Tissue-Type Plasminogen Activator and the Low-Density Lipoprotein Receptor-Related Protein Mediate Cerebral Ischemia-Induced Nuclear Factor- κ B Pathway Activation

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Tissue-type plasminogen activator (tPA) is a serine proteinase found in the intravascular space and the central nervous system. The low-density lipoprotein receptor-related protein (LRP) is a member of the low-density lipoprotein receptor gene family found in neurons and astrocytes. Cerebral ischemia induces activation of the nuclear factor (NF)- κ B pathway. The present study investigated the role that the interaction between tPA and LRP plays on middle cerebral artery occlusion (MCAO)-induced NF- κ B-mediated inflammatory response. We found that MCAO increased LRP expression primarily in astrocytes and that this effect was significantly decreased in the absence of tPA. The onset of the ischemic insult induced activation of the NF- κ B pathway in wild-type and plasminogen (Plg^{-/-})-deficient mice, and this effect was attenuated after inhibition of LRP or genetic deficiency of tPA. Moreover, administration of tPA to tPA^{-/-} mice resulted in activation of the NF- κ B pathway comparable with that observed in wild-type and Plg^{-/-} mice. We also report that inhibition of either tPA activity or LRP or genetic deficiency of tPA resulted in a significant decrease in MCAO-induced nitric oxide production and inducible nitric-oxide synthase expression. In conclusion, our results demonstrate that after MCAO the interaction between tPA and LRP results in NF- κ B activation in astrocytes and induction of inducible nitric-oxide synthase expression in the ischemic tissue, suggesting a cytokine-like plasminogen-independent role for tPA during cerebral ischemia. (*Am J Pathol* 2007, 171:1281–1290; DOI: 10.2353/ajpath.2007.070472)

Tissue-type plasminogen activator (tPA) is a highly specific serine proteinase and one of the two main plasminogen activators.¹ In the intravascular space tPA is primarily a thrombolytic enzyme. However, tPA is also expressed within the central nervous system where it has been associated with learning,² synaptic plasticity,^{2–5} cell death,^{6–10} and regulation of the permeability of the neurovascular unit.^{11,12} Animal studies have demonstrated that after middle cerebral artery occlusion (MCAO) there is an increase in endogenous tPA activity within the ischemic tissue^{9,10,12} and that either genetic deficiency of tPA^{9,13} or its inhibition with neuroserpin^{10,14} are associated with neuronal survival, decrease in the volume of the ischemic lesion, and preservation of the function of the blood-brain barrier.^{11,12} Based on its thrombolytic properties, tPA is the only Food and Drug Administration-approved medication for the treatment of patients with acute ischemic stroke.¹⁵ Thus, it is of paramount importance to understand the mechanisms of tPA's harmful effects on the ischemic brain.

The low-density lipoprotein receptor-related protein (LRP) is a member of the low-density lipoprotein receptor gene family that interacts with multiple ligands including plasminogen activators.¹⁶ LRP has been implicated in cellular signal transduction pathways,¹⁷ and the interaction between tPA and LRP has been demonstrated to have an effect on cerebrovascular tone,¹⁸ matrix metalloproteinase-9 expression,¹⁹ and on the permeability of the neurovascular unit.^{11,12}

The onset of cerebral ischemia is accompanied by the rapid induction of a local inflammatory reaction mediated by activation of the nuclear factor (NF)- κ B pathway.²⁰

Supported in part by the National Institutes of Health (grants NS-49478 to M.Y., AG-023695 to Z.M., and NS-048254 to Z.M.) and The Robert W. Woodruff Health Sciences Center Fund.

Accepted for publication June 29, 2007.

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One of the genes regulated by the NF- κ B pathway is inducible nitric-oxide synthase (iNOS), which has been demonstrated to play a deleterious role during cerebral ischemia.²¹ Recent evidence suggests that LRP mediates NF- κ B pathway activation in some cell systems^{19,22} and the existence of a plasminogen-dependent relation between tPA and nitric oxide (NO) production in an animal model of nonischemic excitotoxic injury.²³ In the studies presented here, we demonstrate that cerebral ischemia induces a rapid cell-specific tPA-dependent increase in LRP expression in the ischemic brain. In addition, the interaction between endogenous tPA and LRP results in activation of the NF- κ B pathway and an increase in the expression of iNOS. Together, our results demonstrate a cytokine-like plasminogen-independent role for tPA during the early phases of ischemic injury.

