Neuroscience Letters 662 (2018) 264–270

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Hsp70 participates in PINK1-mediated mitophagy by regulating the stability of PINK1

Qian Zheng[a,b], Chuxin Huang[a], Jifeng Guo[c,d], Jieqiong Tan[d], Chunyu Wang[a], Beisha Tang[c,d], Hainan Zhang[a,*]

[a] Department of Neurology, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China
[b] Department of Neurology, Guizhou Medical University Affiliated Hospital, Guiyang 550004, Guizhou, China
[c] Department of Neurology, Xiangya Hospital, Central South University, Changsha 41008, Hunan, China
[d] State Key Laboratory of Medical Genetics, Changsha, 41008, Hunan, China

ARTICLE INFO

Keywords:
Parkinson's disease
Mitophagy
PINK1
Hsp70

ABSTRACT

Introduction: Loss-of function mutations in PTEN-induced putative kinase 1 (*PINK1)* is one of the most common causes of autosomal recessive Parkinson's disease (PD). PINK1-mediated mitophagy is critical to mitochondrial quality control and plays an important role in PD pathogenesis. Therefore, identifying the regulatory mechanisms of PINK1 expression may provide novel opportunities for PD therapy. Heat-shock protein 70 kDa (Hsp70) is involved in neuroprotection as a molecular chaperone in neurodegenerative disorders such as PD. Thus far, the interaction between Hsp70 and PINK1 remains unclear.

Objectives: This study aimed to verify the interaction between Hsp70 and PINK1, as well as the role of Hsp70 in PINK1 stability, cell autophagy, and PINK1-mediated mitophagy.

Methods: The interaction and subcellular location of Hsp70 and PINK1 were verified by coimmunoprecipitation and immunofluorescence colocalization. Western blot analysis was used to determine the role of Hsp70 in PINK1 stability. Immunofluorescence and Western blot analyses were performed to determine the role of Hsp70 in PINK1-mediated mitophagy.

Results: We identified the interaction between Hsp70 and PINK1 and revealed that Hsp70 stabilized PINK1 by decreasing PINK1 degradation. Our data demonstrated that Hsp70 participated in PINK1-mediated mitophagy.

Conclusions: Hsp70participated in PINK1-mediated mitophagy by stabilizing PINK1.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder with a significant prevalence in elderly people. It evolves slowly and is distinguished by the appearance of movement disorders characterized by instability, postural rigidity, tremor at rest, and bradykinesia/akinesia [1]. It is associated with the loss of striatal dopamine (DA) and degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [2]. Although a large majority of diagnosed PD cases are idiopathic, autosomal dominant and autosomal recessive familial forms have been identified. Identification of several genetic risk factors has led to a significant advancement in our understanding of PD pathogenesis. Several mutations in PTEN-induced putative kinase 1 (PINK1) gene, which is considered as the second common virulence gene following parkin, have been reported to be associated with autosomal recessive Parkinson's disease (ARPD) [3–6].

Although neuronal death mechanisms remain poorly understood, several studies showed that loss of DA neurons is particularly caused by the alteration of mitochondrial functions [7–9]. Depolarized or impaired mitochondria are cleared by mitophagy, a process of autophagic machinery. Mitophagy is part of a mitochondrial quality control system that coordinates mitochondrial dynamics, biogenesis, fission, and fusion; mitophagy dysfunction is thus associated with human diseases [10,11]. PINK1 and Parkin have been determined to act along similar pathways in regulating mitochondrial quality control in mammalian systems, bolstering previous evidence that mitochondrial damage is involved in PD [12–14]. Under conditions of chemical induction such as treatment with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), PINK1 can also regulate mitophagy in a parkin–dependent fashion. PINK1 accumulates on the outer membrane of damaged/depolarized mitochondria, activates parkin's E3 ubiquitin ligase activity and recruits parkin to the dysfunctional mitochondrion. Afterward, parkin ubiquitinates outer mitochondrial membrane proteins to trigger selective autophagy [15,16].

* Corresponding author.
E-mail address: yyzhn@sina.com (H. Zhang).

http://dx.doi.org/10.1016/j.neulet.2017.10.051
Received 2 April 2017; Received in revised form 20 October 2017; Accepted 23 October 2017

Heat-shock protein 70 kDa (Hsp70) is a stress-induced protein that is expressed in response to various types of central nervous system injuries. It protects proteins from misfolding and assists in the refolding and reactivation of damaged proteins [17,18]. Heat shock cognate 70 (Hsc70) is a constitutive member of the family of Hsp70. Hsp70 and Hsc70 share almost 90% amino acid homology and exhibit quite similar biochemical properties, hence, authors have often used the terms Hsp70 and Hsc70 interchangeably [19,20]. Hsp70 has been shown to be protective in the MPTP model of PD [21]. Increased Hsp70 expression has been found in the substantia nigra of patients with PD [22]. A recent study showed that BAG family molecular chaperone regulator 2 (BAG2), one of the interaction proteins of Hsp70, is a factor that promotes mitophagy [23]. More importantly, our previous study showed that BAG2 directly binds with and stabilizes PINK1 by decreasing its ubiquitination [24].

In this study, we demonstrated that Hsp70 interacts with PINK1 and Hsp70 stabilizes PINK1 by decreasing the degradation of PINK1. Further investigations revealed that Hsp70 participates in cell autophagy and may participate in PINK1-mediated mitophagy. The previous study and our results suggest that Hsp70 may play an important role in the pathogenesis of PD.

