Original Article
The influence of human TDP-43-M337V on ROCK/PTEN signaling pathway in NSC-34 cells

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Received January 5, 2017; Accepted April 5, 2017; Epub April 15, 2018; Published April 30, 2018

Abstract: The mechanism underlying the pathology of amyotrophic lateral sclerosis (ALS) is unclear. Thus, we sought to study the effect of TDP-43-M337V overexpression on the ROCK/PTEN signaling pathway in mouse motor neuron (NSC-34) cells. The hTDP-43-M337V gene reduced cellular protection against oxidative damage, increased intracellular lipid peroxidation, and MTT assay confirmed inhibition of growth in NSC-34 cells transfected with an hTDP-43-M337V overexpression plasmid. Western blot confirmed increased p-PTEN protein and decreased p-Akt protein. PTEN and Akt protein were not different in ALS transfected cells. ROCK activity indicated that Rho-kinase did not change among TDP-43-M337V mutant cells, compared with other cell groups. Transfected hTDP-43-M337V reduced NSC-34 cell activity and oxidative damage resistance, and increased vulnerability of motor neurons to stress-induced injury. Human TDP-43-M337V on NSC-34 cells did not influence ROCK activity or protein expression but modified PTEN and Akt phosphorylation. Thus, a transfected TDP-43 mutant gene may activates apoptosis signaling pathways and induce apoptosis via different mechanisms. These results suggest that hTDP-43-M337V overexpression augments oxidative stress and damages the ROCK/PTEN signaling pathway and suggests a novel mechanism of toxicity that can be used to increase our understanding of signaling during ALS.

Keywords: Amyotrophic lateral sclerosis, NSC-34 cells, TDP-43, ROCK/PTEN signaling pathways

Introduction
Amyotrophic lateral sclerosis (ALS) is a gradually fatal nervous system degenerative disease. As the most common adult-onset motor neuron disease, it is characterized by selective loss of motor neurons in the motor cortex, brainstem, and spinal cord, with a progressive development. ALS or Lou Gehrig’s disease (US) or motor neuron disease (UK), has an annual incidence of 2-3 cases per 100,000. Clinical manifestations of ALS include muscle weakness, atrophy, tremors and tendon hyperreflexia. Approximately 10% of ALS cases are inherited, and the remaining 90% have no known cause. At present, the pathogenesis of ALS is unclear and no effective treatments are available. Patients die from ALS due to respiratory muscle denervation more than 40 months after the initial clinical onset [1, 2].

Rho kinase (ROCK) signaling pathways are key to reconstruction of the cytoskeleton, cell differentiation and migration, and apoptosis. Research suggests that rho kinase activates apoptosis signaling cascades and promotes apoptosis and cell death. Phosphatase and tensin homolog (PTEN) has been confirmed to be a ROCK substrate, and phosphatase activity is elevated after ROCK phosphorylation [3]. PTEN has a negative regulatory role in the PI3K/Akt signaling pathway, and an important role in apoptosis: ROCK/PTEN signaling has been implicated in apoptosis [4-6].

Recently activated ROCK was reported to suppress phosphorylation of Akt via activation of PTEN. Also, in a SOD1 mutant mouse model, ROCK signaling pathways are involved in apoptosis induced by SOD1-G93A mutations [7]. ROCK activation by SOD1-G93A elevates PTEN
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phosphorylation and reduces Akt phosphorylation, inducing neuronal death. Because some ROCK inhibitors are protective against cell death, new target treatments of neurodegenerative disease focused on the ROCK/PTEN signaling pathway have been proposed [8, 9].

Studies indicate that the onset and progress of cardiovascular diseases are associated with elevated ROCK activity [10-12] and ROCK pathways are involved in the occurrence and progress of various diseases [12-16]. Growing concern about ROCK signaling pathway roles in nervous system diseases have stimulated research into ROCK signaling pathways and ALS, and inhibitors of ROCK are expected to provide a new strategy for treatment of ALS.

Numerous studies verified that mutant TDP-43 may could be involved in 2.9% of sporadic ALS cases and 3% of familial ALS [17, 18]. Also, inclusions caused by TDP-43 mutations may initiate neurodegenerative diseases such as ALS [17]. Although TDP-43 inclusions are now recognized as a common characteristic of ALS patients, how this affects ALS is uncertain.

At present, multiple TDP-43 gene mutations have been identified in different populations of sporadic and familial ALS patients [19, 20]. The human TDP-43 mutation expressed in transgenic mice and human ALS have similar clinical manifestations and pathological features, and the human TDP-43 mutant transgenic mouse models is a relatively common for studying ALS (hTDP-43-Q331K and hTDP-43-M337V are typical. Data suggest that TARDBP genetic abnormalities may be tied to the onset and progression of ALS [21]. TDP-43 mutant and SOD1-G93A transgenic mouse models are well established for researching pathogenesis and potential therapies. Here, we studied the effect of the mutant hTDP-43-M337V gene on the ROCK/PTEN signaling pathway in NSC-34 cells transfected with hTDP-43-M337V to better understand ALS and how to treat it.