Materials and Methods

Animal Model

Murine strains were tPA-deficient (tPA^{-/-}), plasminogen-deficient (Plg^{-/-}), and wild-type (WT) C57BL/6J mice. Animals were anesthetized with 4% chloral hydrate. The rectal and masseter muscle temperatures were controlled at 37°C with a homeothermic blanket. Cerebral perfusion in the distribution of the middle cerebral artery was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc., North Royalton, OH), and only animals with a >80% decrease in cerebral perfusion were included in this study. The middle cerebral artery was exposed and occluded with a 10-0 suture as described.^{12,13} Immediately after MCAO, a subgroup of mice was placed on a stereotactic frame and intracortically injected with 2 μ l of phosphate-buffered saline (PBS), the receptor-associated protein (RAP; 9 μ mol/L), or purified goat anti-LRP antibodies (85 μ g/ml). RAP and anti-LRP antibodies were kindly provided by Dr. Dudley Strickland from University of Maryland, Baltimore, MD. A subset of tPA^{-/-} animals was treated with PBS or a combination of tPA (1 μ mol/L; Molecular Innovations Inc., Royal Oak, MI) and RAP (9 μ mol/L). The injections were performed at bregma, -1 mm; mediolateral, 3 mm; and

dorsoventral, 3 mm²⁴ throughout 5 minutes, and the infusion rate was controlled by a microsyringe pump controller (World Precision Instruments, Sarasota, FL) attached to a syringe holder (World Precision Instruments). After the end of the infusion, the needle was left in place for 5 minutes to avoid reflow. Forty-eight hours later, brains were harvested in a subgroup of WT mice ($n = 6$), cut into 1-mm sections, and stained with 2,3,5-triphenyl-tetrazolium chloride to measure the volume of the ischemic lesion as described.¹³ Statistical analysis was performed with the Student's *t*-test. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Definition of Areas of Interest (AOI) and Immunofluorescence Studies

To define the AOI, animals were placed into a stereotaxic headset and on a cradle with a built-in surface coil for brain imaging and a neck coil for cerebral blood flow (CBF) labeling immediately after MCAO. Diffusion and perfusion weighted images were acquired at 30 and 180 minutes after ischemia as described elsewhere.¹¹ The mean difference between the volume of the brain with a >80% decrease in CBF (dark in Figure 1A) and changes in ADC maps (irreversibly damaged brain tissue, dark in Figure 1B and red in Figure 1C) was considered as the area of mismatch (ischemic penumbra, pseudocolored with yellow in Figure 1C). We then divided each coronal section into 16 square areas (150 mm² each) that involved the necrotic core and area of penumbra. Two AOI were chosen in the boundaries between the ischemic penumbra and necrotic core (AOI-1 and AOI-2), whereas a third zone was located in the necrotic core (AOI-3, Figure 1). For the immunohistochemical studies, 20 frozen brain sections 10 μ m each were obtained either 6 or 24 hours after MCAO and stained with monoclonal antibodies that detect NF- κ B p65 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), p65 only when phosphorylated at serine 536 (1:100; Cell Signaling, Boston, MA) or nitrotyrosine-containing proteins (1:100; Cayman Chemicals, Ann Arbor, MI), or with a polyclonal antibody

Figure 1. Definition of the AOI. Animals were subjected to MCAO followed by continuous MRI monitoring of CBF and apparent diffusion coefficient (ADC) of water. **A** and **B**: Changes in CBF (**A**) and ADC of water (**B**) 180 minutes after MCAO. **Arrows** in **A** depict the area of the brain with a >80% decrease in CBF (dark zone). **Arrows** in **B** denote the area of irreversibly injured brain (dark area). **C**: T₂-weighted image of the same brain of **A** and **B** showing the area of the ischemic brain with >80% decrease in CBF (pseudocolored in yellow) and the zone of the ischemic tissue irreversibly affected (pseudocolored in red). The **black squares** represent the areas of the brain where the observations for the immunohistochemical studies were performed.

against iNOS (1:100; Santa Cruz Biotechnology). Some sections were co-stained with a polyclonal antibody that detects glial fibrillary acidic protein (1:200; DakoCytomation, Glostrup, Denmark) and with the nuclear marker 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). Goat anti-rabbit secondary antibodies conjugated to Alexa 488 (Molecular Probes) and donkey anti-mouse antibodies conjugated to rhodamine Red-X (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies. As controls, a separate set of coverslips was incubated with an IgG isotype control or with the secondary antibody only. Observations were made in two AOI chosen in the boundaries between the ischemic penumbra and necrotic core (AOI-1 and AOI-2) and on a third zone located in the necrotic core (AOI-3) as described elsewhere.¹¹ For analysis, images were digitized in an Axioplan 2 (Zeiss, Thornwood, NY) microscope with a Zeiss AxioCam, imported into AxioVision and viewed at 150% of the original $\times 20$ images with Image MetaMorph software (MDS Analytical Technologies, Sunnyvale, CA). Four animals were included in each experimental group.

