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Glucose deprivation reversibly down-regulates tissue plasminogen activator via proteasomal degradation in rat primary astrocytes

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ABSTRACT

Aims: Tissue plasminogen activator (tPA) is an essential neuromodulator whose involvement in multiple functions such as synaptic plasticity, cytokine-like immune function and regulation of cell survival mandates rapid and tight tPA regulation in the brain. We investigated the possibility that a transient metabolic challenge induced by glucose deprivation may affect tPA activity in rat primary astrocytes, the main cell type responsible for metabolic regulation in the CNS.
Main methods: Rat primary astrocytes were incubated in serum-free DMEM without glucose. Casein zymography was used to determine tPA activity, and tPA mRNA was measured by RT-PCR. The signaling pathways regulating tPA activity were identified by Western blotting.
Key findings: Glucose deprivation rapidly down-regulated the activity of tPA without affecting its mRNA level in rat primary astrocytes; this effect was mimicked by translational inhibitors. The down-regulation of tPA was accompanied by increased tPA degradation, which may be modulated by a proteasome-dependent degradation pathway. Glucose deprivation induced activation of PI3K-Akt-GSK3β, p38 and AMPK, and inhibition of these pathways using LY294002, SB203580 and compound C significantly inhibited glucose deprivation-induced tPA down-regulation, demonstrating the essential role of these pathways in tPA regulation in glucose-deprived astrocytes.
Significance: Rapid and reversible regulation of tPA activity in rat primary astrocytes during metabolic crisis may minimize energy-requiring neurologic processes in stressed situations. This effect may thereby increase the opportunity to invest cellular resources in cell survival and may allow rapid re-establishment of normal cellular function after the crisis.

Introduction

Tissue-type plasminogen activator (tPA) is a serine protease originally known for its activity in clot dissolution in peripheral blood. Plasminogen is converted by tPA to plasmin, which is responsible for the cleavage of fibrin clots. Clinically, tPA is the only FDA-approved therapeutic reagent for embolic stroke, although adverse effects of this protease in the later phase of recovery are still a matter of concern. In the central nervous system (CNS), tPA is expressed in almost all cell types, including neurons, microglia and astrocytes, and it regulates a diverse array of neurophysiological and neuropathological processes in the brain by cleaving substrates that include plasminogen, laminin and NMDA receptors. In addition, tPA possesses non-proteolytic neuromodulator- and cytokine-like functions, making it more reasonable to treat tPA as a neuromodulator rather than as a simple protease (Benarroch, 2007; Gravanis and Tsirka, 2005; Tsirka, 2002; Yepes and Lawrence, 2004). tPA has been implicated in a diverse array of neurological functions, such as regulation of synaptic

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plasticity, neural development, neurite outgrowth, cell migration, regulation of neuroinflammation, involvement in addiction, stroke, Alzheimer's disease and fetal alcohol spectrum disorder (FASD). Because tPA regulates both physiological and pathological responses in the brain, the expression and activity of tPA should be tightly regulated through transcription and translational control of tPA expression and regulation by endogenous inhibitors such as plasminogen activator inhibitor-1.

The brain demands metabolic support for proper function, and astrocytes are central to metabolic regulation in the brain (Brown and Ransom, 2007; Pellerin et al., 2007). In metabolically compromised situations, such as glucose deprivation during a stroke, astrocytes are more resistant than neurons and even provide a prolonged period of metabolic support for neurons (Suh et al., 2007; Swanson and Choi, 1993; Whatley et al., 1981). To fulfill this emergency response role, astrocytes are equipped with several intracellular mechanisms in addition to metabolic regulation, making astrocytes like defense coordinators in the brain. One such mechanism would be the stress response to metabolic insults. Specifically, astrocytes halt the translation of proteins non-essential for survival and initiate the catabolism of those proteins to provide building blocks and energy sources for the survival and restoration of the cellular environment. Two of the controlled protein degradation pathways recently gaining much attention are the proteasome and autophagy pathways. Considering the essential role of tPA in brain function and its possibly neurotoxic effects if released in an uncontrolled way, it is reasonable for us to hypothesize that astrocytes regulate tPA activity in metabolically compromised situations.

Prolonged metabolic derangement such as cerebral ischemia induces pan-necrosis of both glial and neuronal cells. However, astrocytes in the penumbral region may have a decisive role in determining the fate of neurons, which makes it important to understand the regulatory mechanisms of astrocyte responses during metabolic insults. Here, we report that glucose-deprived astrocytes reversibly down-regulate tPA activity by a mechanism involving increased proteasome-dependent degradation and decreased translation of tPA mRNA.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), glucose-free DMEM, penicillin/streptomycin, fetal bovine serum (FBS) and 0.25% Trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). U0126, LY294002, SB203580, MG132 and compound C were purchased from Calbiochem, EMD Biosciences (La Jolla, CA), while SP600125 was purchased from Biomol (Plymouth Meeting, PA). Actinomycin D, anisomycin, cycloheximide, chloroquine, NH_4Cl, 3-methyladenine, bafilomycin A1 and AICAR were purchased form Sigma (St. Louis, MO).