Materials and methods

Chemicals

An antibody recognizing anti-β-actin was purchased from Abcam Biotechnology, Inc. (Abcam, Cambridge, MA). Malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MTT Cell Proliferation and Cytotoxicity Assay Kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Total protein extraction kit were purchased from Pulitzer gene technology co., LTD (Beijing, China). A CycLex Rho-kinase Assay Kit was purchased from MBL (CycLex Co. Ltd, Nagano, Japan).

Cell lines and cell cultures

NSC-34 is a hybrid cell line, produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma, which possesses several unique morphological and physiological characteristics of motor neurons. NSC-34 cells stably transfected with the pDEST30-EGFP plasmid, pDEST30-EGFP-TDP-43-WT plasmid and the pDEST30-EGFP-TDP-43-M337V plasmid had been successfully established in our laboratory. Cell lines were removed from liquid nitrogen and thawed quickly in a 37°C water bath. Then, cells were diluted 5-fold with complete medium (containing 90% Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) and seeded to 25-cm² glass culture bottles. Cultures were maintained at 37°C in a 5% CO2 humidified atmosphere. After attachment of cells to culture bottles (4-6 h), the medium was refreshed to remove DMSO. Medium was changed every 2-3 days, depending on growth rate. Once cells reached 90% confluence, they were used for experiments and divided into three groups: empty plasmid transfected with pDEST30-EGFP, TDP-43 WT transfected with pDEST30-EGFP-TDP-43-WT plasmid and TDP-43-M337V transfected with pDEST30-EGFP-TDP-43-M337V plasmid.

Measurement of MDA

MDA is a degradation product of lipid peroxidation that reacts with thiobarbituric acid (TBA) in a colorimetric assay. We measured reacted TBA (532 nm) in confluent cells at room temperature after cell disruption. Then, cells were incubated in a 95°C water bath for 40 min, the reaction products were rapidly cooled with running water. Product absorbance was measured and a BCA method was used to quantify protein
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using BSA as a standard. Data were expressed as nmol/mg prot.

**Measurement of MTT**

The three groups of transfected cells were seeded in 96-well sterile culture plates (2×10³/ml), with three duplications for each group. Cells with no plasmid were blank controls. Once cells were 90% confluent, they were incubated for 4 h in the dark and 20 μl of 5 mg/mL MTT was added. Culture medium was discarded and 150 μl DMSO was added to wells. Cells were vortexed to mix solutions for 10 min. Then, activity was measured by monitoring the change in absorbance at 570 nm.

**Western blot**

NSC-34 cells were lysed with RIPA buffer and proteins were extracted using a total protein extraction kit. Protein was quantified by comparison with a known concentration of BSA using a BCA Protein Assay kit. A total of 60 μg of extracted protein was resolved on 10% SDS-PAGE and resolved proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with the following specific primary antibodies: rabbit monoclonal anti-ROCK1 (1:500), rabbit monoclonal anti-ROCK2 (1:5,000), rabbit monoclonal anti-PTEN (1:500), rabbit monoclonal anti-Akt (1:5,000), rabbit monoclonal anti-phospho-Akt (1:5,000), rabbit monoclonal anti-phospho-PTEN (1:1,000), rabbit monoclonal anti-phospho-Akt (1:5,000), and mouse monoclonal anti-β-actin (1:500). Subsequently, the membranes were incubated with corresponding secondary antibody (the dilution of β-actin secondary antibody was 1:10,000 and the others were 1:5,000) for 1 h at room temperature and immunodetection was performed with an enhanced chemiluminescent substrate. Data were calculated by comparing the density of target protein bands to the density of corresponding β-actin bands.

**ROCK activity assay**

ROCK activity was assayed using a CycLex Rho-kinase assay kit, a single-site, semi-quantitative immunoassay, according to the manufacture’s recommendations. Stably transfected cells were immediately prepared in extraction buffer (25 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 5% glycerol, 10 mM β-mercaptoethanol, and protease and phosphatase inhibitor cocktails) at 4°C, then centrifuged at 12,000×g to obtain lysates. Lysates were added to precoated plates with myosin-binding subunit of myosin phosphate (MSB), including a threonine residue that is phosphorylated by Rho kinase, for 60 min at room temperature. After washing, horseradish peroxidase-conjugated anti-phospho-specific MSB threonine-697 specific antibody was applied into wells and incubated for 1 h at room temperature. Products were devel-
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B.