Cell Cultures

Neurons and astrocytes were cultured from C57BL/6J WT mice (E19 for neurons and 1-day-old/P1 for astrocytes) as described elsewhere.^{11,12,25} Cultures were washed with PBS 10 days later and then incubated under oxygen-glucose deprivation (OGD) conditions for 4 hours in an anaerobic chamber (Billups-Rothenberg, Inc., Del Mar, CA). A $<5\%$ oxygen concentration was confirmed with an oxymeter before starting the experiment. As a control, a similar group of cells was kept under normoxic conditions. To determine the effect of exposure to OGD conditions on cell death, we used a colorimetric assay to quantify the release of lactate dehydrogenase in the media of cultured neurons and astrocytes (Oxford Biomedical Research, Oxford, MI) following the manufacturer's instructions. A subset of astrocytes was incubated with $16\text{ }\mu\text{mol/L}$ neuroserpin, a selective tPA inhibitor, kindly provided by Dr. Daniel A. Lawrence from the University of Michigan, Ann Arbor, MI. After 4 hours of exposure to OGD conditions, cells were returned to the incubator for 20 hours. Quantitative real-time polymerase chain reaction (PCR) analysis for LRP and iNOS was performed with primers and reagents as described below.

Western Blot Analysis

Polyclonal antibodies to phospho-p65 and total p65 were purchased from Cell Signaling Technology and to nitrotyrosine from Cayman Chemical. Monoclonal antibodies to β -actin were obtained from Sigma-Aldrich (St. Louis, MO). WT, tPA^{-/-}, and Plg^{-/-} mice underwent MCAO. WT mice were injected into the ischemic area immediately after MCAO with either PBS or RAP or LRP-blocking antibodies. tPA^{-/-} mice were injected with PBS, murine tPA, or a combination of murine tPA and RAP at the concentrations and coordinates described above. Brains

were extracted 6 hours after MCAO for analysis of NF- κ B activation and 24 hours later for nitrotyrosine studies. Tissue was processed and gels were loaded as described elsewhere.^{25,26} Each observation was repeated five times.

Electrophoretic Mobility Shift Assay (EMSA)

WT, tPA^{-/-}, and Plg^{-/-} mice underwent MCAO as described above, and brains were extracted 6 hours later. Nuclear extracts were prepared from the ischemic tissue using the ProteoExtract subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany) previously demonstrated to be effective to study NF- κ B DNA-binding activity in brain extracts.²⁶ The NF- κ B consensus sense (5'-AGTTGAGGGGACTTTCCAGGC-3') and anti-sense (5'-GCCTGGGAAAGTCCCCTCAACT-3') oligonucleotides were purchased from Operon (Huntsville, AL) and labeled with [³²P]- γ -ATP using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) to produce double-stranded DNA probes. For binding reactions, $5\text{ }\mu\text{g}$ of nuclear extract were first preincubated in EMSA buffer (20 mmol/L HEPES, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 1 mmol/L ethylenediaminetetraacetic acid, and 5% glycerol) containing poly(dI-dC) at room temperature for 5 minutes. ³²P-labeled specific probes were added, and the reaction mixtures were incubated on ice for another 30 minutes. The reaction products were fractionated on a nondenaturing 6% polyacrylamide gel, dried, and subjected to autoradiography. Binding reactions were also conducted in the presence of an unlabeled excess concentration of unlabeled double-stranded probe. For specific complex disruption, $1\text{ }\mu\text{L}$ of anti-NF- κ B polyclonal antibodies (Santa Cruz Biotechnology) was included in the incubation buffer. Each observation was repeated three times.

Quantitative Real-Time PCR Analysis

WT and tPA^{-/-} mice underwent MCAO, followed by the intracerebral injection of $2\text{ }\mu\text{L}$ of RAP ($9\text{ }\mu\text{mol/L}$) in a subgroup of WT mice. Brains were extracted at 5 minutes to 72 hours later. WT neuronal and astrocytic cultures were incubated with PBS or neuroserpin and exposed to OGD conditions as described previously. Control cultures were maintained under normoxic conditions at 37°C. For quantitative measurement of mRNA, $2\text{ }\mu\text{g}$ of DNase I-treated total RNA was used for cDNA synthesis. Reverse transcription was performed with a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA) with random oligonucleotide primers. TaqMan gene expression assays of TaqMan probes and primers for iNOS (Mm00440485-m1) and LRP (Mm00464608-m1) were purchased from Applied Biosystems. Polymerase chain reactions were performed in an ABI Prism 7000 system (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Each observation was repeated six times for each time point. PCR results were analyzed as described elsewhere,²⁷ and statistical analysis was performed with the Student's *t*-test.

