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Essential Role for c-jun N-terminal Kinase on tPA-induced Matrix Metalloproteinase-9 Regulation in Rat Astrocytes

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Abstract: Tissue plasminogen activator (tPA) is used to lyse clots and reperfuse brain in ischemic stroke. However, side-effects of intracerebral hemorrhage (ICH) and edema limit their clinical application. In part, these phenomena has been linked with elevations in matrix metalloproteinase-9 (MMP-9) in neurovascular unit. However little is known about their regulatory signaling pathways in brain cells. Here, I examine the role of MAP kinase pathways in tPA-induced MMP-9 regulation in rat cortical astrocytes. tPA (1-10 μg/ml) induced dose-dependent elevations in MMP-9 and MMP-2 in conditioned media. Although tPA increased phosphorylation in two MAP kinases (ERK, JNK), only inhibition of the JNK pathway by the JNK inhibitor SP600126 significantly reduced MMP-9 upregulation. Neither ERK inhibition with U0126 nor p38 inhibition with SB203580 had any significant effects. Taken together, these results suggest that c-jun N-terminal kinase (JNK) plays an essential role for tPA-induced MMP-9 upregulation.

Key words: Astrocytes, tissue plasminogen activator, matrix metalloproteinase-9, c-jun N-terminal kinase, edema, intracerebral hemorrhage

Reperfusion therapy with tissue plasminogen activator (tPA) is a rational therapy for acute ischemic stroke. Evidence from clinical trials suggests that thrombolysis of the occluding clot with recombinant human tPA can successfully reperfuse ischemic brain (Hacke et al., 1999). However, use of tPA in stroke remains limited, primarily because of the narrow time to treatment windows available for safe and effective therapy (Marler and Goldstein, 2003). Complications may involve increased risks of cerebral hemorrhage and further brain injury (Wardlaw et al., 2003).

Recently, matrix metalloproteinases (MMPs) have been implicated in neurovascular injury after stroke (Rosenberg, 2002; Lo et al., 2003; del Zoppo and Mabuchi, 2003; Wang et al., 2004). MMPs comprise a family of zinc endopeptidases that can modify almost all components of the extracellular matrix (Yong et al., 2001; Lo et al., 2002; Visse and Nagase, 2003). After stroke, MMPs become upregulated, degrade blood-brain-barrier substrates, and promote edema, increased inflammatory infiltration, and parenchymal damage (Rosenberg, 2002; Lo et al., 2003; del Zoppo and Mabuchi, 2003; Lo et al., 2002). Combination treatments using MMP inhibitors plus tPA reduce hemorrhage and improve outcomes in animal mode of embolic stroke (Lapchack et al., 2002; Sumii and Lo, 2002). Previous studies have showed that recombinant tPA elevated MMP-9 levels in human cerebral endothelial cells and promotes MMP-9 upregulation after focal cerebral ischemia in vivo (Wang et al., 2003; Kiyoshi et al., 2005). Hence, it is possible that the deleterious induction of hemorrhage and edema after tPA reperfusion may be related in part to MMPs.

Although tPA induced MMP-9 gene expression and protein secretion (Wang et al., 2003), the regulatory mechanisms involved remain to be fully elucidated. In this study, I aim to address (1) can tPA upregulate MMP-9 in other brain cells, such as astrocyte? (2) Are MAP kinases signal pathways involved on tPA-mediated MMP-9 upregulation? MMP-9 upregulation was induced with tPA in rat cortical astrocytes, and kinase phosphorylation measurements and specific inhibitors experiments demonstrated that JNK pathway was involved in tPA-induced MMP-9 regulation.

MATERIALS AND METHODS

Cell culture

Primary astrocyte cultures were prepared from cerebral cortices of 2-d-old neonatal Sprague-Dawley rats (Abe and Saito, 2000). Briefly, dissociated cortical cells were suspended in Dulbecco's Modified Eagle Medium containing 25 mM

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glucose, 4 mM glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum, and plated on uncoated 25 cm^2 flasks at a density of 600,000 cells/cm^2. Monolayers of type I astrocytes were obtained 12-14 d after plating. Non-astrocytic cells such as microglia and neurons were detached from the flasks by shaking and removed by changing the medium. Astrocytes were dissociated by trypsinization and then reseeded on uncoated 6-, 12-, 24-well plates, or slide chambers at a density of 20,000 cells/cm^2. After the cells reached confluence (7-8 d after seeding), cultures were switched to serum-free Dulbecco's Modified Eagle Medium and experiments were initiated 24 h later. In this system, more than 95% of the cells were identified as type I astrocytes by GFAP staining.

