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Drp1 phosphorylation by MAPK1 causes mitochondrial dysfunction in cell culture model of Huntington's disease

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ABSTRACT

Mitochondrial dysfunction is a major cytopathology in Huntington's disease (HD), a fatal and inherited neurodegenerative disease. However, the molecular mechanisms by which the disease-causing gene, mutant Huntingtin (mtHtt), affects mitochondrial function remains elusive. This study aims to determine the role that Mitogen-activated protein kinase 1 (MAPK1) plays in the over-activation of Dynamin-related protein 1 (Drp1), the mitochondrial fission protein, which leads to mitochondrial dysfunction and neurodegeneration seen in HD. We show that MAPK1 binds to and phosphorylates Drp1 *in vitro*. Drp1 phosphorylation at serine 616 is increased in HD knock-in mouse derived striatal cells, which is abolished by treatment with U0126, a potent inhibitor of MEK1/2. A phosphorylation-deficient mutant of Drp1, Drp1S616A, corrects mitochondrial fragmentation associated with HD. Treatment with U0126 also reduces mitochondrial fragmentation, but has no additional effect in correcting aberrant mitochondrial morphology in cells expressing Drp1S616A. Finally, treatment with U0126 reduces mitochondrial depolarization and mitochondrial superoxide production in HD mutant striatal cells when compared to wildtype cells. This study suggests that in HD, MAPK1 activation leads to the aberrant mitochondrial fission and mitochondrial function by phosphorylating Drp1. Therefore, inhibition of Drp1-mediated excessive mitochondrial fission might be a strategy for development of therapy for treating HD.
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1. Introduction

Mitochondria are vital for energy production, and neurons, that have high electrical activity and cannot use glycolysis as a source of ATP, are extremely dependent on mitochondria for the metabolic demand of regulating calcium, maintaining resting membrane potential, allowing for axonal and dendritic transport as well as the release and re-uptake of neurotransmitters [1,2]. Mitochondrial dysfunction is featured in many neurodegenerative diseases and has therefore become a main hypothesis to explain the cellular mechanism of these diseases [3,4].

Dynamin-related protein 1 (Drp1) is a cytosolic GTPase that regulates the mitochondrial fission, which is important for mitochondrial renewal, proliferation, and redistribution [5,6]. Upon activation Drp1 translocates to the mitochondria, oligomerizes, and binds to its molecular adaptors at the constriction sites, leading to division of the mitochondria [5,6]. Excessive mitochondrial fission causes mitochondrial fragmentation, which leads to permeabilization of the outer mitochondrial membrane, ATP depletion, increase of ROS, and release of apoptotic factors [5,7]. Drp1 can undergo post-translational modification, including phosphorylation, ubiquitination, and sumoylation that can modify its GTPase activity, cellular location, and oligomerization [8,9]. Phosphorylation of Drp1 by different serine/threonine kinases in normal physiology and pathophysiology has different outcomes on the activity of the protein. Phosphorylation of Drp1 at serine 616 promotes Drp1-mediated mitochondrial fission, whereas phosphorylation at serine 637 inhibits both Drp1 enzyme activity and translocation to mitochondria [8,10].

Mitogen-activated protein kinase 1 (MAPK1/ERK2) is a serine/threonine kinase and is a component of the MAP kinase signal transduction pathway. This pathway when activated has many cellular effects including regulation of cell growth, survival, and differentiation. MAPK1 has been implicated in the induction of mitochondrial fragmentation that leads to apoptosis [11] and

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cancer [12,13]. In this study, we aim to determine the role of MAPK1 plays in regulation of Drp1 activation, especially in HD. We have found that MAPK1 bound to and phosphorylated Drp1 at serine 616 residue. Moreover, suppression of MAPK1 by an inhibitor U0126, inhibited mitochondrial fragmentation and mitochondrial dysfunction in HD cell culture model. These data provide a new line of evidence showing the mechanism by which Drp1 hyperactivation mediates mitochondrial dysfunction in HD.

2. Materials and methods

2.1. Antibodies

Antibodies for phospho-(Ser/Thr), phospho-Drp1S616 and phospho-Drp1S637 were purchased from Cell Signaling; Anti-DLP1 was from BD Transduction, anti-ERK1/2 (MAPK1) was from Proteintech. Antibodies for Tom20, actin and Myc were from Santa Cruz technology.

2.2. In vitro phosphorylation of Drp1 by MAPK1

Five hundred nanograms of recombined human Drp1 (GST-tagged protein, Abnova, Tainwan) was incubated with 250 ng of recombined human MARK1 (Prospec) in a 30 μl reaction mixture (40 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM ATP). After incubation at 30 °C for 30 min, the reaction was stopped by boiling in the sample-loading buffer for SDS-PAGE. The phosphorylation of Drp1 was detected by anti-serine/threonine phosphorylation antibody.