Figure 2. Effect of genetic deficiency of tPA on cerebral ischemia-induced NF-κB activation. **A** and **B:** Representative pictures of immunohistochemical analysis of p65 (**A**) and p65 phosphorylated at serine 536 (**B**) in the area of ischemic penumbra in WT (**a–c**) and *tPA*^{-/-} mice (**d–f**) 6 hours after MCAO. **a** and **d:** Combined immunostaining for glial fibrillary acidic protein and DAPI; **b** and **e:** immunostaining for either p65 (**A**) or phosphorylated p65 (**B**); **c** and **f:** merged images. **Arrows** in **b** depict cells with either nuclear translocation of p65 (**A**) or phosphorylated p65 (**B**) indicative of NF-κB activation. **Arrows** in **e** (**A**) point to cells where p65 is primarily located in the cytoplasm indicative of inactive NF-κB pathway. Green is glial fibrillary acidic protein, red is p65 (**A**) or phosphorylated p65 (**B**), and blue is DAPI. Each observation was repeated four times. **C:** Western blot analysis of phospho-p65 and total p65 in brain extracts of WT and *tPA*^{-/-} mice 6 hours after MCAO. Actin expression levels were assayed as a control for protein loading. Each experiment was repeated four times. **D:** Representative gel of an EMSA in brain nuclear extracts from WT sham (**lane 1**), WT sham and an excess of unlabeled probe (**lane 2**), WT 6 hours after stroke (**lane 3**), *tPA*^{-/-} mice 6 hours after MCAO (**lane 4**), and WT sham treated with an anti-NF-κB antibody (**lane 5**). **Black arrow** points toward NF-κB DNA-binding activity. Each observation was repeated four times. Original magnifications, ×100.

Results

Effect of tPA on Cerebral Ischemia-Induced NF-κB Pathway Activation

To study the role of tPA on NF-κB pathway activation during cerebral ischemia, we performed immunohistochemical staining of brain sections from WT and *tPA*^{-/-} mice 6 hours after MCAO with antibodies directed against either p65 protein or p65 phosphorylated at Ser536. We observed that in WT mice there was a significant increase in both the number of cells with p65 immunoreactivity in the nucleus (Figure 2Ab) and phosphorylated p65 (Figure 2Bb) in the AOI-1 and -2, indicating activation of the NF-κB pathway in the zone of ischemic penumbra. This response was significantly decreased in each one of these AOI in *tPA*^{-/-} mice (Figure 2, Ae and Be). To characterize these results further, we performed Western blot analysis for phospho-p65 and total p65 in WT and *tPA*^{-/-} mice 6 hours after MCAO. We observed a rapid phosphorylation of p65 protein in WT mice that was significantly decreased in *tPA*^{-/-} animals (Figure 2C). To corroborate these observations further, we performed an EMSA using nuclear extracts from WT and *tPA*^{-/-} brains 6 hours after MCAO. We found that the ischemic insult induced a significant increase in NF-κB DNA-binding activity 6 hours after the onset of the ischemic insult in WT animals (Figure 2D, lane 3, black arrow)

that was not observed in *tPA*^{-/-} mice (Figure 2D, lane 4). Together, our data demonstrate that the absence of tPA results in a significant decrease of MCAO-induced NF-κB pathway activation.

Role of Plasminogen on tPA-Mediated Cerebral Ischemia-Induced NF-κB Pathway Activation

To determine whether the effect of tPA on NF-κB is mediated by activation of plasminogen, WT and *Plg*^{-/-} mice underwent MCAO followed 6 hours later by Western blot analysis for phospho-p65 and total p65 and EMSA of nuclear extracts. We found that MCAO induces phosphorylation of p65 protein and a significant increase in NF-κB DNA binding in *Plg*^{-/-} mice comparable with that observed in WT animals (Figure 3). These results demonstrate that tPA has an effect on NF-κB pathway activation independent of plasminogen and suggests the presence of a different substrate mediating tPA's effect on the NF-κB pathway.

Effect of Cerebral Ischemia/Hypoxia on LRP Expression

It has been demonstrated that LRP is a substrate for tPA in the central nervous system.^{11,28–31} Thus, to investigate

Figure 3. Effect of genetic deficiency of plasminogen on cerebral ischemia-induced NF- κ B activation. **A:** Western blot analysis of phospho-p65 and total p65 in brain extracts of WT, tPA^{-/-}, and Plg^{-/-} mice 6 hours after MCAO. Actin expression levels were assayed as a control for protein loading. Each experiment was repeated four times. **B:** Representative gel of an EMSA in brain nuclear extracts from WT sham (lane 1), WT sham and an excess of cold probe (lane 2), WT 6 hours after stroke (lane 3), Plg^{-/-} mice 6 hours after MCAO (lane 4), and WT sham treated with an anti-NF- κ B antibody (lane 5). Black arrow points toward NF- κ B DNA-binding activity. Each observation was repeated three times.

