Regulation of the Activity of Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 by Zinc in Rat Primary Astrocytes

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ABSTRACT

Tissue-type plasminogen activator (tPA) is a serine proteinase which plays important roles in functional and structural synaptic plasticity, neural migration, as well as excitotoxic injuries in several pathological situations including ischemic stroke, seizure and Alzheimer's disease (AD). It has been suggested that a divalent cation zinc also plays pathological roles in ischemia and seizure. Interestingly, it has been suggested that zinc and tPA may negatively regulate the activity or the level of each other by mechanism involving physical interaction between the two. In the present study, we investigated the effect of zinc in tPA activity and expression in rat primary astrocyte. Astrocytes were transiently exposed to 20–200 μM Zn²⁺ for 2 h and then were recovered for 24 h. In the culture supernatants, zinc treatment concentration-dependently inhibited the activity of tPA which was determined by casein-plasminogen zymography. There was only marginal changes, if any, in the level of tPA mRNA and protein. On the other hand, the activity of an endogenous inhibitor of tPA, plasminogen activator inhibitor-1 (PAI-1) as well as its expression was increased by zinc treatment in a concentration-dependant manner. These results suggest that zinc-induced decrease in tPA activity was also, at least in part, regulated by indirect way by regulating the level of PAI-1. The decrease in tPA activity may be a part of body's plan to reduce excitotoxic neural injury in a condition of elevated zinc in the brain.

Key words: tissue-type plasminogen activator (tPA), zinc, plasminogen activator inhibitor (PAI)-1, neuronal damage, astrocyte
INTRODUCTION

Tissue-type plasminogen activator (tPA) is a serine proteinase primarily involved in the regulation of thrombolysis, thanks to its ability to convert inactive plasminogen to active plasmin. Recent studies have shown that tPA also plays important roles in central nervous system (CNS). The most well-known example of tPA function in CNS is the regulation of neuronal plasticity during physiological and pathological conditions such as cerebellar motor learning, visual cortical plasticity and addictive behaviors (for review, see Tsirka et al., 2002; Melchor and Strickland, 2004; Samson and Medcalf, 2006). Furthermore, a number of evidence suggests that it may participate in various neurological and neuropsychiatric events such as excitotoxic cell death in neurological conditions including seizures and cerebral ischemia. In a mouse model of excitotoxicity, tPA-deficient mice are resistant to excitotoxic death (Tsirka et al., 1995; 1997). It is suggested that excessive generation of plasmin mediates tPA-induced cell death. Plasmin degrades the layer upon which neurons are laid and form their network, and it contributes to the cell death by anoikis mechanism (Chen and Strickland, 1997; Nagai et al., 1999). More recent studies have shown that tPA mediates excitotoxic cell death through the activation of NMDA-receptor, especially NR1 subunits, leading to a potentiation of NMDA-induced calcium influx into neurons (Nicole et al., 2001; Fernandez-Monreal et al., 2004).

Zinc is one of the most abundant transitional metals in the brain and is regarded as an important component for brain function and development. However, zinc also plays pathological roles in CNS especially, when the level of zinc is above normal range. Approximately 90% of total brain zinc is found in the form of zinc metalloprotein. The rest is presented in the presynaptic vesicle. In presynaptic vesicle, zinc regulates neurotransmission (for a review, see Smart et al., 2004) and upon release, modulates several receptors including the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate, and γ-amino butyric acid (GABA) receptor (Weiss et al., 2000; Smart et al., 2004). During the synaptic transmission, concentration of zinc reached 100~300 μM and is rapidly cleared from synapse (Assaf and Chung, 1984; Howell et al., 1984). However, in pathological condition, excessive release of zinc may participate in neuronal death including ischemic stroke, seizure and traumatic brain injury (Koh et al., 1996; Suh et al., 2000). Although the precise mechanism by which zinc mediates cell death remains to be clarified, there is growing evidence that zinc toxicity is mediated by oxidative stress and excitotoxicity might be involved in the pathogenesis of various neurological diseases such as ischemic stroke, seizure and Alzheimer’s disease. Some researchers (Kim et al., 1999) have reported that zinc produced reactive oxygen species (ROS) and antioxidants attenuated zinc-induced toxicity. Although far from being clarified, several reporters have shown that zinc can permeate glutamate receptor such as AMPA and KA receptors which then mediates excitotoxicity. Furthermore, it has been suggested that zinc can permeate voltage-sensitive calcium channels and it might cause neurotoxicity by direct intracellular action (Freund and Reddig, 1994; Koh and Choi, 1994; Atar et al., 1995; Yin and Weiss, 1995).