2.3. Immunoprecipitation

Soluble protein was incubated with the indicated antibody overnight at 4 °C and protein A/G beads for 1 h. Immunoprecipitates were washed three times with cell lysate buffer and analyzed by SDS-PAGE and immunoblotting with antibodies.

2.4. Mass spectrometry analysis

Recombinant human MAPK1 (500 ng, Prospec) and recombinant human Drp1 (1 μg, Abnova) were incubated in 30 μL of reaction mixture (40 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 100 μM ATP) at 30 °C for 30 min. Samples were then subjected to 10% SDS-PAGE followed by Coomassie blue staining. The bands of Drp1 were excised from the gel, washed/destained in 50% ethanol with 5% acetic acid, dehydrated in acetonitrile, reduced with DTT, alkylated with iodoacetamide, and digested with either trypsin or chymotrypsin. After digestion, the peptides were extracted from the polyacrylamide using 50% acetonitrile with 5% formic acid, and analyzed by capillary column liquid chromatography-tandem mass spectrometry (LC-MS).

2.5. HD cell culture

HdhQ7 and Q111 mouse striatal cells were derived from a knock-in transgenic mouse model with either 7-polyglutamine repeats (Q7, wild-type) or 111-polyglutamine repeats (Q111, HD) in the *Huntingtin* gene. These cells were maintained in HyClone™ Dulbecco's modified Eagle's medium (DMEM, GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS, Corning), 1% penicillin/streptomycin, and 0.4 mg/mL G418. Cultures were maintained at 33 °C in a humidified atmosphere containing 5% CO_2. The cells were maintained below 12 passages.

2.6. Preparation of total cell lysates

HD mouse striatal cells were washed with 1X PBS and then incubated in total lysis buffer (10 mM HEPES-NaOH pH 7.8, containing 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitor, and phosphatase inhibitor) for 20 min at 4 °C. Cells were scraped and then homogenized 5X using a 25-gauge syringe. Samples were centrifuged at 12,000×*g* for 20 min at 4 °C. Supernatants were saved as total lysates (stored at −20 °C).

2.7. Western blot analysis

Protein concentrations were determined by Bradford assay. Thirty micrograms of proteins was resuspended in Laemmli buffer, loaded on SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibody, followed by visualization by ECL.

2.8. Immunocytochemistry

Cells cultured on coverslips were washed with cold PBS, fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100. After incubation with 2% normal goat serum (to block non-specific staining), fixed cells were incubated overnight at 4 °C with antibodies against Tom20 (1:500, Santa Cruz Biotechnology, USA). Cells were washed with PBS and incubated for 60 min with FITC-labeled goat anti-rabbit antibody and rhodamine-labeled goat anti-mouse antibody (1:500, Invitrogen, USA), followed by incubation with Hoechst dye (1:10000, Invitrogen, USA) for 10 min. Coverslips were mounted and slides were imaged by confocal microscopy (Olympus, Fluoview FV100). To determine mitochondrial superoxide production in cultures, cells were incubated with 5 μM MitoSOX™ red mitochondrial superoxide indicator (Invitrogen) for 10 min at 37 °C. To measure the membrane potential of mitochondria in cultures, cells were incubated with 0.25 μM Tetramethylrhodamine (TMRM) (Invitrogen) for 20 min at 37 °C. The staining was imaged by microscope, and quantification was carried out using NIH Image J software.

2.9. Statistical analysis

Results are expressed as mean ± SEM. Unpaired Student's *t*-test was used for differences between two groups to assess significance. One-way ANOVA with *post-hoc* Holm-Sidak test was used for comparison of multiple groups. Statistical significance was considered achieved when the value of p was <0.05.

3. Results

3.1. MAPK1 binds to and phosphorylates Drp1 in vitro

In order to determine whether or not Drp1 is a substrate for MAPK1 phosphorylation, recombinant human Drp1 (GST-Drp1) was subjected to an *in vitro* phosphorylation assay with recombinant human MAPK1 in the presence of ATP. Immunoprecipitation analysis showed Drp1 interacting with MAPK1 (Fig. 1A). Moreover, we found that Drp1 was phosphorylated in the presence of MAPK1 when antibodies recognize phosphor-serine/threonine were used (Fig. 1B). Next, mass spectrometry analysis revealed two conserved sites, Ser637 and Ser616, as a target of MAPK1-dependent phosphorylation (data not shown), the sites which have been found to be phosphorylated by multiple kinases [14–17].

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