Figure 3. Western blot of ROCK (ROCK1, ROCK2), PTEN, p-PTEN, Akt and p-Akt protein in three cell lines. Protein was separated on SDS-PAGE as indicated and probed with specific primary antibodies and β-actin as a control. Protein band densities were measured and their ratio to β-actin was calculated (mean ± SD, n = 3) (EGFP: empty plasmid, WT: hTDP-43-WT, M337V: hTDP-43-M337V). *Statistically different from EGFP, hTDP-43-WT cell lines (*P<0.05).

opposed by incubation with the horseradish peroxidase substrate tetramethylbenzidine at room temperature for 10 min. The reaction was stopped by adding stop solution containing 0.5 M H₂SO₄. Colored products were quantified with spectrophotometry (450 nm). Purified Rho kinase (CycLex) was used as a positive control.

Statistical analysis

Data are expressed as means ± standard deviation. Statistical analyses were performed using one-way ANOVA (P<0.05 statistically significant differences).

Results

Lipid peroxidation

MDA measurement is a proxy for lipid peroxidation so we measured this in NSC-34 cells stably transfected with the hTDP-43-M337V gene. MDA content is depicted in Figure 1. MDA in NSC-34 cells transfected with the hTDP-43-M337V gene was significantly greater than the other two treatment groups and MDA was not statistically significantly different between the other two cell groups.

The hTDP-43-M337V gene and NSC-34 cell viability

An MTT assay was used to measure cell viability (Figure 2). Data show that MTT in NSC-34 cells transfected with the hTDP-43-M337V gene was less than in the other two treatment groups but the other two cell groups were not different from each other. Thus, the hTDP-43-M337V gene did not increase cell viability compared to cells transfected with empty and hTDP-43-WT genes.

The hTDP-43-M337V gene and ROCK protein: downstream factors of the ROCK/PTEN signaling pathway in NSC-34 cells

We measured the effect of hTDP-43-M337V on the ROCK/PTEN signaling pathway in NSC-34 cells, by assessing ROCK protein and the downstream ROCK/PTEN pathway factors using Western blot. p-PTEN protein in NSC-34 cells transfected with the hTDP-43-M337V gene increased, and p-Akt protein decreased compared with the other two cell lines, which showed the opposite effect (Figure 3A and 3B, P<0.05). There was no difference between empty and TDP-43-WT-expressing cells with respect to p-PTEN or p-Akt protein (Figure 3A and 3B, P>0.05). There was also no significant difference between ROCK1, ROCK2, PTEN and Akt protein in these three cell lines (Figure 3A and 3B, P>0.05). Thus, the hTDP-43-M337V gene increased p-PTEN protein expression and inhibited p-Akt protein expression. Therefore, the ROCK/PTEN signaling pathway is likely affected by hTDP-43-M337V.

Effect of the hTDP-43-M337V gene on ROCK activity in NSC-34 cells

Previous studies and our work indicate that the hTDP-43-M337V gene modifies downstream factors in the ROCK/PTEN signaling pathway. Next, we measured differences in ROCK activity to determine if this was the mechanism by which the ROCK/PTEN signaling pathway was manipulated. Data show that ROCK activity was not different in cells transfected with pDEST30-EGFP-TDP-43-WT and pDEST30-EGFP-TDP-43-WT-expressing cells.
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Figure 4. Cells were harvested after incubating for 48 h and ROCK activity was measured in three cell lines (mean ± SD, n = 3) (Mock: blank control, Rho-kinase: positive control, Y-27632: negative control). No difference was noted among EGFP, hTDP-43-WT and hTDP-43-M337V cell lines (P>0.05).

Discussion

Mechanisms underlying ALS pathology are unknown but several hypotheses suggest that ALS onset may be promoted by a series of complex interactions such as oxidative stress [22, 23], gene mutation, apoptosis, excitatory amino acid toxicity, iron metabolism [24-26], mitochondrial damage [27, 28], axonal transport barriers [29], environmental factors, and abnormal protein accumulation [30]. Of these ideas, oxidative stress may be the dominant contributor to chronic motor neuron degeneration as well as a mechanism contributing to selective motor neuron and corticospinal tract degeneration. Studies with spinal cord slices and cerebrospinal fluid of ALS patients suggest that oxidative stress may explain the pathogenesis of ALS [23], and oxidative damage of nucleic acids, proteins, and lipids was noted in SOD1-mutant animal models [31, 32].

Presently, 17 mutant genes associated with ALS have been reported, and the four most common genes are SOD1, FUS sarcoma fusion, C9orf72, and trans activation reaction-DNA binding protein (TARDBP) genes [33]. An ubiquitin abnormal protein aggregation (43 kDa comprised of the TAR DNA-binding protein, TDP-43) was found in the nucleus and cytoplasm of motor neurons and glial cells among ALS patients. TDP-43 contributes to ALS pathogenesis and this gene has been mentioned in this context by another group [19]. To date, TDP-43 gene mutations have been confirmed in different ALS populations—from sporadic to familial ALS, and these mutations are diverse and include missense mutations [19, 20]. TDP-43 mutations can destroy proteostasis and contribute to protein dysfunction [34] and abnormal phosphorylation.

