensity was measured on the presented images using ImageJ and normalized to G3PDH for each time point. The primer pairs used for PCR analysis were as follows: tPA: 5'-CTCTGACTTCGTCTGC-CAG-3' and 5'-TGTCATTGCTTCATGTTGTC-3'; uPA: 5'-CTACACCAAAGGCTGTGGC-3' and 5'-GCCATTACAG-CAGGAGATAG-3'; PAI-1: 5'-ATCCTGCCTAAGTTCTC-TCT-3' and 5'-ATTGTCTCTGTCTGGGTTGTG-3'; matrix metalloproteinase (MMP)-2: 5'-GGACAGTGACACCACGT-GAC-3' and 5'-ACTCATTCCTGCGAAGAACA-3'; MMP-9: 5'-GCACGCTGTCTTTCTTGTGG-3' and 5'-CCCGAC-GCACAGTAAGCATTC-3'.

Immunoblot Analyses

Brain samples for immunoblots were homogenized in TLB buffer (containing 20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 25 mmol/L Na-pyrophosphate, 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L Na₃VO₄, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethyl sulfonyl fluoride, 0.7% protease inhibitor cocktail). The proteins were separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedures, electrotransferred onto a polyvinylidene difluoride microporous membrane (Bio-Rad, Hercules, CA), and immunoblotted with designated antibodies followed by enhanced chemiluminescence detection (Amersham Biosciences, Buckinghamshire, UK). The antibodies used are: anti-occludin (Zymed, South San Francisco, CA) and anti-β-actin (Sigma).

tPA/uPA Zymogram

For detection of tPA/uPA, plasminogen and casein were added to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Protein samples were extracted using RIPA buffer and mixed with an equal volume of the sample buffer (0.5 mol/L Tris-HCl, pH 6.8, 100% glycerol, 0.05% bromophenol blue, 10% sodium dodecyl sulfate) and run at low voltage for up to 5 hours. After electrophoresis, the gel was rinsed twice in 2.5% Triton X-100 for 30 minutes each at room temperature, followed by an incubation in glycine buffer (0.1 mol/L glycine, pH 8.0) at 37°C overnight. The gel was then stained with Coomassie blue solution and destained overnight to reveal the lytic zones of protease activity. Recombinant human tPA (Activase, Genentech) was used as the positive control to reveal the tPA band in zymogram. Quantification was done on the presented image using ImageJ to determine the area of signal for each time point.

MMP Zymogram

For detection of the activity of MMP-2 and MMP-9, 0.15% porcine skin gelatin was added to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Protein samples were extracted and ran as mentioned above. The gels were washed twice with 2.5% Triton X-100, then incubated in reaction buffer (50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl₂) at 37°C overnight, then stained and destained as mentioned above. Purified MMP-2 (M-1552, Sigma) and MMP-9 (M-7942, Sigma) were used for positive controls. Quantification was done on the presented image using ImageJ to determine the area of signal for each time point.

In Situ Gel Zymogram Analysis

Brains were obtained at 4 hours after hypoxia from deeply anesthetized pups and snap-frozen in isopentane cooled with dry ice. Sections were then obtained with a cryostat, and mounted onto poly-L-lysine (Sigma) coated, positive-charged slides kept on dry ice to prevent tissue decay. After thawing the slides, they were coated with 300 μl of a mixture of 2% milk, 1% agar in PBS kept at 50 to 60°C to which was added just before coating plasminogen (80 μg/ml, Hematological Technologies, Essex Junction, VT), tPA-stop (17 μmol/L; American Diagnostica, Stamford, CT), or amiloride (0.1 mmol/L, Sigma). Pictures were taken with dark-field illumination of each section before and after developing at 37°C in a humid chamber for 2.5 hours.

In Situ Hybridization

The *in situ* probe of tPA was designed roughly based on an Allen (mouse) brain atlas riboprobe (RP_050614_03_A10), but adjusted for rat. Using two primers matched to rat tPA transcript (forward: 5'-GCTGACGTGGGAATACTGTG-AC-3', reverse: 5'-TCCTTCTGCCACAGCCGAG-3'), a 760-bp RT-PCR product was cloned directly into PCRII (Invitrogen), linearized, and purified. *In vitro* transcription

