ing that a cellular repair protein or proteins are required for this reaction may have practical ramifications. Such proteins represent a previously unrecognized set of targets for inhibition of this early step in virus replication.

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11. To construct an IN$^-$ variant of R/M, we first subcloned a neo^r marker into the Cla I site of this vector, creating R/Mneo. To introduce the D64E-encoding mutation, we replaced an Hpa I/Asp718 fragment of R/Mneo with that of a SR-B proviral DNA carrying the IN-inactivating D64E mutation [J. Kulkosky, K. S. Jones, R. A. Katz, J. P. G. Mack, A. M. Skalka, *Mol. Cell. Biol.* **12**, 2331 (1992)], creating R/M(D64E)neo, denoted as IN$^-$ virus. A control virus was constructed by replacing an Hpa I/Asp718 fragment of R/Mneo with that of the wild-type SR-B proviral DNA, creating R/M(8KS)neo, denoted as IN$^+$ virus. Producer cells for these viruses were generated first by transfection of Q2bn helper cells [A. W. Stoker and M. J. Bissell, *J. Virol.* **62**, 1008 (1988)] with proviral DNA, followed by infection of DF-1 cells with the supernatant 3 days after transfection. DF-1 cells were selected for G418 resistance 1 day after infection. G418-resistant colonies were then pooled, and the titers of virus produced by these cells were determined by RT assay.
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Nonproteolytic Neuroprotection by Human Recombinant Tissue Plasminogen Activator

Yang-Hee Kim,[1,2] June-Hee Park,[1] Seung Hwan Hong,[2] Jae-Young Koh[1]*

Human recombinant tissue plasminogen activator (tPA) may benefit ischemic stroke patients by dissolving clots. However, independent of thrombolysis, tPA may also have deleterious effects on neurons by promoting excitotoxicity. Zinc neurotoxicity has been shown to be an additional key mechanism in brain injuries. Hence, if tPA affects zinc neurotoxicity, this may provide additional insights into its effect on neuronal death. Independent of its proteolytic action, tPA markedly attenuated zinc-induced cell death in cortical culture, and, when injected into cerebrospinal fluid, also reduced kainate seizure–induced hippocampal neuronal death in adult rats.

Human recombinant tPA can benefit stroke patients by lysing clots (*1*). However, recent evidence suggests that it may also harm neurons by promoting excitotoxic injury (*2*). This detrimental effect of tPA may be mediated by plasmin, the main product of tPA action in thrombolysis, because not only tPA-null but also plasminogen-null mice are resistant to excitotoxic injury (*3*).

Although excitotoxicity is a key mechanism of pathologic neuronal death in many cases (*4*), neurotoxicity mediated by endogenous zinc translocation has recently been shown to be another major mechanism of selective neuronal death in global isch-

[1]National Creative Research Initiative Center for the Study of Central Nervous System Zinc and Department of Neurology, University of Ulsan College of Medicine, 388-1 Poongnap-Dong Songpa-Gu, Seoul 138-736, Korea. [2]Department of Molecular Biology and Institute for Molecular Biology and Genetics, Seoul National University, Seoul, Korea.

*To whom correspondence should be addressed. E-mail: jkko@www.amc.seoul.kr

emia and seizures (*5*). In transient global cerebral ischemia and induction of kainate seizures, zinc accumulation was observed in degenerating neuronal cell bodies (*6*), and an extracellular zinc chelator injected into cerebrospinal fluid (CSF) markedly reduced both zinc accumulation and neuronal death (*7*).

Exposure of cortical cultures to 35 μM zinc for 24 hours resulted in widespread neuronal death the next day (Fig. 1A) (*8, 9*). Addition of tPA (10 μg/ml) completely blocked the zinc-induced cell death (Fig. 1B). In contrast, neuronal death induced by 24 hours of exposure to 30 μM *N*-methyl-D-aspartate (NMDA) was not attenuated by tPA (Fig. 1, C and D). The cytoprotective effect of tPA was seen throughout the zinc concentration range tested, in both the 24-hour and 10-min exposure paradigms (*8*), as quantitatively assessed by the lactate dehydrogenase (LDH) release assay (*10*) (Fig. 1E). tPA completely blocked not only neuronal death at lower zinc concentrations but also additional astrocytic cell death at higher concentrations. In contrast to this protective effect against zinc toxicity, tPA did not alter excitotoxic