2. Materials and methods

2.1. Plasmids, antibodies, and RNA interference

2.1.1. Plasmids

The mammalian expression plasmid pcDNA3.1-Flag-Hsc70 was a kind gift from Dr. Min Wu (College of Life Sciences, Wuhan University), pKH3-HA-PINK1 from Jifeng Guo. Full-length Hsp70 cDNA was amplified from a human fetal brain library using the primers 5′-ATTTGCGGCCGCATGGCCAAAGCCGCGGCG-3′ and 5′-CGGGGTACCC TACTTATCGTCGTCATCCTTGTAATCATCTACCTCCTCAATGGT-3′ and inserted into pcDNA3.1(-) plasma at Not I/kPn I sites. Its fidelity was confirmed by sequencing.

2.1.2. Antibodies

The following antibodies were used for immunoblotting: HA rabbit monoclonal antibody (Santa Cruz Biotechnology), LC3 B rabbit monoclonal antibody (Santa Cruz Biotechnology), PINK1 rabbit monoclonal antibody (Santa Cruz Biotechnology), flag mouse monoclonal antibody (Sigma), β-actin rabbit monoclonal antibody (Sigma). The following antibodies were used for immunofluoresence: HA rabbit monoclonal antibody (Santa Cruz Biotechnology), flag mouse monoclonal antibody (Sigma), Tom20 mouse monoclonal antibody (Sigma). And anti-mouse horseradish peroxidase-conjugated secondary antibody (Millipore) for IP Western blots and regular Western blots. The secondary antibodies labeled by Alexa Fluor 564 for mouse IgG or Alexa Fluor 633 for rabbit IgG were purchased from Life Technologies. All primary antibodies were used at 1:1000 dilution for Western blot analyses. All primary antibodies were diluted 1:200 for immunofluorescent staining. The secondary antibodies for IP were diluted 1:5000. The secondary antibodies for Western blots were diluted 1:10,000. The secondary antibodies for immunofluorescent staining were diluted 1:200.

2.1.3. RNA interference

Oligo RNA was purchased from GenePharma (Shanghai, China) and had the following sequences: Si Hsp70 sense: 5′-GGACGAGUUUGAGCACAAGTT-3′, Si Hsp70 antisense: 5′-CUUGUGCUAAACUCGUCCTT-3′.

2.2. Cell culture, transfection, and drug treatment

HEK293 cells were cultured in Dulbeco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 50 U/mL each of penicillin and streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer for suspended cells in a 24-well plate. For siRNA transfection, 30 pmol siRNA/well was transfected with 1 μL of Lipofectamine 2000 in the 24 wells. For plasmid transfection, 0.5 μg of plasmid was transfected with 1 μL of Lipofectamine 2000 in the 24 wells. At 24 h after transfection, the cells were treated with 20 μM CCCP (Sigma) for up to 2 h to induce oxidative stress and treated with 100 mg/mL cycloheximide (CHX, Sigma) for up to 2 h to inhibit protein synthesis. The expression levels of proteins were analyzed by Western blot.

2.3. Immunofluoresence

HEK293 cells on coverslips were prepared at 24 h prior to transfection. Plasmid DNA/RNA interference and Lipofectamine 2000 in 0.5 mL of OptiMed were used in transfection in accordance with the manufacturer's instructions. At 6 h post-transfection, the cells were replenished with fresh medium. At 24 h post-transfection, the cells were washed with 1 × phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. After three washes with PBS, the cells were permeabilized using 1 × PBS with 1% Triton X-100 for 10 min and blocked using 1% BSA for 1 h. The cells were incubated for 2 h at room temperature with the primary antibody resuspended in 1 × PBS. After three washes with 1 × PBS, the primary antibodies were incubated with their respective fluorescent secondary antibodies for 1 h. After three washes with 1 × PBS, the cells were visualized under the fluorescence microscope.

2.4. Coimmuoprecipitation (CoIP)

HEK293 cells were prepared for 24 h prior to transfection in 6 cm dishes. The transfection was performed with plasmid DNA and Lipofectamine 2000 in 5 mL of OptiMed. At 24 h post-transfection, the cells were washed with cold 1 × PBS and harvested in immunoprecipitation buffer (10 mM Hepes (pH 7.5), 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1% Tritonx-100, 10% Glycerol). The lysate was pre-cleared for 1 h at 4 °C and centrifuged for 30 min at 13,000 rpm. The supernatant was collected and incubated with 5 μL anti-Flag antibody and 40 μL of protein G (Sigma) in a shaker incubator at 4 °C overnight. Protein G beads were pelleted and washed five times with immunoprecipitation buffer. The precipitates were resolved by SDS-PAGE gel and analyzed by Western blot.

2.5. Western blot

HEK293 cells were harvested with trypsin and lysed on ice in 1% (v/v) Triton X-100 in PBS supplemented with protease and phosphatase inhibitors or 0.1% (v/v) sodium dodecyl sulphate (SDS), 10 mM Tris pH 7.4, and 150 mM NaCl supplemented with DNase and protease/phosphatase inhibitors. Protein lysates at 20 μg were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Blocking was performed in a solution composed of 5% skim milk in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBST), and the membranes were incubated overnight at 4 °C with primary antibodies (1:1000 dilution) prepared in the same blocking solution. Afterward, membranes were washed with TBST and then incubated for 1 h at room temperature in PBS containing 5% skim milk with respective horseradish peroxidase-coupled secondary antibodies. Protein bands were visualized with ECL Plus or ECL Western blotting detection reagents (GE Healthcare Life Sciences).

2.6. Statistics

Statistical analyses were performed using SPSS 16.0 software. The data were expressed as mean ± SD of three independent experiments.