was performed using 2 μg of linearized DNA at 37°C for 5 hours with digoxigenin incorporation to obtain sense and anti-sense probes. This was then ethanol-precipitated and separated on an agarose gel for verification. Brain sections were obtained as mentioned above for histology. Sections were first refixed in 4% paraformaldehyde, washed in PBS, and treated for 2 minutes with proteinase K (10 $\mu\text{g}/\text{ml}$; Lambda Biotech, San Diego, CA). These were then washed with triethanolamine (0.125 mol/L, Sigma) and then treated twice for 5 minutes with acetic anhydride in triethanolamine (0.25% in 0.125 mol/L). Next, the sections were washed in PBS, refixed in 4% paraformaldehyde, washed again, taken through ethanol dehydration, and briefly dried. Hybridization was then performed at 55°C in a humid chamber overnight with 5 $\mu\text{g}/\text{ml}$ of antisense and 12 $\mu\text{g}/\text{ml}$ of sense probe in a mix of 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 250 $\mu\text{g}/\text{ml}$ yeast tRNA, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, pH 8, 5 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L Na_2HPO_4 , pH 7.2, and 1% *n*-laurylsarcosine, briefly preheated to 80°C. The next day, slides were washed in 50% formamide/2 $\times$ standard saline citrate (SSC) at 65°C for 30 minutes, washed in RNase buffer (0.5 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5), treated with RNase A (20 $\mu\text{g}/\text{ml}$ in RNase buffer) at 37°C for 30 minutes, washed again with 50% formamide/2 $\times$ SSC at 65°C, 2 $\times$ SSC at 37°C, 0.1 $\times$ SSC at 37°C, maleic acid buffer (0.1 mol/L maleic acid, 0.2 mol/L NaOH, 0.15 mol/L NaCl, pH 7.5) plus 0.1% Tween-20 at room temperature, and then blocked with a solution of 2% blocking reagent (no. 1096176; Roche, Basel Switzerland) + 10% heat-inactivated normal goat serum in MAB for 1 hour at room temperature. Sheep anti-digoxigenin primary antibody (Roche) was then added to the block and incubated overnight at 4°C in a humid chamber. Afterward, slides were washed eight times with MAB + 0.3% Tween-20 for 30 minutes each, 100 mmol/L NaCl/100 mmol/L Tris-HCl, pH 9.5, twice for 20 minutes, and then covered with BM purple (Roche) with levamisole (2 mmol/L). This was allowed to develop at room temperature in a dark, humid chamber for 3 to 7 days to get appropriate signal/background ratio, replacing the BM purple/levamisole solution daily. When finished, slides were washed twice in PBS, dipped in water, dried thoroughly, coverslipped in DPX mounting medium (Sigma), and analyzed with light microscopy.

Statistical Analysis

Values are represented as mean $\pm$ SE. Quantitative data were compared between the lesion side and contralateral side, or between the treatment group and the vehicle group using Microsoft (Redmond, WA) Excel's two-sample *t*-test assuming equal variance.

Results

Neonatal HI Induces Transient Thrombosis and Acute BBB Permeability

A previous study using autoradiography reported the Vannucci model of HI does not induce a significant re-

duction of cerebral blood flow in rat pups within the first 24 hours after the insult.²¹ To confirm this observation, we used fluorescein-conjugated dextran (FITC-dextran) as a tracer of blood perfusion to examine the brains of 7-day-old rat pups at 1 and 4 hours after the Vannucci model of HI.² This analysis revealed patches of vascular obstruction in the hippocampus and the cerebral cortex on the carotid-occluded side of brain at 1 hour of recovery (Figure 1, A and B). The perfusion index (the ratio of FITC-filled pixels to those of the total area) is at 53% of the contralateral side ($P < 0.0001$ by *t*-test, $n = 8$) (Figure 1Q). However, at 4 hours of recovery, the patches of vascular obstructions disappeared, and the cerebral perfusion index rose to 74% of the contralateral side ($P = 0.03$, $n = 8$) (Figure 1, C, D, and Q). These results confirmed the previous report,²¹ indicating that HI only causes a brief period of vascular obstruction in the newborn brain. The HI-induced transient hypoperfusion in newborn brains is in contrast to its effect of persistent deficits of cerebral perfusion in adult brains.²²

In adult brains, the cerebral blood flow reduction is primarily caused by HI-induced fibrin and platelet deposition inside the blood vessels.²² Thus, we set out to examine whether HI has the same effects in the newborn brain. To do so, rat pups were sacrificed at 1 and 4 hours after HI by transcardial perfusion of saline—to remove the intravascular fibrinogen and platelets—and fixative, followed by processing the brains for immunohistochemical analysis. This analysis revealed unilateral fibrin(ogen) and platelet (detected by antibodies against the platelet-specific integrin subunit αIIb) deposition in the blood vessels at 1 hour of recovery on the carotid-occlusion side of brain (Figure 1, E, F, I, and J; $n = 5$). However, the extent of intravascular fibrin(ogen) and platelet deposition markedly diminished at 4 hours of recovery (Figure 1, G, H, K, and L; $n = 5$), consistent with the recovery of cerebral perfusion at this time (Figure 1, C and D).