Antibodies were purchased as follows: anti-PI3K, anti-Akt, anti-GSK3β, anti-p38, anti-Erk, anti-JNK, anti-AMPK, anti-phospho-PI3K (Tyr458), anti-phospho-Akt (Ser473), anti-phospho-GSK3β (Ser9), anti-phospho-p38 (Thr180/Thr182), anti-phospho-Erk (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185) and anti-phospho-AMPK (Thr172) were purchased from Cell Signaling (Hitchin, UK); and anti-β-actin was purchased from Sigma (St. Louis, MO).

Rat primary astrocyte culture

Rat primary astrocytes were cultured from the frontal cortices of 2-day-old Sprague Dawley (SD) rat pups according to a previously described method with slight modifications (Shin et al., 2001). Briefly, the frontal cortex was dissected out, digested with trypsin-DNAse I and followed by trituration. Cells were then seeded onto a 20 μg/ml poly-D-lysine (PDL)-coated 75 cm^2 flask. The cells were grown in DMEM containing 10% FBS until they reached confluence. Confluent cells were subcultured with trypsin-EDTA on 6-well culture dishes and were grown in the same medium for 6–7 days before the experiments. The purity of astrocytes was approximately 97%, with microglia representing most other cells; cell types were determined by immunocytochemistry against specific markers, such as GFAP for astrocytes, OX-42 for microglia and MAP2 for neurons.

Casein zymography

The activity of tPA was assayed by direct casein zymography as described previously (Shin et al., 2004). Briefly, samples were mixed with zymography buffer and loaded on a 10% polyacrylamide gel containing casein (1 mg/ml; Sigma, St. Louis, MO) and plasminogen (13 μg/ml; American Diagnostica, Stamford, CT). The gel was washed with 2.5% Triton X-100 for 30 min and incubated in 0.1 M Tris buffer (pH 8.0) overnight at room temperature. The gel was then stained with Coomassie brilliant blue (R250) and destained with 20% methanol and 10% acetic acid. The caseinolysis band detected at 68 kDa was specific for tPA and corresponded to a band of purified tPA that was run on the same gel. To detect PAI-1 activity by one-phase inverse zymography, gels were processed as above and were incubated in 0.1 M Tris buffer (pH 8.0) containing uPA (0.5 IU/ml, American Diagnostica, Stamford, CT). The uPA digests the casein in the gel, and PAI-1 inhibits the proteolytic action of uPA, leaving dark bands of casein at a molecular weight of ~48 kDa after Coomassie blue staining.

Western blot analysis

Cells were washed twice with phosphate-buffered saline (PBS) and lysed with 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (4% w/v SDS, 20% glycerol, 200 mM dithiothreitol (DTT), 0.1 M Tris–HCl [pH 6.8] and 0.02% bromophenol blue). The samples were fractionated by 10% SDS-PAGE and electrically transferred to nitrocellulose (NC) membranes. The NC membranes were blocked with 0.1% polyvinyl alcohol in PBS containing 0.2% Tween-20 for 30 min. The membranes were incubated with primary antibody overnight at 4 °C and then incubated with peroxidase-labeled secondary antibody (Invitrogen, Carlsbad, CA) for 2 h at room temperature. Specific bands were detected using the ECL system (Amersham, Buckinghamshire, UK) and exposed with an LAS-3000 image detection system (Fuji, Japan).

Semi-quantitative RT-PCR

Total RNA was extracted from rat primary astrocytes using Trizol® reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed for 45 min at 42 °C with 2 μg of total RNA using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seoul) according to the manufacturer's protocol. Oligo $(dT)_{18}$ was used as a primer for this reaction. The samples were then heated at 94 °C for 5 min to terminate the reaction. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification of tPA (accession number: M23697) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, M17701) mRNA as described previously (Shin et al., 2004). The PCR amplification consisted of 35 cycles (94 °C, 1 min; 60 °C, 1 min; and 72 °C, 1 min) with the following oligonucleotide primer sets:

For tPA:	5′-TCA GAT GAG ATG ACA GGG AAATGC C-3′ (sense)
	5′-ATC ATA CAG TTC TCC CAG CC-3′(antisense)
For GAPDH:	5′-TCC CTC AAG ATT GTC AGC AA-3′(sense)
	5′-AGA TCC ACA ACG GAT ACA TT-3′(antisense)

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) and were analyzed for statistical significance by one-way