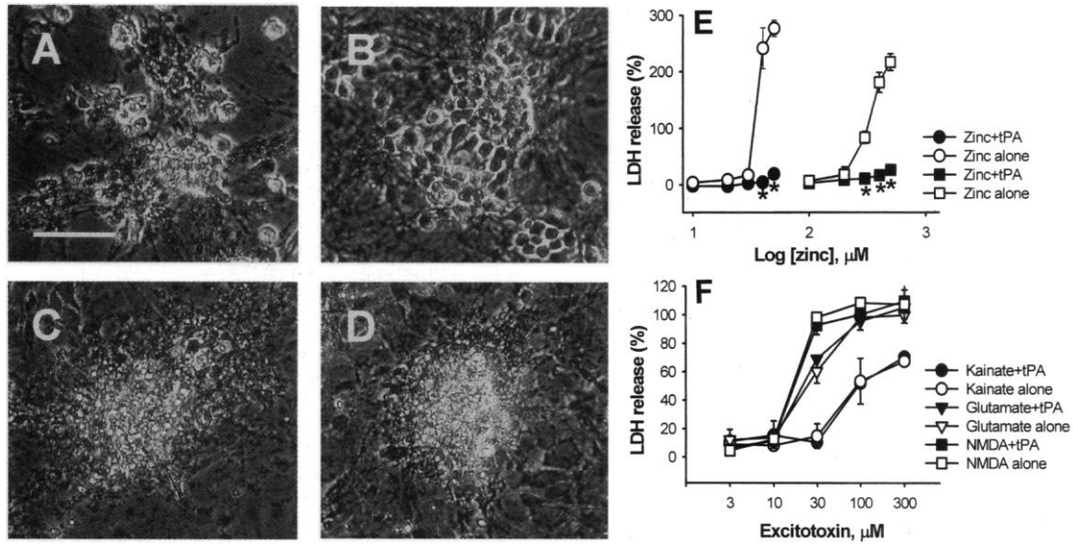

Fig. 1. tPA attenuates Zn toxicity but not calcium-overload excitotoxicity. (**A** to **D**) Phase-contrast photomicrographs of sister cortical cultures after 24 hours of exposure to 35 μM zinc alone (A) or with addition of tPA (10 μg/ml) (B), and to 30 μM NMDA alone (C) or with addition of tPA (D). Scale bar, 50 μm. (**E**) Data represent LDH release (mean ± SEM, $n = 4$) in cultures, 24 hours after 10 min of exposure (squares) or after 24 hours of exposure (circles) to indicated concentrations of zinc without (open symbols) or with addition of tPA (10 μg/ml) (solid symbols). In both conditions, tPA was present in culture medium from the beginning of zinc exposure until the LDH assay. Asterisks denote difference from zinc alone ($p < 0.05$, two-tailed t test). (**F**) LDH release in cultures (mean ± SEM, $n = 4$) after 24 hours of exposure to indicated concentrations of glutamate, NMDA, or kainate without (open symbols) or with addition of tPA (10 μg/ml) (solid symbols).