Studies using ALS disease models caused by a TDP-43 mutation suggest that 2.9% of ALS cases are sporadic and 3% are familial [17]. TDP-43 mutations in ALS patients are common but how they function is unclear. Huang's group reported that mutant TDP-43 expression in rat motor neurons triggered the onset and progression of ALS [35] but how the TDP-43 gene mutations degenerates motor neurons is not well understood.

To investigate the toxic effects of TDP-43-M337V on motor neurons, NSC-34 cell lines transfected with hTDP-43-M337V and the ROCK/PTEN pathway was observed. hTDP-43-M337V reduced cellular protection against oxidative damage and increased intracellular lipid peroxidation and cellular damage. Oxidative damage is documented to occur according to biochemical and histopathological studies of postmortem tissue of ALS patients, and this was correlated to disease severity. These studies verified that nucleic acid oxidation, lipid peroxidation, protein nitration and protein glycosylation in ALS patients was significantly elevated [36, 37]. Oxidative damage also has been reported to be present in the cerebral cortex and CSF of ALS patients. We used empty, hTDP-43-WT and hTDP-43-M337V transfected cells to measure oxidative damage, and MDA in hTDP-43-M337V cells was significantly increased compared with the other two cell treatments (Figure 1). Thus, antioxidant detoxification in hTDP-43-M337V gene transfected cells decreased, which may be a potential mechanism by which mutant NSC-34 cells are vulnerable to external stimuli and subsequent injury.

To observe the influence of hTDP-43-M337V on NSC-34 cell vitality, an MTT assay was used to measure viability and hTDP-43-M337V-treated cells had fewer viable cells compared to the other two cell groups (Figure 2). Thus, in NSC-34 cells, hTDP-43-M337V gene overexpression inhibited motor neuron growth so the hTDP-43-
M337V gene could inhibit motor neuron cells viability.

To understand the role of TDP-43-M337V on ROCK/PTEN signaling pathways, we compared empty- or hTDP-43-WT-expressing, and TDP-43-M337V-expressing NSC-34 cells and noted upregulation of phosphorylated PTEN and downregulation of phosphorylated Akt. However, total protein expression of PTEN or Akt was not different in either group of cells (Figure 3A and 3B). Moreover, there was no significant difference among empty-, hTDP-43-WT- and hTDP-43-M337V-expressing NSC-34 cells on ROCK1 or ROCK2 protein (Figure 3A and 3B). This is consistent with results obtained by Tanaka’s group [7] in motor neuron cells of human SOD1 mutant transgenic mouse models.

ROCK activity in hTDP-43-M337V cells was not different than in the other cell groups (Figure 4), but these data differed from published results [7]. It is suggested that elevated ROCK activity suppressed phosphorylation of protein kinase B (Akt) though activation of phosphatase and tension homolog deleted on chromosome 10 (PTEN) [4], which was activated by SOD1-G93A, in vivo and in vitro. In contrast, when cells were exposed to oxidative stress, the mutant TDP-43 gene transformed phosphorylation of downstream effectors PTEN and Akt, but not via activating ROCK. Thus, a TDP-43 gene mutation may participate in the pathogenesis of ALS using different means or mechanisms. ROCK appears to be implicated in positive and negative regulation of PI3K/Akt signaling pathway, and outcomes may depend on cell types or stimuli [6]. Previous research indicates that the ROCK/PTEN/Akt pathway regulates cell death in neurons [38] and the PTEN/Akt pathway accounts for motor neuron survival in human SOD1-related ALS [39]. In a SOD1-G93A transgenic mouse model, ROCK activated by SOD1-G93A downregulates phosphorylated Akt by enhancing phosphorylated PTEN, causing apoptosis. Thus, TDP-43 mutant transgenic mouse models might undergo different pathogenesis from that of SOD1, inducing apoptotic signaling pathways and contributing to apoptosis in ways that are currently unclear.

In conclusion, oxidative stress was elevated during ALS and the hTDP-43-M337V gene damaged the ROCK/PTEN signaling pathway, thereby decreasing cell viability and reducing cell defenses against oxidative damage. This caused increased vulnerability of injury induced by stress in motor neurons. Transfection of the hTDP-43-M337V gene in NSC-34 cells activated apoptosis signaling pathways and induced apoptosis but this was not via elevated ROCK activity.

Acknowledgements

This work was supported in part by a grant from Shandong Provincial Natural Science Foundation, China (ZR2010HM041) and a grant from Shandong Provincial Post-doctoral Innovation Foundation, China (201102004). We thank LetPub for its linguistic assistance during the preparation of this manuscript.

Disclosure of conflict of interest

None.

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