On the other hand, Siddiq and Tsirka (2004) have demonstrated that zinc toxicity has been attenuated for hippocampal neuron in the presence of exogenous tPA and vice versa. Previous reports have been suggested that tPA also has neuroprotective effect against zinc-induced neuronal death (Kim et al., 1999), which might be mediated direct binding and sequestration of free-zinc from extracellular space.

Even with the reported strong interaction between zinc and tPA as well as the suggestion that zinc may regulate tPA-induced toxicity, it is unclear whether zinc may affect the level of tPA by mechanisms other that direct binding to tPA. To answer these questions, we examined the activity and expression level of plasminogen activator inhibitor (PAI)-1, an inhibitor of tPA, as well as that of tPA in rat primary astrocytes exposed to ZnCl₂.

MATERIALS AND METHODS

Materials

Reagents for culturing rat primary astrocytes including Dulbecco’s modified Eagle medium (DMEM)/F12, heat-inactivated fetal bovine serum (FBS) and
trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY). Zinc chloride (ZnCl₂) was purchased from Sigma (St. Louis, MO, USA) and reagents for reporter construct transfection including Lipofectamine 2000 was obtained from Gibco BRL (Grand Island, NY). Reagents for RNA isolation was obtained from Invitrogen (Carlsbad, CA) or Qiagen (GmbH, Germany) and those for PCR amplification were the products of Introntbio (Daejeon, Korea). All other chemicals including electrophoresis-grade casein were purchased from Sigma-Aldrich Co. (St. Louis, MO) and were highest grade commercially available.

**Rat primary astrocyte culture**

Rat primary cortical astrocytes were prepared from the frontal cortices of 1~2 day-old Sprague-Dawley rat pups as previously described (Shin et al., 2001). Briefly, frontal cortices were dissected-out and digested with porcine pancreatic trypsin. Tissues were incubated at 37°C and cells were triturated and seeded onto poly-D-lysine coated culture plates. Cells were cultured in DMEM/F12 with 10% heat inactivated fetal bovine serum (FBS). Culture media was changed every 3 days. Confluent cells were subcultured with trypsin-EDTA on 24 well or 6 well culture dishes and 13~14 DIV (days in vitro) cells were used for this study. The purity of astrocytes was checked by immunocytochemistry using antibodies against specific markers (glial fibrilary acidic protein) for astrocytes, which was typically 95% with others mostly microglia (Shin et al., 2001). Zinc chloride was treated for 2 h and then cells were washed and changed with serum free culture media for 24 h. The culture supernatants were harvested and frozen at −70°C until analyzed for the tPA and PAI-1 level by zymography (Shin et al., 2004).

**Casein zymography**

The activity of tPA was assayed by direct casein zymography as described previously (Shin et al., 2004). Briefly, samples were electrophoresed on 10% polyacrylamide gel containing casein (1 mg/ml; Sigma, St. Louis, MO) and plasminogen (13 μg/ml; American Diagnostica) under non-reducing condition. The gel was washed and re-natured with 2.5% Triton X-100 and incubated in 0.1 M Tris buffer for 12~24 hr at room temperature. After Coomassie Brilliant Blue (G-250) staining, PA activity was visualized as light bands resulting from casein degradation. The caseinolysis band detected at 68 kDa is specific for tPA and corresponds to the band of purified tPA run in the same gel. To detect PAI-1 activity by one-phase inverse zymography, gels were incubated with uPA (0.5 IU/ml, American Diagnostica, Greenwich, CT) after re-naturation step (Masos and Miskin, 1997). The incubation with uPA digested casein on the gel and PAI-1 inhibited the proteolytic action of uPA leaving dark bands of casein at molecular weight of ~48 kDa after Coomassie Blue staining. On the gels without casein, no bands were appeared suggesting the band was not originated from the staining of other nonspecific proteins in the sample.

