Zinc-Triggered Induction of Tissue Plasminogen Activator and Plasminogen in Endothelial Cells and Pericytes

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Cerebral amyloid angiopathy (CAA) is common in patients with Alzheimer’s disease (AD) and may contribute to cerebral hemorrhage. We previously demonstrated that tissue plasminogen activator (tPA) and plasminogen (PLG) accumulated at the periphery of compact amyloid-cored plaques and in the walls of CAA-containing blood vessels in the brains of Tg2576 mice, a widely used AD mouse model. We had also observed that zinc-triggered tPA and PLG induction were observed in mouse cortical cultures. Because zinc also accumulates in amyloid plaques and blood vessel walls in AD brains, we examined whether zinc increases mRNA and protein levels of tPA and PLG in brain endothelial cells and pericytes. Four hours after the exposure of brain endothelial cells (bEnd.3) to 40 μM zinc, the mRNA and protein expressions of tPA and its substrate PLG were significantly increased. In the case of brain pericyte cultures, increases in tPA and PLG expression were also detected 2 hr after treatment. However, amyloid-β (Aβ)1-42 oligomers did not augment tPA and PLG expression in bEnd.3 cells and pericytes, suggesting that zinc but not Aβ induces tPA and PLG accumulation in CAA found in the AD brain.

Key words: zinc, amyloid-β (Aβ), tissue plasminogen activator (tPA), plasminogen (PLG), cerebral amyloid angiopathy (CAA)
Tissue plasminogen activator (tPA) is a serine protease involved in thrombolysis [18-20]. tPA converts plasminogen (PLG) to plasmin, the most potent and reactive serine protease [18]. The tPA-PLG-plasmin cascade system is involved in various physiological and pathological processes, including thrombolysis, embryo development, wound healing, and cancer progression [18, 21-23]. In the central nervous system, the tPA/PLG/plasmin system exerts additional beneficial and detrimental functions. Whereas tPA induces neurite outgrowth and neuronal survival [24, 25], tPA exacerbates mild excitotoxicity by proteolytically up-regulating N-methyl-D aspartate receptor function [26]. Because of the potent protease action of the tPA/PLG/plasmin system, the expression levels of tPA and its substrate, PLG, are tightly regulated [18]. However, zinc neurotoxicity, a possible underlying neuronal death mechanism for brain ischemia and seizure [27, 28], was markedly attenuated by tPA [29]. In addition, the tPA/PLG/plasmin system is involved in long-term potentiation [30, 31] via cleavage of pro-brain-derived neurotrophic factor (BDNF) to mature BDNF [32], proteolytic degradation of laminin [33, 34], and lipoprotein receptor-mediated induction of matrix metalloproteinase (MMP) [35]. We previously reported that tPA/PLG co-accumulated at the periphery of amyloid deposits and in CAA-containing blood vessel walls in Tg2576 mice [36]. Although tPA/PLG/plasmin system degrades Aβ fibrils and reduces the overall Aβ burden [37, 38], it also increases the risk of hemorrhage.

In mouse cortical cultures, we found that mRNA and protein levels of tPA and PLG were regulated by zinc via MMP activation and BDNF secretion [39]. However, it is not clear how tPA and PLG are upregulated in CAA. In the present study, we investigated whether zinc or Aβ that is co-accumulated into CAA with tPA and PLG play a key role in regulating tPA and PLG expression in endothelial cells and pericytes.

MATERIALS AND METHODS

Cultures of mouse brain endothelial cells (bEnd.3) and primary mouse brain pericytes

Immortalized mouse brain endothelial bEnd.3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco/Invitrogen, Carlsbad, CA USA) containing 10% FBS (Hyclone, Logan, UT, USA), and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified 5% CO2 atmosphere.

Mouse brain pericytes were obtained from 8-week old mouse cortical tissue [40]. Dissociated cortical tissues were digested with an enzymatic solution (15 units/ml papain [Worthington Biochemical Corp., Lakewood, NJ, USA], 2,000 units/ml DNase I [Worthington], 1.1 mM EDTA, 0.067 mM mercaptoethanol, and 5.5 mM cystein-HCl in Earle’s Balanced Salt Solution [EBSS; Gibco/Invitrogen, USA]) in a 37°C water bath for 30 min, and then homogenized by passage through an 18-gauge needle five times, followed by five passages through a 21-gauge needle. The homogenized cortical tissues were mixed with 1.7 volumes of Dulbecco’s phosphate-buffered saline (DPBS) containing 22% bovine serum albumin (BSA) and then centrifuged at 4,000 rpm for 10 min. The cell pellet was re-suspended in endothelial growth medium (ECGM; endothelial basal medium supplemented with 2% FBS, hydrocortisone, human epidermal growth factor, vascular endothelial growth factor, human fibroblast growth factor-B, R3-insulin-like growth factor-1 [R3-IGF-1], ascorbic acid, heparin, and gentamicin/amphotericin [all from LONZA, Basel, Switzerland]) and then plated onto two wells of a 6-well plate that had been coated with 300 μg/cm2 collagen (Sigma, St. Louis, MO, USA). The cells were kept in ECGM during the first two passages, and then incubated in 2% FBS-pericyte medium (ScienCell Research Laboratories, Carlsbad, CA, USA) for the next 3-4 passages and then finally maintained in 10% FBS-DMEM at 37°C in a humidified 5% CO2 atmosphere. Primary mouse brain pericytes that were between 10 and 30 passages were used for experiments. Before use, each culture was assessed for purity by performing immunocytochemistry with anti-CD31 (an endothelial cell marker) and anti-CD146 (a pericyte marker); we confirmed that 100% of the cells were pericytes, and endothelial cells were absent in these cultures (Fig. 1).