the effect of cerebral ischemia on LRP expression and whether endogenous tPA plays a role on this outcome, we performed quantitative real-time polymerase chain reaction (RT-PCR) analysis for LRP in brain extracts from WT and tPA^{-/-} mice 5 minutes to 72 hours after MCAO. We found a significant increase in LRP expression in the ischemic tissue of WT mice 3 hours after MCAO (1.7 ± 0.17 -fold increase in mRNA, $P < 0.05$) that peaked 48 to 72 hours later (2.7 ± 0.2 -fold increase in mRNA, $P < 0.05$; Figure 4A). In contrast, this early increase in LRP expression was absent in tPA^{-/-} mice. Indeed, we observed an increase in LRP expression in tPA^{-/-} mice only 72 hours after MCAO (2.01 ± 0.2 -fold increase) but still significantly decreased when compared with WT animals at the same time point. To determine what cell(s) in the brain up-regulate LRP after the ischemic insult and the role of tPA on this response, the expression of LRP was studied in primary neuronal and astrocytic cultures from WT animals exposed to OGD conditions in the presence or absence of the selective tPA inhibitor neuroserpin. To determine the effect of exposure to OGD conditions on cell death, we measured the release of lactate dehydro-

Figure 4. tPA mediates cerebral ischemia-induced LRP expression in the ischemic brain. **A:** Quantitative RT-PCR analysis of LRP expression in brain extracts from WT (black bars) and tPA^{-/-} mice (white bars) 0.1 to 72 hours after MCAO. Error bars described SEM. $n = 7$ for each observation. * $P < 0.05$ when compared with WT animals sacrificed 0.1 hour after MCAO. ^ $P < 0.05$ when compared with tPA^{-/-} mice at each time point. † $P < 0.05$ when compared with tPA^{-/-} mice sacrificed 0.1 to 48 hours after MCAO. **B:** RT-PCR analysis of LRP expression in primary murine astrocytic and neuronal cultures exposed to OGD conditions for 4 hours and either PBS (black bars) or neuroserpin (NS, white bars). $n = 3$ for each observation. * $P < 0.05$ when compared with NS-treated cells.

genase into the media in both sets of cultures. We found that in our system exposure to OGD conditions results in $<5\%$ release of lactate dehydrogenase from astrocytes and $<15\%$ from neurons compared with cultures treated with the cell lysing reagent provided by the manufacturer. We found that, compared with cultures maintained under normoxic conditions, exposure of astrocytes to OGD conditions induced a 4.0 ± 0.9 -fold increase in LRP mRNA (Figure 4B; $P < 0.05$) that was significantly decreased on incubation with neuroserpin (1.3 ± 0.80 -fold increase). In contrast, we failed to detect a significant change on LRP expression when neuronal cultures were exposed to OGD conditions in absence of neuroserpin (1.1 ± 0.03 -fold increase). However, incubation of neurons with neuroserpin under OGD conditions resulted in a 3.0 ± 0.5 -fold increase in LRP mRNA (Figure 4B, $P < 0.05$). Finally, incubation of either neurons or astrocytes with $1 \mu\text{mol/L}$ recombinant tPA under normoxic conditions failed to induce a significant increase in LRP expression.

Figure 5. Effect of LRP inhibition on the volume of the ischemic lesion after MCAO. Quantitative analysis of infarct volume 48 hours after MCAO in WT mice treated with PBS (black bar), RAP (white bar), or anti-LRP antibodies (gray bar). $n = 6$. Lines denote SEM. * $P < 0.05$ compared with PBS-treated animals.

Figure 6. Effect of LRP inhibition on cerebral ischemia-induced NF- κ B activation. **A** and **B**: Representative gel of a Western blot analysis of phospho-p65 and total p65 in brain extracts of WT (**A**) and tPA-deficient (tPA^{-/-}) mice 6 hours after MCAO and the intracortical injection of the RAP or anti-LRP IgG in WT mice (**B**), and recombinant tPA or a combination of recombinant tPA and RAP in tPA^{-/-} animals. Actin expression levels were assayed as a control for protein loading. Each experiment was repeated four times.

Effect of LRP Inhibition on the Volume of the Ischemic Lesion after MCAO

To study the effect of LRP inhibition after MCAO, the volume of the ischemic lesion was measured in WT animals sacrificed 48 hours after the onset of the ischemic insult and treatment with either PBS, RAP, or anti-LRP antibodies as described in Materials and Methods. We found that the volume of the ischemic lesion decreased from 17.2 ± 1.1 mm³ in WT littermate controls to 8.25 ± 1.8 mm³ in RAP-treated mice and 9.33 ± 2.1 mm³ in animals treated with anti-LRP antibodies (48% and 46% reduction in the volume of the ischemic lesion, respectively, $n = 6$, $P < 0.05$ compared with untreated mice; Figure 5).