**Western blot**

Astrocytes were lysed with 100 μl of 2×sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris-HCl, pH 6.8, and 0.02% bromphenol blue) and the samples were fractionated by 10% SDS-PAGE. Western blot was performed with antibodies against tPA (Molecular Innovations, Southfield, MI) and PAI-1 (American Diagnostica) which were diluted at 1:1,000 in 5% Blotto. Briefly, protein bands were electrophoretically transferred onto nitrocellulose (NC) membrane. Nonspecific binding sites on NC membrane were blocked by incubating the membrane with polyvinylalcohol solution (PVA, 1 μg/ml, Sigma, MO). The membrane was washed with PBS and incubated with the first antibody overnight at 4°C. After three 10 min washes with PBS containing 0.2% Tween-20 (PBS-T), the NC membranes were incubated with peroxidase-labeled goat anti-mouse IgG at room temperature for 2 h. After washing, NC membranes were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) using LAS-3000 imaging system (Fujifilm, Tokyo, Japan). As a loading control, Western blot was performed using an antibody against β-Actin (Sigma, MO) in 1:10,000 dilution.

**Semiquantitative RT-PCR**

Astrocytes were lysed with Trizol reagents (GibcoBRL) and total RNA was extracted following manufacturer’s instruction. Reverse transcription was performed for 1 h at 42°C with 2 μg of total RNA
using 1 unit/μl of superscript II reverse transcriptase (GibcoBRL) according to the manufacturer’s protocol using Oligo (dT)₁₈ as a primer. The cDNA obtained from 0.5 μg of total RNA was used as a template for PCR amplification of tPA, PAI-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as described previously (Lee et al., 2003). The PCR amplification consisted of 30~35 cycles (94°C, 20 sec; 60°C, 1 min; 72°C, 1 min) with the following oligonucleotide primer sets.

for tPA, 5’-TCA GAT GAG ATG ACA GGG AAA TGC C-3’ (sense)
5’-ATC ATA CAG TTC TCC CAG CC-3’ (antisense)

for PAI-1 5’-GCT CCT GGT CAA CCA CCT TA-3’ (sense)
5’-CCC CAC AAA ATT CAA GAC CA-3’ (antisense)

for GAPDH, 5’-TCC CTC AAG ATT GTC AGC AA-3’ (sense)
5’-AGA TCC ACA ACG GAT ACA TT-3’ (antisense)

After the last cycle, all samples were incubated for an additional 10 min at 72°C. Final PCR products were analyzed by 1% agarose gel electrophoresis. After staining the gel with ethidium bromide, gels were photographed under ultraviolet light using BioRad GelDoc system (Biorad, Hercules, CA).

Transcript transfection and PAI-1 promoter reporter assay
Astrocytes were co-transfected with pGL3 control or pGL3-PAI-1 promoter luciferase reporter plasmid, which was kindly provided by Dr. SG Kim (College of Pharmacy, Seoul National University), using Lipofectamine reagent as reported previously (Kim et al., 2007). Next day, primary astrocytes were treated with zinc chloride for 2 h and then washed and changed with new culture media. Luciferase activities were assayed by using luciferase enzyme assay system (Promega, Madison, WI), respectively.

Statistical analysis
Data are expressed as the mean±standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test and a p value <0.05 was considered significant.

RESULTS
Primary cultures of rat brain astrocytes were exposed to increasing concentration of ZnCl₂ for 2

A. tPA zymography

![Zinc inhibits the proteolytic activity of tPA. (A) tPA activity decreased by zinc in a concentration dependent-manner. Cultured primary astrocytes were exposed to ZnCl₂ for 2 h and recovered for 24 h in serum-free media. The supernatants were collected and analyzed by casein zymography for tPA activity as described in Material and Methods. (B) MTT analysis of cell toxicity. Zinc induced toxicity was determined by MTT assay as described in Materials and Methods. The decrease of tPA activity was not accompanied with cell toxicity. Each data indicates the mean±S.E.M. (n=4). *Significant difference compared with control (p<0.05).](image-url)
h. After 2 h, cells were recovered for 24 h in serum-free media and the culture media was collected for the analysis of tPA activity. tPA activity was attenuated by zinc in a concentration dependent-manner (Fig. 1A), in agreement with the study conducted with hippocampal neuronal cells (Siddiq and Tsirka, 2004). To check whether the observed decrease in tPA activity is mediated by zinc-induced toxicity in astrocytes, we performed MTT assay after zinc-treatment. There was no significant cell death observed in astrocyte cultures even at the highest concentration of zinc used in this study (200 μM) (Fig. 1B). These results suggest that decreased tPA activity was not due to the nonspecific cell toxicity induced by zinc. Consistent with these results, there was no difference in matrix metalloproteinase-2 and MMP-9 activity in zinc-stimulated astrocytes suggesting zinc did not induce nonspecific modulation of proteinase activity in astrocytes in our condition (data not shown).

