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Critical role of PTEN in the coupling between PI3K/Akt and JNK1/2 signaling in ischemic brain injury

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Abstract **JNK pathway is an important pro-apoptotic kinase cascade mediating cell death in response to a variety of extracellular stimuli including excitotoxicity, which results in selective and delayed neuronal death in the hippocampal CA1. On the contrary, activation of the protein kinase Akt, which is controlled by the opposing actions of PI3K and PTEN, contributes to enhanced resistance to apoptosis through multiple mechanisms. We here demonstrate that the temporal pattern of Akt activation reversely correlates with JNK1/2 activation following various time points of ischemic reperfusion. However, the activation of JNK1/2 could be decreased by the elevation of Akt activation via increasing the tyrosine phosphorylation of PTEN by bpv(pic), a potent PTPases inhibitor for PTEN, or by intracerebroventricular infusion of PTEN antisense oligodeoxynucleotides (AS-ODNs). In contrast, JNK1/2 activation was significantly increased by preventing PTEN degradation after pretreatment with proteasome inhibitor. The neuroprotective effects of bpv(pic) and PTEN AS-ODNs were significant in the CA1 subfield after transient global ischemia. In conclusion, the present results clearly show that PTEN plays a key regulatory role in the cross-talk between cell survival PI3K/Akt pathway and pro-death JNK pathway, and raise a new possibility that agents targeting phosphatase PTEN may offer a great promise to expand the therapeutic options in protecting neurons form ischemic brain damage.**

Keywords: PTEN; Akt; JNK; Hippocampus; Cerebral ischemia; Neuronal death

1. Introduction

The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is one of the mitogen-activated protein kinase (MAPK) modules activated by variety of extracellular stimuli and environmental stress including proinflammatory cytokines, oxidative stress, heat shock, and ischemia [1,2]. The signals in stress-activated JNK pathway are transmitted through three core modules: MAP3Ks such as members of the mixed lineage kinases (MLKs) or apoptosis signal-regulated kinase 1 (ASK1), a MAP2K such as MAPK kinase 4 (MKK4) or MKK7, and MAPK such as JNK family members [3,4]. The activated MAP3K phosphorylates and activates MKK7 or MKK4, which in turn phosphorylates and activates JNK. JNK may influence the cell death process both by transcriptional inducing death-promoting genes and by modulating the mitochondria-dependent pathway via non-transcriptional mechanisms [5,6]. Therefore, one mechanism of cell survival may be to block JNK signaling pathway.

The cellular decision to undergo either cell death or cell survival is determined by the integration of multiple survival and death signals. The activation of phosphatidylinositol 3-kinase (PI3K) correlates with increased cell survival, and this effect is largely mediated through the activation of a serine/threonine kinase Akt (also known as PKB). In previous studies, phosphorylation of Akt (Thr-308 and Ser-473) by phosphoinositide-dependent protein kinase-1 and serine/threonine kinases, including an integrin-linked kinase, was responsible for Akt activation [7,8]. The PI3K/Akt pathway promotes cellular survival, in part, by phosphorylating and inhibiting death-inducing proteins, including glycogen synthase kinase 3 (GSK-3), Bcl-2/BclxL-associated death protein (BAD), caspase 9, Forkhead transcription factors [9–12]. Recently, active Akt has been reported to phosphorylate and inactivate components of the JNK pathway, including ASK1, MLK3 and MKK4 [13–16], thereby inducing antiapoptotic effects.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a ~58–60 kDa lipid phosphatase and tumor suppressor protein that is frequently mutated in tumor cells [17]. It plays a key role in cell migration, survival and apoptosis by negatively regulating phosphoproteins in the PI3K/Akt pathway. PTEN is a major negative regulator of the PI3K/Akt signaling pathway by catalyzing degradation of the phosphatidylinositol (PI)-3,4,5-triphosphate (PIP3) to PI-4,5-diphosphate [18]. Structurally, PTEN protein is composed of an N-terminal dual specificity phosphatase-like enzyme domain and a C-terminal regulation domain. The latter has been

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Abbreviations: MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PTPases, protein tyrosine phosphatases; PTEN, tumor suppressor phosphatase and tensin homolog deleted on chromosome 10; bpV(pic), bisperoxo(pyridine-2-carboxyl)oxovanadate; AS-ODNs, antisense oligodeoxynucleotides; PMSF, phenylmethylsulfonyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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shown to be important in the regulation of the stability and half-life of the molecule [19]. It has been reported that the protein kinase casein kinase 2 (CK2) could phosphorylate PTEN at its C terminal and affect PTEN protein stability and function. The phosphorylation of three specific residues (S380, T382, and T383) on noncatalytic regulatory domain is required to maintain PTEN in a stable yet relatively inactive state, and may play an important role in control of its biological activity [20]. In addition, PTEN contains 23 tyrosine residues; previous data demonstrated that the phosphorylation of PTEN tyrosine residues could negatively regulate the PTEN function [21,22].

Recently, there has been increased interest in the role of PTEN in cellular function, particularly in neurons [23,24]. Although studies suggest that downregulation of PTEN may play a neuroprotective role in neurodegenerative disorders that involve excitotoxicity and apoptosis [25,26], the underlying mechanisms still need to be established. The present study was undertake to examine the regulation of the balance between Akt and JNK pathways through modulating the phosphorylation and expression of PTEN in rat hippocampal CA1 region, and to determine the potential mechanism underlying PTEN downregulation against ischemic neuronal injury.