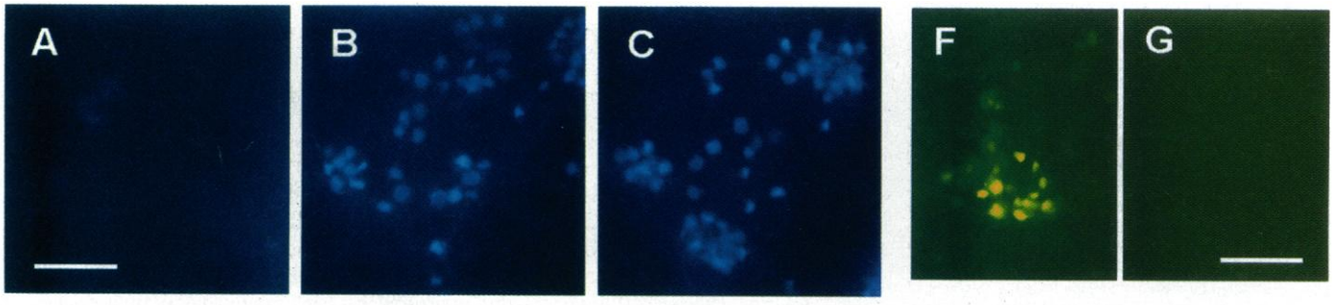

Fig. 2. The site of tPA protection may be downstream of zinc influx, probably somewhere in the oxidative injury cascade. (**A** to **C**) Fluorescence photomicrographs of TSQ-stained cortical cultures, immediately after 10 min of exposure to HBSS alone (A) or to 300 μM zinc without (B) or with addition of tPA (10 μg/ml) during the zinc exposure (C). Scale bar, 50 μm. (**D**) LDH release in cultures 24 hours after 10 min of exposure to 300 μM zinc alone (CTRL) or with addition of tPA (10 μg/ml) both during and after (Co) or only after (Post) zinc exposure. Asterisks denote difference from CTRL ($p < 0.05$, two-tailed t test with Bonferroni correction for two comparisons). (**E**) Bars represent LDH release (mean ± SEM, $n = 4$) in cultures after 24 hours of exposure to 100 μM Fe^{3+} (Fe), 100 μM H_2O_2, 1 mM BSO, 2 μM TPEN, or 100 nM staurosporine (STSP) alone or in the presence of tPA (10 μg/ml). Asterisks denote difference from toxin alone ($p < 0.05$, two-tailed t test). (**F** and **G**) Fluorescence photomicrographs of sister cultures stained with DCF 18 hours after 10 min of exposure to 300 μM zinc alone (F) or with addition of tPA (10 μg/ml) (G). Scale bar, 50 μm. (**H**) Bars denote increases in the membrane thiobarbituric acid–reactive substances (TBARS; mean ± SEM, $n = 3$) over that of sham wash controls, after 14 hours of exposure to 35 μM zinc or 100 μM Fe^{3+} (Fe), alone or with addition of tPA (10 μg/ml). Asterisks denote difference from zinc or Fe^{3+} alone, respectively ($p < 0.05$, two-tailed t test with Bonferroni correction for two comparisons).

neuronal death induced by 24 hours of exposure to 3 to 300 μM NMDA, kainate, or glutamate (Fig. 1F).

Because zinc neurotoxicity is triggered by the influx of zinc into neurons (*11*), we examined the possibility that tPA attenuates the toxic zinc influx. However, visualization of zinc influx with a zinc-specific fluorescent dye, 6-methoxy-8-quinolyl-*p*-toluenesulfonamide (TSQ) (*12*), revealed no difference in zinc accumulation in cultured neurons exposed for 10 min to 300 μM zinc with or without the addition of tPA (Fig. 2, A to C). Furthermore, if the protective effect of tPA were based on the reduction of zinc influx, then tPA given after the 10-min zinc exposure should not be protective, as zinc influx cannot occur after zinc washout. However, tPA given after zinc exposure was as protective as tPA given both during and after zinc exposure (Fig. 2D).

Previously, we showed that zinc neurotoxicity is mainly mediated by oxidative stress in cortical culture, because it is accompanied by increases in lipid peroxidation and cytosolic 2,7-dichlorofluorescein diacetate (DCF) fluorescence, and is blocked by antioxidants (*8*). tPA attenuated oxidative stress–related neuronal death induced by exposure to Fe^{3+}, H_2O_2, or a γ-glutamyl-cysteine synthetase inhibitor, buthionine sulfoximine (BSO) (*13*). On the other hand, protein synthesis–dependent but antioxidant-insensitive neuronal apoptosis induced by staurosporine or *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (*14*) was not altered by addition of tPA (Fig. 2E). Consistent with its antioxidative effect, tPA markedly attenuated DCF fluorescence (Fig. 2, F and G) and membrane lipid peroxidation (Fig. 2H) increased by zinc exposure (*15*).

By cleaving plasminogen, tPA produces plasmin, the final effector protease in the thrombolytic cascade (*16*). Plasmin aggravated cell death induced by mild zinc exposure (*3*) but did not alter NMDA- or kainate-induced neuronal death (Fig. 3A). Intriguingly, the protective effect of tPA (10 μg/ml) was not reversed by excessive amounts (30 μg/ml) of plasminogen activator inhibitor–1 (PAI-1) (*17*), indicating that the protection was unlikely mediated by its proteolytic action (Fig. 3B). tPA has an epidermal growth factor (EGF)–homologous domain (*18*) and induces endothelial cell proliferation by activating protein kinase A (PKA) (*19*). However, EGF did not attenuate zinc-induced cell death, nor did the PKA inhibitor H-89 or EGF reverse the