Besides thrombosis-fibrinolysis events, we also used immunocytochemistry and immunoblot analyses to examine the timing of HI-induced BBB damage in newborn brains. Immunocytochemistry revealed immunoglobulin staining, an indication of increased BBB permeability,^{23,25} at 4 hours on the carotid-occluded side of brain (Figure 1, M and N), which was intensified at 24 hours (Figure 1, O and P). Moreover, immunoblot showed the reduction of occludin, an endothelial tight junction-associated protein, at 4 hours after HI (Figure 1R). Together, these results suggest HI induces progressive BBB damage and rapid thrombosis in neonatal brains, similar to its effect in adults,²² but the immature brain has a countermechanism to rapidly remove the thrombi.

Neonatal HI Triggers Persistent Induction of the Plasminogen System

To search for the mechanism of acute recovery of cerebral blood flow and progressive damage to BBB in HI-challenged neonatal brains, we used RT-PCR, zymography, and *in situ* zymography to examine the expression and activity of tPA and urinary-type plasminogen activa-

Figure 1. HI causes transient thrombosis, cerebral perfusion deficits, and acute BBB damage in the neonatal brain. **A–D:** Intracardial injection of FITC-conjugated dextran showed patches of vascular hypoperfusion on the carotid-occluded side of the brain at 1 hour after HI (**B**), which diminished at 4 hours after HI (**D**). Areas of hypoperfusion are more commonly found in the dorsolateral cerebral cortex (Ctx) and hippocampus (Hip) than in the thalamus (Th). The quantification of cerebral perfusion compared to the contralateral side (**A**, **C**) is shown in **Q**. **E–I:** Immunostaining showed intravascular fibrin deposition (**F**) and platelet aggregation (via anti-integrin glycoprotein GPIIb subunit stain). **J:** At 1 hour after HI on the carotid-occluded side of the brain, and a reduction of such staining at 4 hours close to the levels of the contralateral side of the brain (**H**, **L**). Shown are typical results in five animals for each time point. **M–P:** Immunostaining showed extravasation of immunoglobulins (IgG) in the hippocampus (Hip) and the cerebral cortex (Ctx) on the carotid-occluded side of brain at 4 hours (**N**), which intensified at 24 hours (**P**) ($n > 6$ for each time point). **Q:** Quantification showed the perfused area on the carotid-occluded side was reduced to 53% of the contralateral side at 1 hour after HI ($P < 0.001$), which improved to 74% at 4 hours ($P = 0.03$). Data shown are mean $\pm$ SE ($n = 8$ for each time point) and P values were determined by two-sample t -test assuming equal variance. **R:** Immunoblots showed reduction of the tight junction-specific protein occludin on the carotid-occluded (**right**) side of the brain at 4 hours (R4). **L:** The contralateral (**left**) side of brain. UN, unchallenged brain. Shown is the representative blot of three sets of experiments. Scale bars: 400 μ m (**A–D**); 160 μ m (**E–H**); 80 μ m (**I–L**); 400 μ m (**M–P**).

Figure 2. HI causes rapid and persistent induction of the plasminogen system. **A:** RT-PCR analysis of tPA, uPA, PAI-1, MMP-2, and MMP-9 expression using RNA extracted from the hippocampus. This analysis showed a general decrease of transcripts of these genes at 1 hour (R1) and an increase of tPA, uPA, and PAI-1 at 4 hours (R4) on the lesion side. There was also an increase of tPA at 1 hour (L1) and 4 hours (L4) after HI on the contralateral side, and induction of PAI-1 at L1. The numbers are the ratio of optical intensity normalized to the G3PDH signal. Similar results were obtained in more than three animals for each time point. **B:** Plasminogen/casein gel zymogram showed the tPA activity [64 kDa; corresponding to recombinant tPA (rt-PA) used as positive control] was induced from 1 to 24 hours after HI on the carotid-occluded side, and to a greater extent on the lesion side. The induction of uPA activity (46 kDa) was more pronounced at 4 and 24 hours, and limited on the lesion side (R4 and R24). **C:** Gelatin-zymogram gel showed an induction of the MMP-9 activity on the lesion side at 24 hours after HI (R24), with no obvious increase in the MMP-2 activity. Similar data were found in four sets of animals. **D:** Plasminogen/casein-zymogram gel using the adult rat brains showed no obvious induction of tPA or uPA activity within 4 hours after the same HI insult. Same results were found in three sets of animals. The numbers in **B–D** are the optical intensity of indicated signals in each lane using immunoblot against β -actin for loading control.