LRP and Cerebral Ischemia-Induced NF- κ B Pathway Activation

The existence of a relationship between LRP and NF- κ B activation has been suggested.¹⁹ In addition, it has been demonstrated that inhibition of the NF- κ B pathway after MCAO results in a significant decrease in the volume of the ischemic lesion.^{20,32,33} Thus, we decided to investigate the role of LRP on cerebral ischemia-induced NF- κ B pathway activation. WT mice underwent MCAO and treatment with either RAP or anti-LRP IgG followed 6 hours later by Western blot analysis of p65 phosphorylation in

the ischemic tissue. We found that compared with PBS-injected animals, inhibition of LRP resulted in a significant decrease in p65 phosphorylation (Figure 6A). To investigate whether tPA needs to interact with LRP to induce NF- κ B activation, tPA^{-/-} mice underwent MCAO followed by the intracerebral injection of either tPA alone or tPA in combination with RAP. We found that the intracerebral injection of tPA into tPA^{-/-} mice after MCAO resulted in an increase in p65 phosphorylation similar to that observed in WT mice and significantly increased compared with that observed in untreated tPA^{-/-} animals. The effect of tPA on p65 phosphorylation was inhibited in animals treated with a combination of tPA and RAP (Figure 6B), demonstrating that the interaction between tPA and LRP is necessary for the effect of tPA on NF- κ B pathway activation.

tPA and Cerebral Ischemia-Induced NO Production

One of the consequences of increased NO production is the accumulation of nitrotyrosine on nearby proteins. To investigate the role of tPA on ONOO⁻ production during cerebral ischemia, we performed immunohistochemical staining for nitrotyrosine in both WT and tPA^{-/-} mice 48 hours after MCAO. We found a large number of nitrotyrosine-positive cells scattered in each one of the AOI in the ischemic hemisphere of WT mice that was notably decreased in tPA^{-/-} animals (Figure 7A). These results indicated a relation between endogenous tPA and NO production during cerebral ischemia. To confirm our results and to study the relation between tPA and LRP on NO production, we performed Western blot analysis for nitrotyrosine 48 hours after MCAO in brain extracts from tPA^{-/-} and WT mice treated with either RAP or anti-LRP IgG immediately after MCAO. We found that cerebral ischemia induced an increase in nitrotyrosine formation in WT animals that was significantly decreased in both tPA^{-/-} mice and WT animals treated with either anti-LRP IgG or RAP (Figure 7B).

Figure 7. Role of tPA and LRP on cerebral ischemia-induced nitrotyrosine production. **A**: Representative pictures of immunohistochemical analysis of nitrotyrosine formation in the area of ischemic penumbra (AOI-2) in WT (**a**) and tPA^{-/-} (**b**) mice 48 hours after MCAO. **Arrows** point to examples of nitrotyrosine-immunoreactive cells. Blue is DAPI; red is nitrotyrosine. Each observation was repeated four times. **B**: Western blot analysis of nitrotyrosine formation 48 hours after MCAO in WT and tPA^{-/-} mice. A subgroup of WT mice was treated with either anti-LRP IgG (α -LRP) or the RAP. Each observation was repeated three times. Original magnifications, $\times 40$.

Figure 8. Role of tPA and LRP on cerebral ischemia-induced iNOS expression. **A:** Representative picture of immunohistochemical analysis of iNOS expression in the area of ischemic penumbra (AOI-2) in WT (**a** and **b**) and tPA^{-/-} (**c** and **d**) mice 24 hours after MCAO. Green is iNOS, and blue is DAPI. NC, necrotic core. Each observation was repeated four times. **B:** Quantitative real-time PCR (RT-PCR) analysis of iNOS expression in brain extracts from WT (black bars), tPA^{-/-} (white bars), and WT mice treated with the RAP (gray bars) 0.1 to 72 hours after MCAO. Error bars described SEM. $n = 7$ for each observation. * $P < 0.05$ when compared with treated and untreated WT animals sacrificed at each time point after MCAO. † $P < 0.05$ when compared with untreated WT mice at each time point. **C:** RT-PCR analysis of iNOS expression in primary murine astrocytic cultures exposed to OGD conditions for 4 hours and incubation with either PBS (black bars) or PBS and neuroserpin (NS, white bars). $n = 4$ for each observation. * $P < 0.05$ when compared with NS-treated cells. Original magnifications, $\times 40$.

Effect of tPA and LRP on iNOS Expression

The onset of cerebral ischemia is followed by a sharp increase in the expression of iNOS.^{21,34,35} In addition, NF- κ B has been identified as an essential requirement for the expression of this gene.³⁶ To study the role of tPA on iNOS production after cerebral ischemia, we performed