Next, the level of endogenous tPA inhibitor, plasminogen activator inhibitor-1 (PAI-1) was determined. The supernatants were collected and analyzed by one-phase inverse zymography for PAI-1 activity. In contrast to tPA activity, PAI-1 activity was increased by zinc in a concentration-dependent manner (Fig. 2). These results suggest that zinc-induced regulation of tPA activity is modulated by the regulation of the activity of PAI-1.

To investigate whether zinc also modulates tPA at genetic level, RT-PCR was performed. Unlike the tPA activity analysis, the mRNA level encoding tPA was not changed significantly by zinc, although there was a tendency of decreased expression. In Western blot analysis, tPA protein expression in cellular fraction was slightly increased by 50~200 μM of zinc, which might reflect the decreased release of tPA and/or increased protein expression of tPA (Fig. 3). In contrast, PAI-1 mRNA was up-regulated by zinc (Fig. 3A). In addition, the PAI-1
Fig. 4. Zinc modulates PAI-1 promoter activity. Cultured primary astrocytes were transfected with either pGL3 plasmid (negative control) or pGL3 plasmid containing PAI-1 promoter as described in Materials and Methods. After 24 h, cells were lysed, collected, and the luciferase activities were measured using luminometer as described. There was a significant increase in luciferase activity with PAI-1 promoter vector. Each data indicates the mean ± S.E.M. (n=4). *Significant increase as compared with control (p < 0.05).

luciferase promoter activity was also increased by zinc treatment in rat primary astrocytes (Fig. 4). These results suggest that the increased activity and expression of PAI-1 regulates the overall activity of tPA in zinc-stimulated rat primary astrocytes.

DISCUSSION

Tissue-type plasminogen activator (tPA) is the only USA Food and Drug Administration-approved treatment of acute ischemic stroke. In patients with ischemic stroke, tPA restored reperfusion and reduced morbidity and mortality when applied within 3 hours after the stroke onset (NIHDS rt-PA stroke study group, 1995). Although tPA can be a treatment of ischemic stroke, there is only a narrow time-window and limited patient population in which thrombolytic agents are appropriate. In recent years, animal experiments pointed out that tPA had unfavorable side effect in ischemic stroke through the generation of plasmin and induction of MMPs (Tsirka et al., 1995; Wang et al., 1998). In opposition to these reports, previous studies demonstrated that tPA also had neuroprotective effect against zinc toxicity in cell culture model by mechanism independent of proteolysis. Consistent with the role of oxidative injury in zinc-induced neuro-toxicity, tPA attenuated zinc-induced and other oxidative stress induced neuronal death, but not excitotoxic or apoptotic neuronal death (Kim et al., 1999). Interestingly, the neuroprotective effect of tPA was not altered by excessive amounts (30 μg/ml) of plasminogen activator inhibitor-1 (PAI-1), suggesting the protective effect was not mediated by its proteolytic action. Mechanistically, tPA facilitates zinc import into neurons through voltage-sensitive Ca²⁺ channels and Ca²⁺-permeable AMPA/KA channel. Hence, tPA decreases extracellular free zinc levels as well as zinc-induced toxicity by promoting the transport of zinc into hippocampal neuron, thereby sequestering zinc from inducing excitotoxicity (Siddiq and Tsirka, 2004). Due to the binding activity between zinc and tPA, which was also reported in the purification procedure of tPA using zine-chelate agarose chromatography (Rijken and Collen, 1981), one can envision that appropriate concentration of zinc may inhibit tPA activity. In fact, Siddiq and Tsirka showed that zinc inhibits tPA proteolytic activity concentration-dependently (2004), which may underlie the potential reciprocal inhibition of neurodegeneration induced by each other.

