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4-Phenylbutyrate Attenuates the ER Stress Response and Cyclic AMP Accumulation in DYT1 Dystonia Cell Models

Jin A. Cho1, Xuan Zhang2, Gregory M. Miller3, Wayne I. Lencer1,4, Flavia C. Nery2*

1 Division of Gastroenterology/Cell Biology, Boston Children’s Hospital, Harvard Medical School, Boston, MA, United States of America, 2 Neuroscience Center, Department of Neurology, and Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital and Program in Neuroscience, Harvard Medical School, Boston, MA, United States of America, 3 Department of Pharmaceutical Sciences and Center for Drug Discovery, Northeastern University, Boston, MA, United States of America, 4 Harvard Digestive Diseases Center, Harvard Medical School, Boston, MA, United States of America

Abstract

Dystonia is a neurological disorder in which sustained muscle contractions induce twisting and repetitive movements or abnormal posturing. DYT1 early-onset primary dystonia is the most common form of hereditary dystonia and is caused by deletion of a glutamic acid residue (302/303) near the carboxyl-terminus of encoded torsinA. TorsinA is localized primarily within the contiguous lumen of the endoplasmic reticulum (ER) and nuclear envelope (NE), and is hypothesized to function as a molecular chaperone and an important regulator of the ER stress-signaling pathway, but how the mutation in torsinA causes disease remains unclear. Multiple lines of evidence suggest that the clinical symptoms of dystonia result from abnormalities in dopamine (DA) signaling, and possibly involving its down-stream effector adenylate cyclase that produces the second messenger cyclic adenosine-3', 5'-monophosphate (cAMP). Here we find that mutation in torsinA induces ER stress, and inhibits the cyclic adenosine-3', 5'-monophosphate (cAMP) response to the adenylate cyclase agonist forskolin. Both defective mechanins are corrected by the small molecule 4-phenylbutyrate (4-PBA) that alleviates ER stress. Our results link torsinA, the ER-stress-response, and cAMP-dependent signaling, and suggest 4-PBA could also be used in dystonia treatment. Other pharmacological agents known to modulate the ER stress cascade, and ER stress may also be therapeutic in dystonia patients and can be tested in the models described here, thus supplementing current efforts centered on the dopamine pathway.

Introduction

The DYT1 early-onset primary dystonia is the most severe form of hereditary dystonia and is caused by a mutation in the gene, TOR1A (DYT1), encoding torsinA protein [1]. Current treatments include high dose anticholinergics, which are only partially effective and can compromise learning and memory [2]. Another available treatment for dystonia is deep brain stimulation (DBS), which involves an implant of stimulating electrodes into the basal ganglia [3]. Although DBS can be effective in treating some DYT1 dystonia patients, it is a neurosurgical procedure with concomitant risks and high cost. The DYT1 dystonia-associated protein, torsinA, is a ubiquitously expressed protein in many organs, including the central nervous system (CNS). Intriguingly, in patients with DYT1 dystonia, only the CNS is affected. The clinical phenotype of dystonia is thought to result from decreased function of torsinA causing defects in neuronal function, including abnormal dopaminergic neurotransmission in the basal ganglia [4]. However, the role of torsinA and the correlation between the dysfunction caused by the mutation and the dystonic phenotype remain unclear. Many studies at the biochemical, structural, and cell biological levels have been performed in order to characterize torsinA [5,6]. These studies, together with the generation of several animal models [7–9], have contributed to the identification of cellular compartments and pathways affected by mutant torsinA, including the nuclear envelope (NE) [10,11], cytoskeleton [12,13], cell migration [12,14], secretory pathway [15–18], endoplasmic reticulum (ER) stress [23,24], and endoplasmic reticulum-associated degradation (ERAD) [24,25], where torsinA’s function may be crucial for the cell homeostasis.

To define therapeutic pathways to target dystonia, it is important to identify cellular processes affected in the brain. Our studies with dystonia animal models and fibroblast cells from DYT1 patients have implicated decreased intracellular levels of cAMP in these DYT1 dystonia models when compared to controls. Alterations of the second messenger cAMP affect normal brain function such as in synapses, neuronal memory, and striatal plasticity [26–28], and can also be found in several neurological...
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Dopa|se and reduced intracellular cAMP levels. While it is not clear observed that our DYT1 dystonia cell models have increased ER somehow contributes to ER stress load [23,24]. We recently in dystonia, in which torsinA may play a new role.