immunohistochemical analysis for iNOS in WT and tPA^{-/-} mice 48 hours after MCAO. We found a significant increase in iNOS immunoreactivity in WT animals in the AOI-1 and -2 that was significantly decreased in tPA^{-/-} mice (Figure 8A). To characterize these results further and to study the role of the interaction between tPA and LRP on iNOS expression after MCAO, we performed quantitative RT-PCR analysis for iNOS in brain extracts from WT and tPA^{-/-} mice 5 minutes to 72 hours after MCAO. A subgroup of WT mice was treated with RAP. We observed a significant increase in iNOS mRNA expression in the ischemic tissue of WT mice 24 hours after MCAO (2.5 ± 0.198 -fold increase, $n = 6$; $P < 0.05$) that was maximal 72 hours later (2.8 ± 0.31 , $n = 6$; $P < 0.05$) and significantly decreased after inhibition of LRP or genetic deficiency of tPA (Figure 8B). To determine the cell(s) in the brain that up-regulate iNOS in response to the ischemic insult and the role of tPA on this response, the expression of iNOS was studied in primary neuronal and astrocytic cultures from WT animals exposed to OGD conditions in the presence or absence of neuroserpin as described in Materials and Methods. We found that exposure to OGD conditions failed to increase iNOS expression in neurons (data not shown). In contrast, there was a 4.0 ± 0.8 -fold increase in iNOS expression in astrocytes exposed to OGD conditions (Figure 8C; $P < 0.05$, $n = 4$) that was significantly decreased when astrocytes were incubated with neuroserpin (1.3 ± 0.76). Moreover, as observed with LRP, incubation of neurons and astrocytes with $1 \mu\text{mol/L}$ recombinant tPA under normoxic conditions failed to induce a significant increase in iNOS expression.

Discussion

TPA is a serine proteinase found in the intravascular space and the central nervous system. In the vascular system tPA's principal substrate is plasminogen,³⁷ and its main role is as a thrombolytic enzyme. The presence of tPA in the intravascular space during cerebral ischemia is deemed as beneficial,³⁸ and treatment of acute ischemic stroke patients with recombinant tPA results in a 30% chance of complete or near complete recovery 90 days later.¹⁵ However, the ischemic insult also induces an increase in tPA activity in the ischemic brain^{9,10} that has been associated with worsening of the volume of the ischemic lesion,^{9,10,13} cell death,^{6,7} and increase in the permeability of the neurovascular unit.^{11,12} In addition, some studies have demonstrated the passage of tPA from the intravascular space into the brain under ischemic and nonischemic conditions.^{39,40} Because tPA is the only Food and Drug Administration-approved medication for the treatment of patients with acute ischemic stroke,¹⁵ it is important to investigate the mechanisms of tPA's deleterious effects during cerebral ischemia.

There is a growing body of evidence indicating that in the brain tPA acts through a plasminogen-independent mechanism.^{11,12} Here we demonstrated that tPA mediates cerebral ischemia-induced NF- κ B pathway activation. This is a plasminogen-independent event that is

mediated by the interaction of tPA with LRP. LRP is assembled by a 515-kD heavy chain noncovalently bound to an 85-kD light chain containing a transmembrane and a cytoplasmic domain.¹⁶ LRP mediates the internalization of apoE-enriched lipoprotein particles,⁴¹ α -2-Macroglobulin-protease complexes⁴² and several other ligands including plasminogen activators, proteinase-inhibitor complexes, clotting factors, and the amyloid precursor protein.¹⁶ LRP is expressed in smooth muscle cells, and specific deletion of the LRP gene from vascular smooth muscle cells on a background of low-density lipoprotein receptor deficiency causes smooth muscle cell proliferation, increased susceptibility to cholesterol-induced atherosclerosis, and aneurysm formation.⁴³ In the brain LRP is found in neurons and in perivascular astrocytes.^{11,44} In neurons, LRP mediates events such as long-term potentiation⁴⁵ and calcium influx via NMDA receptors.⁴⁶ In contrast, its function in astrocytes is less well characterized.

We found that MCAO induces a rapid and sustained tPA-dependent increase in LRP expression in the ischemic brain. This effect seems to be cell-type specific. Indeed, after exposure to OGD conditions, LRP expression increases in astrocytes and decreases in neurons. In both cases, the effect of OGD on LRP expression is blocked by inhibition of tPA with neuroserpin. Analysis of lactate dehydrogenase release into the media demonstrates that the effect observed on neurons is not attributable to OGD-induced cell death. Together, our results demonstrate that cerebral ischemia has a tPA-dependent, cell-type-specific effect on the expression of LRP in the ischemic tissue and suggests the possibility that under ischemic conditions LRP may play multiple cell type-specific roles. The lack of effect of recombinant tPA on LRP and iNOS expression under normoxic conditions suggests the presence of an unknown hypoxia/ischemia-induced co-factor for tPA. In addition, the divergent effect of tPA on neurons and astrocytes indicates the existence of different cell type-dependent regulatory mechanisms mediating the effect of tPA on LRP and iNOS expression. Our data demonstrate that LRP inhibition after MCAO results in a significant decrease in the volume of the ischemic lesion. This suggests that the up-regulation of LRP after the onset of the ischemic insult may have a deleterious effect on cell survival. It has been demonstrated in *in vitro* neuronal cultures that anti-LRP antibodies can dimerize LRP leading to increased influx of calcium in the cell.⁴⁶ This does not seem to be the case in our animal model of cerebral ischemia. In fact, our *in vivo* experiments demonstrated that inhibition of LRP with anti-LRP antibodies is protective and that this effect is also observed when animals are treated with RAP.