tor (uPA), as well as matrix metalloproteases (MMP-2 and MMP-9, thought to be the key proteases damaging the BBB in ischemic brain injury²⁵) on the carotid-occluded (right, R) side and the contralateral (left, L) side of the brain after the insult. For RT-PCR analysis, we extracted total RNA from the hippocampus because it is an easily separable structure consistently showing initial perfusion deficits and eventual tissue destruction (Figure 1, A–D; and Figure 3E). This analysis showed an initial reduction of mRNA for all nonhousekeeping genes at 1 hour on the carotid-occluded side of brain (R1)—this may relate to reduced RNA stability immediately after an ischemic-hypoxic insult—followed by a great increase of tPA transcript at 4 hours on the lesion side (R4), and to a lesser degree at 1 and 4 hours on the contralateral side (L1 and L4) (Figure 2A) ($n > 3$ for each time point). The uPA transcript was markedly increased at R4. So was the plasminogen activator inhibitor-1 (PAI-1) mRNA up-regulated at L1 and R4 (Figure 2A). In contrast, there was no obvious induction of MMP-2 or MMP-9 transcripts on either side of the brain within 4 hours after HI (Figure 2A). Because RT-PCR analysis showed expression of both tPA/uPA and PAI-1 after HI, which could cancel out the activation of plasminogen, we used zymograms to assess the activity of these proteases.

Gel zymography using the proteins extracted from cortical hemispheres showed a marked increase of the tPA activity on the carotid-occluded side of brain from 1 to 24 hours, and to a lesser degree on the contralateral side. The uPA activity also appeared on the carotid-occluded side of the brain between 4 to 24 hours after HI (Figure 2B, shown are typical results in four sets of experiments). In contrast, there was no marked induction of the MMP-2

activity after HI. The MMP-9 activity was only increased at 24 hours of recovery on the carotid-occluded side, much later than tPA and uPA induction (Figure 2C). This pattern of delayed MMP-9 activation, consistent with a recent report,²⁵ suggest that it is unlikely an important factor of initial BBB damage after neonatal HI.

We also examined the tPA and uPA activity in adult brains following the Vannucci model of HI. Interestingly, although adult rat brains already possess a basal tPA activity, it was not obviously increased by the same insult of HI (Figure 2D, repeated in three sets of animals). This result is consistent with many previous reports showing a general lack of robust induction of endogenous tPA/uPA activity by ischemic injuries in adult brains.^{14–16}

Next, we performed *in situ* plasminogen-zymography using unfixed brain sections to delineate the regions of PA induction after HI. This analysis revealed a large area of caseinolysis in the hippocampus and dorsolateral cortex on the carotid-occluded (R) side of the brain at 4 hours of recovery (Figure 3A). Confirming the specificity of this reaction, omission of plasminogen in the overlay totally abolished the caseinolytic activity (Figure 3B). Addition of a tPA-inhibitor (17 μ mol/L tPA-Stop)²⁶ or a uPA-inhibitor (0.1 mmol/L amiloride)²⁷ in the overlay partially reduced the level of caseinolysis (Figure 3, C and D). Interestingly, when the rat pups were sacrificed after 7 days of recovery, severe tissue destruction was noticed in the brain regions showing acute PA activity after HI (Figure 3E). The patterns of brain damage include obliteration of the hippocampus, destruction of the corpus callosum, and dilation of the cerebral ventricle (arrows), as well as cystic degeneration of the dorsolateral cortex (Figure 3E, arrowheads). Together, these results indi-

Figure 3. Induction of the plasminogen system correlates with HI-damaged brain regions. **A:** *In situ* gel zymogram using unfixed brain sections showed increased PA activity on the carotid-occluded side (R) at 4 hours after HI. The PA activity is concentrated in the dorsal cerebral cortex (Ctx) and hippocampus (Hip) than in the basal thalamus (Th). **B:** Omission of plasminogen in the overlay abolished the *in situ* proteolysis activity. **C** and **D:** Inclusion of a synthetic tPA inhibitor tPA-STOP (17 μ mol/L) or a uPA inhibitor amiloride (0.1 mmol/L) reduced the extent of proteolysis. Images shown are representative results from at least three sets of samples. **E:** Representative examples of Nissl stain indicating damage in the posterior part of the forebrain at 7 days of recovery after HI. Note the typical pathology of obliteration of the hippocampus and unilateral dilation of the cerebral ventricle (arrows), as well as cystic degeneration of the dorsolateral cerebral cortex (arrowheads).

cated a remarkable spatial correlation between acute PA induction and eventual tissue destruction after HI in the neonatal brain.