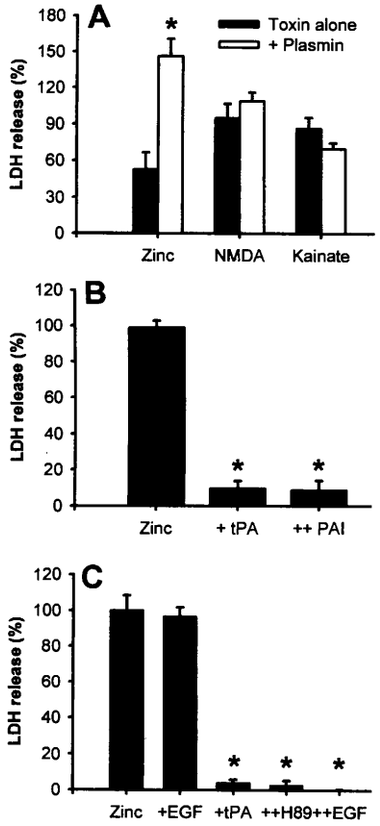

Fig. 3. Plasmin production or other proteolytic effect may not be the protective mechanism. (**A**) LDH release (mean ± SEM, $n = 4$) in cultures after 24 hours of exposure to 30 μM zinc, 30 μM NMDA, or 100 μM kainate alone or in the presence of plasmin (100 μg/ml). Plasmin potentiated zinc-induced cell death selectively; lower concentrations (1 to 30 μg/ml) of plasmin did not alter zinc neurotoxicity. (**B**) LDH release (mean ± SEM, $n = 4$) in cultures after 24 hours of exposure to 35 μM zinc alone or with addition of tPA (10 μg/ml) (+tPA) or tPA plus PAI-1 (30 μg/ml) (++PAI). (**C**) LDH release (mean ± SEM, $n = 4$) in cultures exposed for 24 hours to 35 μM zinc alone or with addition of EGF (1 μg/ml) (+EGF), tPA (10 μg/ml) (+tPA), tPA plus 3 μM H-89 (++H89), or tPA plus EGF (1 μg/ml) (++EGF). Asterisks denote difference from respective controls ($p < 0.05$, two-tailed *t* test with Bonferroni correction for multiple comparisons).

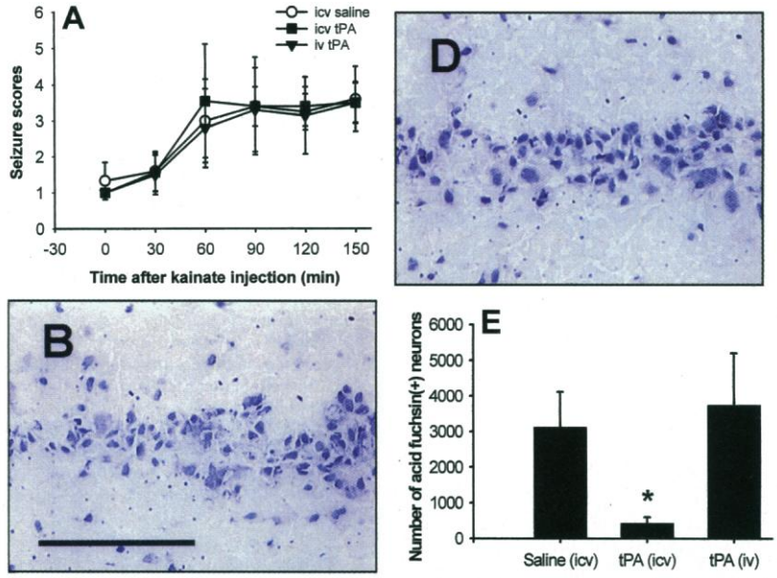

Fig. 4. Intracerebroventricular tPA injection attenuates kainate seizure–induced neuronal death in the hippocampus. (**A**) Time course of seizure intensity after kainate injection. Data denote seizure scores (*23*) (mean ± SEM, $n = 3$) after intraperitoneal kainate injection (10 mg/kg) in rats given 3 μl of saline or tPA in saline (1 mg/ml) into lateral ventricle (icv), or tPA (3 mg/kg) as bolus into tail veins (iv). No difference in seizure scores was noted among conditions. (**B** to **D**) Photomicrographs of cresyl violet-stained hippocampal CA1 regions of rats with injection of icv saline (B), icv tPA (C), or iv tPA (D), taken 24 hours after the kainate injection. Note the marked sparing of CA1 pyramidal neurons relative to severely injured ones in (B) and (D), as compared with (C). Scale bar, 100 μm. (**E**) Bars denote acid fuchsin–positive neurons (mean ± SEM, $n = 3$) counted in three sections (10 μm thickness, 800 μm apart) of the hippocampus in the above conditions. Although iv tPA did not alter kainate seizure–induced neuronal degeneration in the hippocampus, icv tPA markedly attenuated it. Asterisk denotes difference from the others ($p < 0.05$, ANOVA with post hoc Student-Newman-Keuls test).

protective effect of tPA (Fig. 3C).