It is unknown whether tPA regulates directly the expression of LRP. However, a relation between the composition of the extracellular matrix and LRP expression has been demonstrated.^{47,48} MCAO induces a rapid change in the composition of the extracellular matrix⁴⁹ associated with local increases in tPA activity.^{12,31} Therefore, it is possible to postulate a model whereby LRP expression increases in response to cerebral ischemia-induced tPA-mediated degradation of the extracellular

matrix, suggesting that after MCAO the expression of LRP is posttranscriptionally regulated in response to changes in the composition of the extracellular matrix.

The NF- κ B family includes five structurally related proteins that bind to a specific DNA motif and regulate gene expression.^{50,51} Activation of NF- κ B has been observed in transient and permanent models of cerebral ischemia.^{20,52} *In vitro* experiments have demonstrated that the effect of NF- κ B under ischemic conditions is cell-type-specific. Indeed, whereas activation of NF- κ B in glial cells results in cell death, activation of NF- κ B in neurons is protective.^{53,54} In contrast, inhibition of NF- κ B in *in vivo* models of cerebral ischemia by either genetic deficiency of p50^{20,33} or administration of a recombinant adenovirus expressing a dominant-negative form of I κ B α ⁵⁵ have been shown to be protective.

In earlier work we demonstrated that the onset of the ischemic insult induces a tPA-dependent shedding of LRP's ectodomain from perivascular astrocytes into the basement membrane associated with increase in the permeability of the neurovascular unit and development of cerebral edema.¹¹ Based on these observations and the data presented here, we postulate the hypothesis that cerebral ischemia-induced tPA-mediated shedding of LRP's ectodomain from astrocytes precedes the release of LRP's intracellular domain, which then may activate the NF- κ B pathway. This possibility is strongly supported by our results indicating a significant decrease in MCAO-induced NF- κ B activation after inhibition of LRP or genetic deficiency of tPA. It should be kept in mind that in the EMSA studies the binding of the antibody to the NF- κ B subunit (p65) may prevent the association of p65 and the labeled probe so that there is a decrease or absence of the shifted band. In our studies we demonstrate that genetic deficiency of tPA inhibits MCAO-induced NF- κ B DNA-binding activity, which remains unchanged in Plg^{-/-} mice. The fact that the intracortical injection of tPA into the brain of tPA^{-/-} mice after MCAO induced a rapid and sustained phosphorylation of p65, and the observation that this effect was inhibited when tPA was co-administered with RAP demonstrates that the effect of tPA on NF- κ B is mediated by its interaction with LRP.

The onset of the ischemic insult induces an NF- κ B-mediated inflammatory reaction characterized by the generation of free radicals and release of proinflammatory cytokines.^{20,32,33,56} One of these free radicals is NO, which is generated in response to the ischemic insult.^{21,34,35} A plasminogen-dependent relation between tPA and NO production after nonischemic excitotoxic injury has been demonstrated by others.²³ Here we demonstrate that under ischemic conditions tPA mediates the production of NO by a plasminogen-independent, LRP-mediated mechanism.

In the brain there are three isoforms of nitric-oxide synthase (NOS): neuronal (nNOS, constitutively expressed in neurons), inducible (iNOS, expressed by glial cells on exposure to different stimuli), and endothelial (eNOS, found in endothelial cells).⁵⁷⁻⁶⁰ The onset of the ischemic insult induces a progressive increase in iNOS expression that peaks 24 to 48 hours later.⁶¹ Moreover,

inhibition of iNOS results in a significant decrease in the volume of the ischemic lesion 48 hours after MCAO.^{21,35} The increase in the expression of iNOS after MCAO is originated in infiltrating cells, astrocytes, and microglia.⁶² However, induced neutropenia does not have an effect on iNOS activity, the volume of the ischemic lesion,⁶³ suggesting that astrocytes and microglia are the main source of iNOS. Our results demonstrating an increase in iNOS expression in astrocytes exposed to OGD conditions supports the hypothesis that astrocytes are one of the main sources of iNOS during cerebral ischemia. Activation of the NF- κ B pathway has been identified as an essential requirement for the expression of iNOS,³⁶ and exposure to low-density lipoprotein increases NO production in astrocytes.⁶⁴ Our results demonstrate that after a hypoxic/ischemic injury there is a sustained increase in iNOS mRNA and that this rise is significantly attenuated by inhibition of either tPA or LRP, or genetic deficiency of tPA. Together, our results demonstrate that during cerebral ischemia the interaction between tPA and LRP induces the expression of iNOS in astrocytes. In summary, we propose a model in which in response to the ischemic injury there is a rise in tPA activity in the ischemic area. This tPA interacts with LRP on astrocytes, inducing activation of the NF- κ B pathway and an increase in the expression of iNOS. Our results suggest a cytokine-like role for tPA during cerebral ischemia.

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