HI Induces Extravascular tPA Expression by Astrocytes and Macrophages

Because the zymogram data indicated tPA induction precedes uPA and MMP-9 activation after cerebral HI, we used *in situ* hybridization to examine the distribution of tPA transcripts in newborn brains. At 4 hours of recovery, the highest expression of tPA mRNA was in the dorsolateral cortex, striatum and hippocampus, and near the lateral ventricle (LV) on the carotid-occluded side of brain (Figure 4, A and B), corresponding to the pattern of *in situ* PA activity (Figure 3A). In control experiments, sense riboprobes showed no signal (Figure 4C). Closer inspection suggested the tPA mRNA was synthesized both within and outside the blood vessels (Figure 4, E and F) and in the white matter (WM) adjacent to lateral ventricles at 4 hours after HI (Figure 4, H and I). The expression of tPA mRNA in the hippocampus was located both within and outside the pyramidal neuron layer, and increased after 24 hours of recovery (Figure 4, K and L).

Next, we used two approaches of fluorescent double-labeling to determine the identity of tPA-producing cells

after HI. In the first method, we combined tPA *in situ* hybridization and anti-glial fibrillary associated protein (GFAP) immunocytochemistry to test whether astrocytes express tPA after HI. This is because astrocytes with extensive end-feet surrounding blood vessels and projecting into the subependymal zone (also called the germinal matrix) are thought to be important regulators of ischemic brain injury.²⁸ Consistent with this view, we found many instances in which tPA transcript was localized within the cell body of GFAP-positive astrocytes in the vicinity of blood vessels (Figure 5, A–C) and the lateral ventricle wall (Figure 5, D–F). In the second approach, we paired anti-tPA antibody with various cell-type markers for fluorescent double-labeling. This analysis revealed a high degree of co-localization of anti-tPA immunoreactivity within the GFAP-positive astrocytes (Figure 5, G–I) or CD11b/OX42-positive macrophage-microglia (Figure 5, J–L). Together, our data were similar to previous reports of excitotoxin-induced tPA expression by astrocytes and microglia,^{9,10} and raised the possibility of tPA/plasmin-mediated extravascular toxicity in neonatal HI brain injury.

tPA/Plasmin Activity Is a Critical Factor of HI Brain Injury in Neonates

To test whether the HI-induced tPA/plasmin activity contributes to brain damage, we injected α 2-antiplasmin (also known as α 2 plasmin inhibitor) into the cerebral ventricle of rat pups after HI to test if it offered any protection. The intracerebroventricular (ICV) route of α 2-antiplasmin delivery was chosen to inhibit plasmin-mediated proteolysis in the extracellular space, while preserving its fibrinolytic potential inside the blood vessels. In the first set of experiments, we infused saline or antiplasmin (6.25 to 50 μ g) into lateral ventricles immediately after the recovery of 90 minutes of HI. By quantifying the size of the residual brain areas compared to contralateral counterparts at 7 days of recovery, we calculated the percentage of tissue loss in the saline-injected animals to be $48 \pm 2\%$ in the striatum, $46 \pm 3\%$ in the cerebral cortex, and $61 \pm 2\%$ in the hippocampus (mean $\pm$ SE, $n = 15$). The ICV-injection of antiplasmin in the range of 6.25 to 25 μ g showed a dose-responding protection, and reduced the extent of tissue loss to $24 \pm 3\%$ in the striatum, $19 \pm 3\%$ in the cerebral cortex, and $27 \pm 3\%$ in the hippocampus with 25 μ g of antiplasmin ($P < 0.005$ by *t*-test, $n = 10$). The protective effect was attenuated with 50 μ g of antiplasmin injection (Figure 6A). This may have been attributable to nonspecific effects or interference with intravascular fibrinolysis by the high dose of antiplasmin injected.

A second set of experiments was performed to investigate the therapeutic window of antiplasmin treatment. We found that ICV infusion of 20 μ g of antiplasmin at 2 hours after HI still showed a trend of reduction of brain damage (Figure 6B). However, the protective effect was lost by further delaying the injection of antiplasmin to 4 hours of recovery, indicating that this was beyond the therapeutic window (Figure 6C).

In the third set of experiments, we tested whether administration of exogenous tPA into the extravascular

Figure 4. Extravascular and periventricular expression of tPA mRNA after HI. **A–L:** *In situ* hybridization using tPA antisense riboprobes showed increased signals on the carotid-occluded (**right**) side of the brain at 4 hours of recovery, specifically in regions matching the eventual damage that include the striatum (St), hippocampus (hip), and the dorsolateral cerebral cortex (Ctx) (**A, B**). The sense probes produced no specific signal in control experiments (**C**). Close inspection revealed that the transcript of tPA was mostly located both within and surrounding the cerebral blood vessels (**E, F**), and in the subependymal zone and the white matter (WM) (**H, I**) adjacent to lateral ventricles (LV) at 4 hours after HI. By 24 hours after HI, the transcript of tPA was detected within the pyramidal neuronal layer in the hippocampus (Hip) (**K, L**). Shown are the representative results from three animals for each time point. Scale bars: 25 μ m (**F, I, L**); 50 μ m (**D, E**); 100 μ m (**G, H**); 200 μ m (**J, K**).