Exposure to zinc or Aβ

Mouse brain endothelial cells (bEnd.3) and pericytes were exposed to zinc (ZnCl2) or Aβ (Aβ1-42 [BACHEM, Bubendorf, Switzerland]) in serum-free MEM (Eagle’s minimal essential medium, Gibco/Invitrogen). To prepare Aβ oligomers [41], 1 mg Aβ1-42 was suspended at an initial concentration of 1 mM in 222 μl hexafluoroisopropanol (HFIP; J.T. Baker, Phillipsburg, NJ, USA), using a Hamilton syringe and then incubated at room temperature (RT) for 2 hr. HFIP was evaporated off in a fume hood. The peptide was resuspended at a concentration of 5 mM by addition of 44 μl dimethyl sulfoxide (DMSO; ATCC). The peptide solution was vortexed and sonicated with a water ultrasound sonicator at RT for 10 min. After oligomerization, Aβ1-42 peptide was diluted in 440 μl EBSS at a concentration of 500 μM and then incubated at 4°C for at least 24 h. Then, Aβ1-42 Peptide solution was centrifuged at 11,000 g for 2 min, and the supernatant was collected for experimental use. Immediately before their use, oligomers were...
sonicated with a water ultrasound sonicator for 10 min at RT.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde at 4°C for 30 min and permeabilized with 0.1% Triton X-100 in DPBS. After blocking with normal serum in DPBS, cultures were incubated with CD31 (endothelial cell marker; Abcam, Cambridge UK) or CD146 (pericytes marker; Abcam) antibodies at 4°C overnight. Cultures were washed and incubated with an Alexa Fluor 488-conjugated secondary antibody for 2 hr. Cells were observed under a fluorescence microscope (EVOSfl, Advanced Microscopy Group, Bothell, WA, USA).

**mRNA isolation and reverse-transcription polymerase chain reaction**

Total RNA was isolated with a TRIzol Reagent (Invitrogen) according to the manufacturer's manual and reverse-transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA USA). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with the following primer sets specific for mouse sequences: tPA primer (forward, 5’-TGCTTCTGCTTCTTCTCAG-3’; reverse, 5’-AGATACCCCTTCTCTTGTTGG-3’), PLG primer (forward, 5’-CACAGCTTGTTCTTCTCCAC-3’; reverse, 5’-CCGTCAGGGTTGGCGGAATAAT-3’), β-actin primer (forward, 5’-TCTCAAAATGTTGCTGGGAC-3’; reverse, 5’-CTTTGCCCATTCAAGATA-3’). Primer sequences were designed based on published cDNA sequences. PCR was carried out for 25 cycles (94°C for 60 sec, 60°C for 90 sec, and 72°C for 60 sec) for tPA and 35 cycles (94°C for 60 sec, 61°C for 60 sec, and 72°C for 60 sec) for PLG.

**Western blot**

Cell lysates were prepared in lysis buffer (62.5 mM Tris [pH 6.8], 6 M urea, 2% sodium dodecyl sulfate [SDS], 5% mercaptoethanol, 20 mM dithiothreitol, 0.005% bromophenol blue, 10% glycerol) containing freshly prepared protease and phosphatase inhibitors (2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF; 1 mM NaVO$_4$, and 10 mM Na$_4$P$_2$O$_7$). For each sample, 30 μg total protein was separated by SDS-polyacrylamide gels (8%) under reducing conditions and immunoblotted with antibodies against mouse tPA (ab28374, Abcam), PLG (ab154560, Abcam), or actin (A5060, Sigma). Enhanced chemiluminescence reagents (Intron Biotechnology, Sangdaewon-dong, South Korea) were used to visualize the immunoreactive bands.

**RESULTS**

To determine whether zinc increases tPA and PLG expression in brain endothelial cells, we treated bEnd.3 cultures with sub-lethal doses of zinc (10–40 μM). Six hours later, increases in mRNA and protein levels of tPA and PLG were detected for zinc concentrations greater than 10 μM (Fig. 2A). To determine the time points of tPA
and PLG induction by zinc, we examined tPA and PLG expression levels from 2 to 8 hr after bEnd.3 cells were treated with zinc. We observed substantial inductions of tPA and PLG beginning 4 hr after zinc exposure to bEnd.3 cultures (Fig. 2B).