We then examined whether tPA had protective effects on neuronal death in vivo. To circumvent the potential injury-promoting action of plasmin, we injected tPA (3 μl, 1 mg/ml in saline) directly into the CSF of adult rats (*20*). In CSF, plasminogen concentration is reportedly very low (*21*). For comparison, other rats were given saline alone (3 μl) into lateral ventricles, or tPA (3 mg/kg body weight) intravenously (iv) (*22*). In all animal groups, seizure intensity was identical and reached stage 3 and 4 of the Zhang *et al.* classification (*23*) 1 hour after the kainate injection (Fig. 4A). Examination of brain sections taken 24 hours after kainate injection revealed that intracerebroventricular (icv) injection of tPA markedly attenuated kainate seizure–induced death of hippocampal neurons (CA1–4), whereas icv saline or iv tPA injection did not (Fig. 4, B to D) (*24*). Counting numbers of acidophilic neurons in hippocampus (CA1–4) revealed greater neuronal death in saline-injected control and iv tPA-injected rats than in icv tPA-injected rats (Fig. 4E).

We observed the neuroprotective effect of tPA against zinc-induced and other oxidative neuronal death, but not against excitotoxic or apoptotic neuronal death in cortical culture. Because zinc neurotoxicity is mainly mediated by oxidative injury (*8*) and tPA also attenuated other oxidative neuronal injury in our study, tPA may act at certain common steps in the oxidative injury cascade. Interestingly, our results indicate that the proteolytic action of tPA may not be the mechanism for its cytoprotective effect. Although EGF homology and PKA activation are potential mechanisms of the nonproteolytic effect of tPA (*18, 19*), neither EGF nor the PKA inhibitor reversed tPA protection. Our results appear to be in conflict with the reported cytotoxic effect of tPA in vivo (*2, 3*). However, this difference is likely due to the lack of plasminogen in the exposure medium. In fact, although tPA markedly attenuated zinc toxicity in cortical culture, plasmin potentiated it. Another line of evidence supporting this scenario is that the injection of tPA into CSF, where plasminogen is quite low (*21*), reduced zinc influx–associated neuronal death in rats subjected to kainate seizures (*6, 7*). Of note, tPA injection into CSF did not alter the intensity of seizures, which suggests that the neuroprotection by tPA is not the result of fewer seizures. By contrast, injection of tPA into veins was not protective (*2*).

tPA and urokinase-type plasminogen activator are up-regulated after brain injury (*25*), and this has been considered a pro-injurious event (*2, 3*). However, our results suggest that they may play a direct cytoprotective role in brain injury. The elucidation of targets of the tPA cytoprotective action may provide insights into the molecular cascade of oxidative injury.

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20. Male adult Sprague-Dawley rats (250 to 300 g) were used for experiments. The Animal Welfare Guidelines of University of Ulsan College of Medicine were followed. Under stereotactic guidance and halothane anesthesia, one group was injected into lateral ventricles with 3 μl of saline alone (icv saline group, $n = 3$), and another group with 3 μl of tPA (1 mg/ml) in saline (icv tPA group, $n = 3$). The final group received injection of tPA (3 mg/kg) into the tail veins (iv tPA group, $n = 3$). Anesthesia was discontinued immediately after the injections. After 30 min, rats were intraperitoneally injected with kainate (10 mg/kg). Three hours after the kainate injection, seizures were halted by intraperitoneally injecting sodium diphenylhydantoin (50 mg/kg).
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24. Twenty-four hours after kainate injection, brains were harvested, stained with cresyl violet or acid fuchsin (*7*), and examined under the microscope. No hemorrhage was seen in any specimen. Neuronal cell death in the hippocampus (CA1–4) was quantified by counting acid fuchsin–positive neurons in three brain sections, 10 μm thick and 800 μm apart, starting at 3.5 mm from bregma. Numbers obtained by two independent evaluators, who were blind to the experimental conditions, were averaged and statistically compared with one another using analysis of variance (ANOVA) with post hoc Student-Newman-Keuls test.
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