space after HI could have deleterious effects in the neonatal brain. To do so, we injected tPA (50 μ g) or vehicle into the lateral ventricle of rat pups subjected to a milder insult of HI. By shortening the duration of hypoxia from 90 to 70 minutes, we reduced the extent of tissue loss to $17 \pm 12\%$ in the striatum, $28 \pm 11\%$ in the cortex, and $36 \pm 15\%$ in the hippocampus in vehicle-injected animals (mean $\pm$ SE, $n = 5$). The injection of tPA significantly increased tissue loss in

the striatum ($46 \pm 7\%$) and the hippocampus ($68 \pm 7\%$, $P < 0.05$ by t -test, $n = 5$), and showed a trend of greater damage in the cerebral cortex ($46 \pm 11\%$, $P = 0.096$) (Figure 6D). In control experiments, ICV injection of the same dose of tPA in sham-operated animals did not cause obvious tissue loss (not shown, $n = 2$). Together, these results suggest the tPA/plasmin activity in the extravascular space contributes to HI brain injury in neonatal animals.

Figure 5. Astrocytes and macrophage-microglia express tPA after HI in neonatal brain. **A–F:** Fluorescent double-labeling of *tPA* mRNA (red) and glial fibrillary associated protein (GFAP) (green) revealed the expression of tPA by astrocytes surrounding the blood vessels (V) (**A–C**, **arrows**) and near the lateral ventricle (LV) (**D–F**, **arrows**). **G–I:** Double-labeling with anti-tPA (red, **G** and **J**) and anti-GFAP (green, **H**) or anti-CD11b/OX42 (green, **K**) antiserum showed co-localization in astrocytes (**G–I**, **patches** and **arrows**) and macrophage-microglia (**J–L**, **arrows**) after HI in the neonatal brain. The rat brains were collected at 4 hours after HI. Shown are typical results in more than three sets of experiments. Scale bars: 10 μ m (**A–C**); 20 μ m (**D–F**); 30 μ m (**G–L**).

We also compared the response to cerebral HI by 10-day-old wild-type and *tPA-null* mice backcrossed to the C57BL/6 strain. This analysis showed *tPA-null* mice have a higher mortality rate (81.8%, $n = 11$) than wild-type mice (12.5%, $n = 8$) during or shortly after a 50-minute HI insult (Figure 6E). Although the exact cause of the high mortality of *tPA-null* mice under HI remains to be determined, one possibility is that it may relate to decreased intravascular fibrinolytic ability of these mutants.²⁴

Inhibition of Plasmin Reduces HI-Induced Gray Matter and White Matter Injury

To examine the mechanisms of antiplasmin-mediated protection, we prepared another group of rat pups to receive a 90-minute HI challenge followed by immediate ICV injection of saline or 25 μ g of antiplasmin ($n = 5$ for each group, shown are the representative results). These

Figure 6. Antiplasmin protects, and tPA injection enhances HI brain injury. **A:** Intracerebroventricular injection of $\alpha 2$ -antiplasmin immediately after 90 minutes of HI showed a dose-responding reduction of tissue loss compared to the contralateral side in the dose range up to 25 μ g. Shown are mean $\pm$ SE. * $P < 0.05$; ** $P < 0.005$ compared to saline-injected animals using *t*-test assuming equal variance. **B and C:** Injection of 20 μ g of $\alpha 2$ -antiplasmin at 2 hours after HI showed a trend of reduction of tissue loss, but injection at 4 hours showed no protection. **D:** Injection of tPA (50 μ g) after a shorter duration of HI (70 minutes) increased the extent of brain injury, when compared to the vehicle (3.85 mg L-arginine) injection. **E:** Genetic background-matched *tPA*-null mice showed a higher mortality rate (81.8%) than wild-type C57BL/6 mice (12.5%) during or shortly after a 50-minute hypoxic period after unilateral carotid artery ligation. The number of injected animals for each experiment is indicated.

animals were sacrificed at 24 hours of recovery when neither antiplasmin- nor saline-treated animals displayed gross tissue damage. However, the antiplasmin-treated group showed marked preservation of cortical neurofilaments over saline-injected animals (Figure 7, A–C). In addition, the white matter oligodendrocyte processes (detected by anti-myelin basic protein) were less fragmented or scrambled in antiplasmin-treated than in saline-injected animals (Figure 7, D–F). Furthermore, the antiplasmin treatment markedly decreased the number of TUNEL-positive nuclei in the cerebral cortex than saline injection, forming clusters or columns of dying cells surrounding the blood vessels (Figure 7, G–I). Together, these results suggest the extravascular plasmin activity is an important factor of HI-induced gray matter and white matter injury.