MMP gelatin zymography

The culture medium was collected and centrifuged at 14,000 rpm for 5 min at 4°C to remove cells and debris. The cleared medium was concentrated 10-fold using Microcon (Millipore) with a 10 kDa pore diameter cut-off, then each sample was mixed with equal amounts of SDS sample buffer (Novex) and electrophoresed on 10% SDS-polyacrylamide gels (Novex) containing 1 mg/ml gelatin as the protease substrate. Following electrophoresis, gels were placed in 2.7% Triton X-100 for 1 hour to remove SDS, and then incubated for 10 hours at 37°C in developing buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Briji 35; Novex) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% w/v coomassie brilliant blue for 1 hour followed by destaining. Mixed human MMP-2 and MMP-9 standards (Chemicon) are used as positive controls. Gelatinolytic activity was manifested as horizontal white bands on a blue background. Relative gelatinolytic activity (MMP-2 and MMP-9) was quantified and expressed as a ratio of the loaded positive controls via measurement of optical density using the NIH image analysis software.

Western blot analysis

Cultures were rinsed twice with ice-cold phosphate-buffered saline and the cells were collected into cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphospate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF). Cell lysates were then homogenized and centrifuged at 14,000 rpm for 10 min at 4°C and protein concentration in the supernatant was determined with the Bradford assay (Bio-Rad). Samples were heated with equal volumes of SDS sample buffer (Novex) and 10 mM DTT at 95°C for 5 min then each sample (2 μg per lane) was loaded onto 4-20% Tris-glycine gels. After electorophoresis and transferring to polyvinylidene difluoride membranes (Novex), the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 0.2% I-block (Tropix) for 90 minutes at room temperature. Membranes were then incubated overnight at 4oC with anti-p-ERK1/2, anti-pp38, anti-p- SAPK/JNK antibody (Cell Signaling Technology, New England Biolabs), or anti-ERK1/2 antibody (1 : 2000, promega) after incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

Statistical analysis

Quantitative data were analyzed using ANOVA followed by Tukey's honestly significant difference tests between individual groups. Data were expressed as mean ± SD. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Although it has now been over 8 years since tPA was approved for stroke therapy by the FDA, it remains limited in its use to only about 2-3% of stroke patients. In part, this may be related to the narrow 3 hours time window available for effective and safe therapy. Properly titrated use of tPA in selected patients is beneficial in reperfusing rescuing ischemic brain tissue. However, under some circumstances, use of tPA in delayed times after stroke onset incurs elevated risks of brain hemorrhage and injury (Wardlaw et al., 2003). Emerging data from experimental stroke models suggest the involvement of the extracellular protease family of MMPs. MMPs can degrade critical basal lamina and blood brain barrier substrates, thus leading to edema and vascular rupture (Rosenberg, 2002; Lo et al., 2003; del Zoppo and Mabuchi, 2003; Lo et al., 2002). An emerging hypothesis therefore states that neurovascular complications of tPA reperfusion is due to tPA-induced MMP-9 dysregulation in the neurovascular unit (Wang et al., 2004;

Fig. 1. Fluorescent stained cells of cultured rat astrocytes. Cells were stained for the astorcytic marker glial fibrillary acidic protein (GFAP).