Next, we examined whether zinc could also augment the expression levels of tPA and PLG in mouse brain pericytes. Sub-lethal dose of zinc (10–40 μM) also increased mRNA and protein levels of tPA and PLG in pericytes (Fig. 3A). We detected increased mRNA and protein levels of tPA 2 hr after pericytes were treated with zinc (Fig. 3B), which is earlier than observed for bEnd.3 cells (Fig. 2B). Distinct induction of PLG by zinc in pericytes was observed from 4 hr on (Fig. 3B).

Because we previously reported co-accumulation of tPA, PLG, and zinc, as well as Aβ in amyloid plaques in the brains and blood vessel walls of Tg2576 AD model mice [36], we investigated whether tPA and PLG induction was triggered by Aβ in bEnd.3

Fig. 2. Induction of tPA and PLG by zinc in mouse brain endothelial cells (bEnd.3). (A) RT-PCR (upper) and western blot analysis (lower) showing concentration-dependent zinc induction of tPA and PLG expression. mRNA and protein samples were prepared from bEnd.3 cultures 6 hr after exposure to the indicated concentrations of zinc. Actin was used as a loading control for RT-PCR and western blots. (B) RT-PCR (upper) and western blot analysis (lower) of the time course of zinc-induced tPA and PLG expression. mRNA and protein samples were prepared at the indicated time points after exposure of bEnd.3 cultures to 40 μM zinc.

Fig. 3. Induction of tPA and PLG by zinc in mouse brain pericytes. (A) RT-PCR (upper) and western blot analysis (lower) of zinc concentration-dependent induction of tPA and PLG expression in mouse brain pericytes. mRNA and protein samples were prepared from pericycle cultures 6 hr after exposure to the indicated concentrations of zinc. (B) RT-PCR (upper) and western blot analysis (lower) of the time course of zinc-induced tPA and PLG expression in mouse brain pericytes. mRNA and protein samples were prepared from pericycle cultures at the indicated time points after exposure to 30 μM zinc.
or pericyte cultures. In contrast with zinc-induced expression of tPA and PLG, 10 μM Aβ treatment did not increase tPA or PLG expression levels in bEnd.3 or pericyte cultures (Fig. 4). These results indicate that induction of tPA and PLG in endothelial cells and pericytes are largely dependent on zinc and are not influenced by Aβ.

**DISCUSSION**

Our results demonstrate that mRNA and protein levels of tPA and PLG were noticeably increased by zinc in both brain endothelial cells and pericytes. However, although Aβ co-accumulates with zinc, tPA, and PLG in amyloid deposits and CAA in Tg2576 mice, Aβ treatment did not affect tPA or PLG expression in bEnd.3 endothelial cultures or primary brain pericytes (Fig. 4). Although tPAs protease activity is stimulated by assembled Aβ in vitro [42], we found that tPA and PLG expression levels in bEnd.3 endothelial cells and pericytes were not affected by treatment with Aβ1–42 oligomers. This result suggested that the major function of tPA/PLG induction in bEnd.3 endothelial cells and pericytes may not be the degradation of Aβ. One of the possible roles of tPA induction could be the attenuation of zinc-induced damage, because zinc neurotoxicity was almost completely blocked by tPA [29, 43]. Emmetsberger and colleagues showed that reduction of zinc toxicity by tPA was mediated by promotion of zinc uptake into cells via ZIP4 [43]. Taken together, tPA/PLG system in bEnd.3 endothelial cells and pericytes may be largely related to zinc homeostasis rather than Aβ clearance.

However, it is still possible that tPA/PLG/plasmin cascade system may help clear Aβ. While hippocampal injections of Aβ into mice lacking either tPA or PLG persist and induce neuronal damage, Aβ injected into normal mice is cleared and does not cause neuronal damage [44]. tPA and PLG may also increase the risk of hemorrhage in CAA. Upregulation of plasminogen activator inhibitor-1 (PAI-1), an inducible protein inhibitor of tPA, contributes to the protective effects of tPA/PLG/plasmin system in wild-type mice. To understand whether zinc-induced tPA and PLG expression in endothelial cells and pericytes have beneficial or detrimental effects, zinc-triggered PAI-1 induction should be examined in future studies.

ZnT3 and synaptic zinc play key roles in brain parenchymal and vascular Aβ deposition; deletion of ZnT3 dramatically reduced amyloid plaques and CAA in Tg2576 AD model mice [12, 17]. To assess the relevance of zinc-triggered induction of tPA and PLG in vascular cell cultures to CAA in AD, tPA and PLG expression levels should be examined in ZnT3-disrupted Tg2576 mice (APP+/ZnT3−/−). Because residual amyloid deposits and CAA in APP+/ZnT3−/− mice still contain histochemically reactive zinc [10], co-localization of tPA and PLG with zinc could be confirmed in this mouse model.

We previously demonstrated that zinc-triggered tPA and PLG induction in mouse cortical cultures was mediated by MMP activation and a subsequent increase of pro-BDNF cleavage [39]. Upregulation of BDNF induces tropomyosin receptor kinase (Trk) signaling, which then increases tPA and PLG expression in cortical neuronal cultures [13]. Further studies are needed to understand which signaling pathways are involved in zinc-triggered tPA and PLG induction in endothelial cells and pericytes. Such research will provide important insights into AD research and elucidate the underlying mechanisms of CAA.

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