2. Materials and methods

2.1. Materials

Antibodies to phospho-Akt (Ser473, #4058), Akt (#9272), PTEN (#9559), phospho-PTEN (Ser380, #9551) and phospho-Akt (Ser473, #3787, IHC Specific) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-active (diphosphorylated) JNK1/2 antibody, BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitroblue tetrazolium) were from Promega (Madison, WI, USA). Anti-JNK1/2 antibody, calpain Inhibitor I (A6185), alkaline phosphatase conjugated goat anti-rabbit IgG and goat anti-mouse IgG were all from Sigma (St. Louis, MO, USA). Bpv(pic) (ALX-270-205) was obtained from Alexis (Dianova, Hamburg, Germany). LY294002 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). All other chemicals were from Sigma unless indicated otherwise.

2.2. Animal model of ischemia

Adult male S.D. rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 250–300 g were used. Cerebral ischemia was induced by four-vessel occlusion (4-VO) as described before [27,28]. Briefly, under anaesthesia with chloral hydrate (350 mg/kg, i.p.), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. Ischemia was induced by occluding the common arteries with aneurysm clips. Rats which lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during ischemia were selected for the experiments. Rats with seizures were discarded. An EEG was monitored to ensure isoelectricity within 30 s after carotid artery occlusion. Carotid artery blood flow was restored by releasing the clips. Rectal temperature was controlled at 36.5–37.5 °C before and after ischemia-reperfusion and after treatment with drugs via a temperature-regulated heating pad. Sham control animals received the same surgical procedures except that carotid arteries were not occluded.

2.3. Drug administration

When necessary, animals were given bisperoxo(pyridine-2-carboxyl)oxovanadate (bpv(pic)) at a dose of 20 μg/100 g for four times with an interval of 3 h by intraperitoneal injections, and ischemia was induced 20 min after the last injection. Control rats received intraperitoneal injections of vehicle (0.9% saline). LY294002 or A6185 (25 μg in 10 μl 1% DMSO diluted in saline) was administrated to the rats 20 min before ischemia through intracerebroventricular (i.c.v.) injection. For i.c.v. injection, the rats were placed on ear bars of a stereotaxic instrument under anesthesia. Drug infusion was performed using a stepper-motorized microsyringe (Stoelting, Wood Dale, IL) at a rate of 1 μl/min through a preimplanted cannula in the left cerebral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm). To investigate the possible role of PTEN in response to ischemic injury, 10 nmol of end-phosphorothioated PTEN AS-ODNs in 10 μl TE buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA) were administrated to the rats every 24 h for 3 days by means of i.c.v. infusion. The same dose of missenses (MS) or vehicle (TE) was used as controls. The sequences for PTEN AS-ODNs used in this study were 5′-CTGCTAGCCTCTGGATTTGA′, and missenses were 5′-CTTCTGGCATCCGGTTTAGA-3′, as previously described [29].

2.4. Tissue preparation

For brain tissue preparation, rats were sacrificed under anesthesia at several time points of reperfusion after 15 min of global cerebral ischemia. Whole brains were removed for dissections and the hippocampal CA1 regions were microdissected from both sides of the hippocampal fissure and immediately frozen in liquid nitrogen. Tissues were homogenized in ice-cold homogenization medium consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 12 mM β-glycerophosphate, 3 mM dithiotheitol (DTT), 2 mM sodium orthovanadate (Na_3VO_4), 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and inhibitors of protease and enzymes. The homogenates were centrifuged at 15 000 × *g* for 20 min at 4 °C, supernatants were collected and stored at −80 °C for use. The protein concentrations were determined by the method of Lowry with bovine serum albumin as standard.

2.5. Immunoprecipitation and Western blotting

For immunoprecipitation, the cytosolic fractions (each containing 400 μg of proteins) were diluted fourfold with HEPES buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM each of EGTA, EDTA, PMSF and Na_3VO_4. Samples were pre-incubated for 1 h with 20 μl protein A/G then centrifuged to remove any protein adhered nonspecifically to the protein A/G. The supernatant was incubated with 2–5 μg proper antibodies for 4 h at 4 °C. After the addition of Protein A/G-sepharose, the mixture was incubated at 4 °C for an additional 2 h. Samples were triple washed with HEPES buffer and eluted by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer then boiling at 100 °C for 5 min.

Western blot analysis was carried out on 10% SDS–PAGE. Briefly, proteins were electrotransferred onto nitrocellulose filter (NC, pore size, 0.45 μm). After blocking for 3 h in PBS with 0.1% Tween 20 (PBST) and 3% BSA, the membranes were incubated over night with primary antibody in PBST containing 3% BSA. Detection was carried out by the use of proper alkaline phosphatase conjugated IgG (1:20 000) and developed with NBT/BCIP assay kit (Promega, Madison, USA).

3. Immunohistochemistry

Rats were anesthetized with chloral hydrate and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS). Brains were removed, post-fixed overnight in paraformaldehyde, processed and embedded in paraffin. Coronal brain sections (6 μm-thick) were cut on a microtome (Leica RM2155, Nussloch, Germany). Sections were de-paraffinized in xylene and rehydrated in a gradient of ethanol and distilled water. High-temperature antigen retrieval was performed in 1 mM citrate buffer. To block endogenous peroxidase activity, sections were incubated for 6–8 min in 3% H2O2. After being blocked with 5% (v/v) normal goat serum in 0.01 M PBS (pH 7.4) for 1 h at 37 °C, sections were incubated with corresponding primary antibodies at 4 °C for 3 days. Sections were washed three times with 0.01 M PBS (pH 7.4) after each step followed. The sections were then incubated with biotinylated secondary antibody overnight and subsequently with stretavidin-peroxidase for