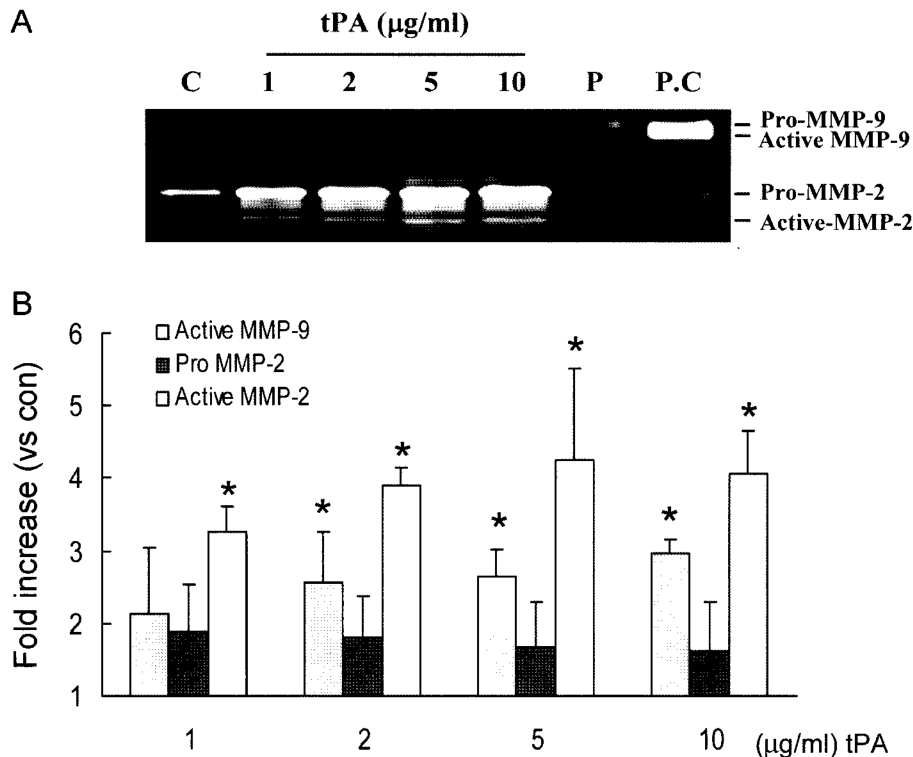

Fig. 2. tPA induced MMP-9 upregulation in cultured rat astrocytes. (A) Gelatin zymograpy shows upregulation of MMP-9 and MMP-2 after exposure to tPA in a dose dependent manner. P indicates PMA-treated conditioned medium as a positive control. (B) Quantification of changes of MMP-9 and MMP-2. *$P < 0.05$.

Fernandez-Monreal et al., 2004). It has been documented that tPA administration amplified MMP-9 levels after animal focal cerebral ischemia (Tsuji et al., 2005) and tPA upregulated MMP-9 expression in human primary microvascular endothelial cells and fibroblasts mediated by one of tPA receptor, LRP (Wang et al., 2003; Hu et al., 2006). However, the signaling pathways regulate MMP-9 in brain cells remain to be fully elucidated. Here, to investigate the involvement of tPA in MMP-9 regulation and mechanisms of tPA-mediated MMP-9 regulation in other brain cells, a rat cortical astrocyte culture system was used. In this system, more than 95% of the cells were identified as type I astrocytes by GFAP immunocytochemical staining as well as their flattened, polygonal morphology (Fig. 1). Exposure to tPA (1-10 μg/ml for 24 hrs) resulted in a two- to four fold elevation in MMP-2 secretion. A dose dependent elevation of active MMP-9 was observed over 24 hrs (Fig. 2). At all dose of tPA tested, there was no detectable cytotoxicity (data not shown).

The promoter region of the MMP-9 gene contains AP-1 sites, thus implicating a role for MAP kinases as signal transduction cascades involved in transcriptional regulation. MAP kinase pathways have been shown to mediate MMP-9 regulation in many non-brain cell lines. In the monocytic cells, THP-1 or HL60, inhibition of ERK by PD98059 blocks TNF-a-induced MMP-9 secretion (Kim and Lee, 2005; Heidinger et al., 2006). In the OVCAR-3 cell, the JNK inhibitor SP600125 inhibited PMA-dependent secretion of MMP-9 (Shin et al., 2002). In this model, the involvement of 3 major MAP kinases (ERK, p38 kinase, JNK) in tPA-induced MMP-9 regulation was examined. Treatment of tPA induced phosphorylation in ERK1/2 and JNK, with no change in total levels. Phospho-ERK1/2 levels reached a peak at 30 min after tPA treatment, then declined but maintained up to 4 hrs. JNK was markedly phosphorylated at approximately 30-60 min post-tPA, whereas p38 kinase activity was not changed (Fig. 3). To determine whether ERK or JNK signaling pathway are necessary for tPA-induced MMP-9 secretion, I tested U0216, SB203580 and SP600125 to inhibit these pathways, respectively. Pretreatment with the SP600125 (5 μM) for JNK inhibition significantly reduced tPA-induced MMP-9 expression. However, no statistically significant reductions were achieved by ERK inhibition with U0126 (10 μM) and p38 inhibition with SB203580 (10 μM) (Fig. 4). These results suggest that tPA can upregulate MMP-9 via JNK pathway in astrocytes.