Discussion

It is often said that infants are not miniature adults. Because adult brains and the immature brain of newborns have different properties in physiology and energy me-

tabolism, they often exhibit distinct responses to similar insults.⁴ For example, previous studies indicated the immature brains have a greater susceptibility to combined HI than adult brains, as shown in severe brain damage in rodent pups after the Vannucci model of HI² and in the high incidence of HIE in premature infants.⁵ Interestingly, although the Vannucci model of HI causes rapid thrombosis and persistent hypoperfusion in adult brains,²² the same insult only briefly impairs cerebral blood flow in newborns.²¹ These contrasting consequences between adult and newborn animals suggest either HI does not induce thrombosis in neonatal brains, or the immature brain has unique mechanisms to eliminate thrombosis, yet remains vulnerable to HI-induced damage. The present study aims to determine the neonate-specific responses to cerebral HI.

The results of our study indicated HI triggers fibrin and platelet deposition in the blood vessels of neonatal rat brains, similar to its effects in adult brains (Figure 1). However, the HI-induced thrombosis and vascular obstruction was only short-lived in the neonatal brain. It was also accompanied by rapid induction of endogenous tPA and uPA activity, which was not observed in adult animals (Figure 2). These results indicated acute and persistent induction of the plasminogen system is a neonate-specific response to cerebral HI. Interestingly, the HI-induced tPA and uPA activities persisted in newborn brains long after the recovery of cerebral perfusion, and followed the progression of increased BBB permeability. Although future studies are needed to determine the timing of transition from the neonate-specific to adult brain responses to HI, our findings have important implications for the growing awareness of tPA neurotoxicity and the etiology of HIE.

Paradoxical Roles of tPA in Ischemic Brain Injury

Accumulating evidence indicates tPA has complex functions in the central nervous system physiology and pathology.^{29,30} Although tPA is primarily synthesized in liver and circulates inside the blood vessels to maintain hemostasis, the brain parenchyma normally has a low level of tPA-plasmin activity, which has neuroprotective functions via acceleration of the degradation of amyloid- β peptides.^{8,31} However, excitotoxins also induce neurons, astrocytes, and microglia to express tPA, leading to plasmin-mediated extracellular proteolysis and cell degeneration.^{9,10} Furthermore, tPA has several plasmin-independent functions, including activation of microglia as a cytokine,¹² escalation of *N*-methyl-D-aspartate receptor-mediated signaling,¹³ and induction of matrix metalloproteinases.⁷ These effects are harmful to the neurovascular unit and impose a threat to the survival of neurons. Hence, it is well-known that exogenously administered tPA in thrombolytic therapy increases the risk of hemorrhagic transformation and may cause further brain damage.^{29,32}

In contrast, the roles of endogenous tPA and plasminogen in ischemic brain injury are controversial.^{17–20} This uncertainty is primarily attributable to the fact that, except

Figure 7. Antiplasmin treatment reduces axonal loss, white matter injury, and apoptosis before the onset of cystic degeneration in neonatal brains after HI. **A–I:** Immunostaining at 24 hours after 90 minutes of HI in saline-injected rat pups showed great reduction of neurofilaments in the dorsolateral cortex (Ctx) (**B**), myelin-basic protein-positive oligodendrocyte processes in the white matter (WM) (**E**), and numerous TUNEL-positive nuclei in the dorsolateral cortex toward the pia surface (Pia) (**H**), when compared to the contralateral side (**C**, **F**, **I**). Injection of 25 μ g of α 2-antiplasmin immediately after HI greatly attenuated the destruction of neurofilaments (**A**) and oligodendrocyte processes (**D**), and reduced TUNEL-positive nuclei to isolated clusters and columns (**G**). Shown are representative results in five animals for each treatment. Scale bars: 400 μ m (**A–C**, **G–I**); 100 μ m (**D–F**).

for permanent focal ischemia, the majority of studies only found a low or negligible level of tPA/plasmin induction after transient focal ischemia in adult animals.^{14–16} Moreover, there was a dispute over whether tPA deficiency reduces or enlarges the infarct volume in transient cerebral ischemia.^{17–19} Here, we show a rapid, strong, and persistent induction of the endogenous plasminogen system after HI in the newborn, but not the adult brains (Figure 2). The robust induction of tPA/plasmin activity in neonatal brains allows us to examine its functions. Our results indicate the endogenous plasminogen system has complex effects in neonatal HI brain injury, depending on the timing and the location of its activity (Figure 8).