In addition to its binding activity with tPA, we reported here that the activity of tPA is inhibited by zinc by regulating PAI-1 expression in rat primary astrocytes. We presented data indicating that zinc modulates the expression and thereby the activity of plasminogen activator inhibitor-1 (PAI-1), which is the major physiological inhibitor of tPA. Even though the mechanism(s) regulating tPA activity and expression are poorly understood, it has been reported that several factors are involved in the regulation of tPA including transcriptional (Qian et al., 1993), translational (Shin et al., 2004), and posttranslational control (Mühännen and Vaheri, 2004). In this study, we demonstrated that tPA was regulated by zinc in posttranslational level through the induction of PAI-1. It has been reported that several factors modulate plasminogen activator inhibitor-1 (PAI-1) including bacterial endotoxin, inflammatory cytokines, angiotensin II, lipoprotein, transforming growth factor-β, hypoxia and clinical situations that are associated with increased risk of ischemic stroke (Heaton et al., 2003; Dallas and...
In addition to these factors, we now report that zinc can modulate the expression of PAI-1 in rat primary astrocytes.

The most eminent question arising from the present study is the mechanism of up-regulation of PAI-1 expression by zinc in astrocytes. In astrocytes, TGF-β strongly up-regulated PAI-1 expression without affecting tPA expression, which might underlie the protective effect of TGF-β in ischemic stroke (Buisson et al., 1998). In cultured femoral-diaphyseal and metaphyseal tissues, 100 μM zinc significantly upregulated TGF-β (Ma et al., 2001), which may suggest that zinc up-regulates PAI-1 in rat primary astrocytes by the expression of TGF-β, which remains to be clarified in the future study.

Several groups of researchers including us reported that prolonged treatment of high concentration of zinc induced astrocyte toxicity (Ryu et al., 2002; Sheline et al., 2004; Bishop et al., 2007) even though the toxicity was milder than neuron (Dineley et al., 2000). For example, we reported that 24 h treatment of 200 μM zinc induced astrocyte cell death characterized by shrunken and fragmented cell nuclei, which was mediated by ROS production and depletion of intracellular glutathione (Ryu et al., 2002). In this study, we also found that overnight treatment of 200 μM zinc induced astrocyte toxicity. However, when we treated astrocytes with zinc for 2 h and changed the medium with fresh one, we didn’t find any obvious toxicity as shown in Fig. 1. These results clearly suggest that astrocytes have strong capacity to combat with oxidative stress (Dringen et al., 2000) and the response observed in this study is not mediated by simple toxic response in astrocyte.

Although majority of studies reported the zinc-induced neurotoxicity, it has been also suggested that zinc may be neuroprotective in certain experimental setting. For example, Hussein et al. (1996) showed that treatment of zinc induced the expression of metalloprotein in rat, which mediate neuroprotection by scavenging free radicals (Hussein et al., 1998). Considering neurotoxic effects of high concentration of tPA, it would be an interesting topic to examine whether zinc-induced decrease in tPA activity may underlie this type of zinc-induced neuroprotection.

There are growing numbers of reports suggesting that zinc inhibits serine protease by binding to enzyme molecules including tPA. Siddiq and Tsrika have shown that zinc bind to tPA and inhibit its activity in a dose-dependent manner, thus confer neuroprotection from tPA’s proteolytic neurotoxic action (2004). Our data are consistent with this previous report for a potential inhibitory role of zinc on tPA activity. Moreover, the activity of other serine proteases can be modulated by zinc. It was reported that zinc inhibited kallikrein 2. In addition, Zn(II)-mediated inhibition of trypsin-like proteases has also been reported (Katz et al., 1998). Crystallographic evidence has indicated that zinc can bind to trypsin-like proteases (such as tPA) at physiological concentrations and act as a scaffold for binding of serine protease inhibitors, which underlies its potential inhibitory activity on serine proteases (Katz and Luong, 1999).

Many serine proteases are targets for therapeutic intervention because they often play key roles in disease onset and progress. In particular, the regulation of excessive tPA is important not only for the neurobiological aspects but also in clinical situations such as ischemic stroke and seizure. Therefore, the inhibitor of serine proteases has been especially spotlighted as plausible targets of therapeutic agents. The present study showed that zinc can regulate tPA activity by modulating PAI-1 expression in rat astrocytes. Next questions would be whether similar things happen in other cell types producing tPA in brain such as neuron and microglia. However, although it may need further investigation, the results from the present study suggest that the interaction of between zinc and tPA in astrocyte contributes to minimize neuronal damage in conditions of elevated zinc in brain such as ischemia and to provide a better treatment strategy in neurodegenerative condition.

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