There are a few caveats in the present study. First, in vitro model may not fully predict what happens in vivo. Activation of MAP kinases and efficacy of their inhibitors used in this experimental system need to be further defined, and extended into animal in vivo study. Second, many other

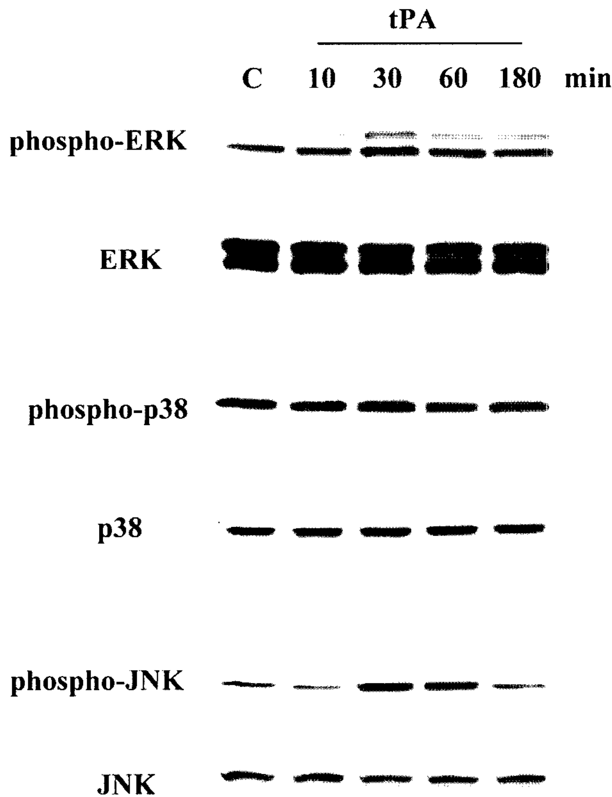

Fig. 3. tPA mediated phosphorylation of MAP kinases. Western blots of phospho or total MAP kinases from rat astrocyte cultures after exposure tPA (5 μg/ml) for the indicated times showed both ERK and JNK were significantly activated.

MMPs besides MMP-9 may be involved in brain injury and it is known that astrocytes can produce a wide spectrum of these proteases (Muir et al., 2002). It will be critical for future studies to examine how multiple MMPs interact in astrocytes in response to stimuli and injury.

In conclusion, I have demonstrated that JNK signaling pathway is essential for MMP upregulation in astrocytes after tPA stimulation. The utility of targeting these pathways in brain may allow us to lengthen the time window for tPA and improve safety and efficacy in thrombolysis associated hemorrhage and edema in stroke.

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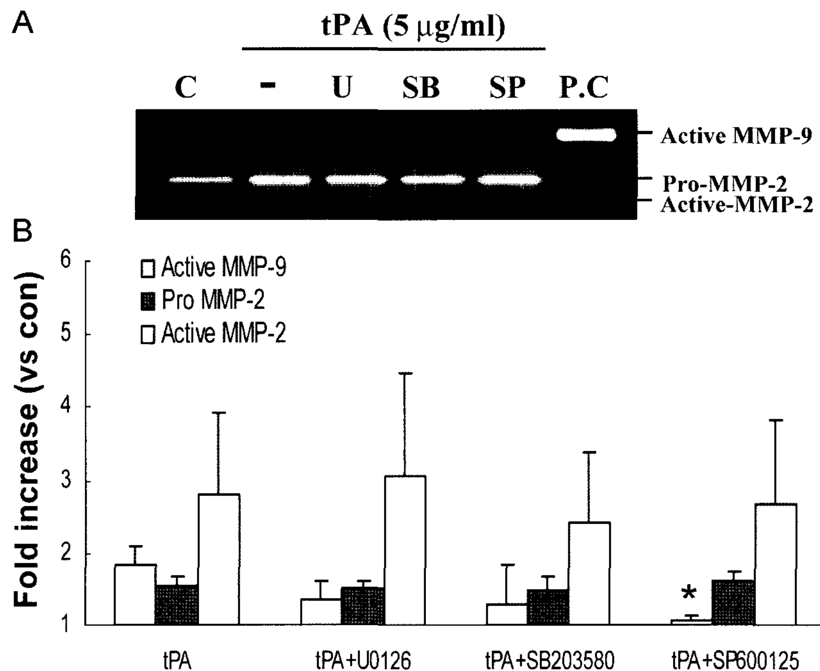

Fig. 4. Effects of MAP kinase inhibitors on MMP-9 upregulation. Gelatin zymograpy (A) and quantitative analysis (B) of MMP-9 and MMP-2 in conditioned media at 24 hrs after exposure to tPA (5 μg/ml). Cells were pre-treated with the ERK inhibitor U0126 (10 μM), the p38 kinase inhibitor SB203580 (10 μM), or the JNK inhibitor SP600125 (5 μM) for 30 min. *$P < 0.05$ vs tPA treated cells.

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