We showed that cerebral HI triggers acute induction of endogenous tPA/uPA activity in specific regions displaying initial thrombosis and rapid recovery of the blood flow (Figures 1 to 3). Moreover, the HI-induced tPA mRNA was concentrated both within and around the blood vessels (Figure 4, A–F; and Figure 5, A–C). These results suggest the acute tPA/uPA induction by HI may contribute to rapid

Figure 8. tPA/plasmin has paradoxical roles in neonatal HIE. Our results suggest HI causes a unique response in neonatal brains through acute and persistent induction of the plasminogen activators. Further, our results show macrophage-microglia and astrocytes surrounding blood vessels and cerebral ventricles express tPA after HI in the newborn brain. The persistent tPA/plasmin activity may damage the extracellular matrix (ECM), BBB, neurons, and oligodendrocytes, leading to the onset of HIE.

fibrinolysis in neonatal brains. Consistent with this view, *tPA-null* mice show a higher mortality rate than wild-type mice during or shortly after HI (81.8% versus 12.5%, Figure 6E). Future studies are needed to compare the response to HI among several strains of mice with a mutation in components of the plasminogen system (including *tPA-null*, *uPA-null*, and *plasminogen-null* mice^{10,24}) to determine their relative functions for acute fibrinolysis in neonatal brains.

Although the induction of tPA/plasmin in the acute phase after HI may be protective, our study provided strong evidence that their persistent activity is a critical factor of brain injury in newborns (Figure 8). First, there is a high degree of spatial correlation between HI-induced tPA expression and brain regions that are most susceptible to this insult, including the hippocampus, the periventricular white matter, and the dorsolateral cerebral cortex (Figures 3 and 4). Furthermore, the double-labeling data indicate perivascular-periventricular astrocytes²⁸ and microphage-microglia are principle tPA-producing cell types after HI (Figure 5). This pattern of tPA expression, similar to the situation in excitotoxin-challenge,^{9,10} suggests the persistent tPA/plasmin activity after HI may induce extravascular proteolysis leading to brain damage. Consistent with this hypothesis, we showed that inhibition of extravascular proteolysis, by cerebroventricular injection of antiplasmin in the range of 6.25 to 25 μ g, provided dose-responding reduction of brain damage at 7-day recovery of HI (Figure 6). The less efficacy of 50 μ g of antiplasmin in ventricular injection may be attributable to nonspecific effects or drug diffusion into blood vessels at this high dose. Furthermore, we showed that ventricular injection of antiplasmin markedly protects against HI-induced neurofilament and oligodendrocyte damage before the onset of gross tissue degeneration (Figure 7). In contrast, ventricular injection of tPA significantly amplified the extent of HI brain damage (Figure 6). Together, these results strongly suggest persistent activation of endogenous tPA/plasmin is a critical factor of HI-induced brain damage through extravascular proteolysis.

Extracellular Proteolysis in HIE

HIE attributable to placenta insufficiency or birth asphyxia is an important cause of perinatal mortality and long-term neurological morbidity, including cerebral palsy, mental retardation, and epilepsy.^{1,33} Previous studies on the etiology of HIE focused on the risk of energy failure and the cell death signaling pathways within neurons. However, except for hypothermia therapy, none of these neuroprotective strategies have been translated to clinical practice against HIE.^{4,5} Consequently, the current therapy of HIE remains mainly supportive, and directed toward the systemic complications. Hence, better understanding the pathological mechanism of HIE and development of new therapies remains an urgent issues in neonatology.

In this regard, the present study is significant because it suggests a novel mechanism and a new therapeutic

target in HIE, namely tPA/plasmin-mediated extracellular proteolysis. Although this hypothesis is currently based on studies using a rodent model of neonatal HIE (the Vannucci model),² several lines of evidence suggest it is relevant to clinical settings. First, imaging studies rarely identify thrombosis in infants diagnosed with HIE, similar to the rapid recovery of cerebral blood flow after HI in the animal model.²¹ Moreover, the characteristic pathological finding of HIE in humans is cystic encephalomalacia, which is more consistent with an extracellular proteolysis-based mechanism of tissue injury than apoptosis-based cell elimination that does not typically lead to cystic cavitations.³³ Furthermore, the locations of *tPA* transcript in the Vannucci model correlate with the white matter lesions (periventricular leukomalacia) and germinal matrix hemorrhage often observed in the HIE infants.¹ Finally, tPA has been shown to increase oxygen-glucose deprivation-induced neuronal death *in vitro*,³⁴ and injection of tPA into the white matter induces oligodendrocyte damage in neonatal brains.^{35,36} Together, these circumstantial evidences suggest our findings are not limited to the rodent model, but may be relevant to HIE in clinical settings.

In conclusion, we suggest future studies are warranted to examine whether infants diagnosed with HIE have an elevated level of tPA and plasmin activity in the brain or the cerebrospinal fluid. If so, anti-tPA or anti-plasmin treatment in the extravascular space of neonatal brain may offer direct brain protection against this devastating disorder.

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