Our previous studies suggest that the presence of torsinA somehow contributes to ER stress load [23,24]. We recently observed that our DYT1 dystonia cell models have increased ER stress and reduced intracellular cAMP levels. While it is not clear how the mutant torsinA affects ER stress and cAMP production, we find these defects are rescued by treatment with 4-phenylbutyrate (4-PBA), a small molecule that suppresses the ER stress response to misfolded proteins. The findings open new perspectives on repairing disrupted ER stress and cAMP signaling in dystonia, in which torsinA may play a new key role.

Materials and Methods

Ethics Statement

All human cell lines and protocols in the present study were carried out in accordance with the guidelines approved by institutional review boards (IRB) at Partners Human Research Committee (Protocol #: 2007P001632/MGH). Fibroblast cell lines received from Dr. Xandra Breakefield laboratory were previously collected through a Yale Medical School research protocol between 1974–1984. Individuals enrolled in this research protocol had skin biopsies taken, and signed consent forms. All animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 2004N000271/MGH). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Animals

Heterozygous Tor1A knockout mice (Tor1A<sup>+/−</sup>) [9], and Heterozygous Tor1A knock-in mice (Tor1A<sup>+/−</sup> ; [7,9] were received from Dr. Yuqing Li (University of Florida, Gainesville, FL, USA). We crossed Tor1A<sup>+/−</sup> and Tor1A<sup>+/−</super> mice to generate timed pregnancies. The day of vaginal plug detection was considered embryonic day 0 (E0). Embryonic neuron cultures were prepared from each embryo on embryonic day 15 (E15). The genotype of each embryo was identified as reported previously [7,9].

Mixed cortical and striatal neuron culture

Mixed cortical and striatal neuron cultures were prepared from in-vitro E15 mouse embryos. Briefly, embryos were removed by Caesarean section from a pregnant mouse. Cortices and striata from embryonic mice were dissected and stored in ice-cold 1x phosphate-buffered saline (PBS). The cortices and striata of individual embryos were dissociated with 0.625% trypsin at 37°C in neurobasal media (NBM, supplemented with B27, N2, 500 mg/ml streptomycin, 100 IU/ml penicillin and 2 mM L-glutamine, all from Invitrogen, Carlsbad, CA, USA). Trypsin was removed and Dulbecco’s Modified Eagle Medium plus nutrient mix F12 (DMEM/F12, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), N2 supplement and penicillin/streptomycin added to inactive the trypsin. Cortices and striata were treated with 12.5 mg/ml DNase I (Sigma, St. Louis, MO, USA) in DMEM/F12+ FBS for 5 min at 37°C and homogenized. Cell suspension was then diluted in NBM supplemented with with 2B27, 0.5 mM L-glutamine. The cells were counted and then plated on a 96-well plate coated with polyethylenimine (Sigma, St. Louis, MO, USA) in 0.15 M Borate Buffer at a density of 4x10^4 cells/well. For each embryo, we generated approximately 8 wells. Half the media was replaced with fresh media every two days. The cells were treated with 5 µM cytosine arabinoside (ARA-C) four days after being plated and the total media was changed after 24 hours. The density of immunoreactive cells positive for βIII tubulin (a neuronal marker), and GFAP (a marker of astrocytes) was performed in parallel at 5-8 days in vitro (DIV). Results showed that the majority of cells (99.5%) were positive only for βIII tubulin. Note that cultures from individual neurons (an average of 10 wells per embryos) were maintained for 10 days, and were then used for cAMP assay.

Mouse embryonic fibroblast cells (MEFs)

MEF cultures were prepared as described [12,41] from single embryos (embryonic day 15) of mating between heterozygous torsinA knockout mice or torsinA knock-in mice. Cells were grown in DMEM (GIBCO, Rockville, MD) as described [41]. MEFs cells were seeded, and incubated overnight before the cAMP assay.

Human fibroblast cell culture

Primary human skin fibroblasts of control fibroblasts (HF6, HF19) and of DYT1 patient’s fibroblasts (HF46) were generated in Dr. Breakefield’s laboratory as described previously [42], under IRB approved guidelines. Fibroblast control cell lines GM00023, GM00024, GM01651, GM02912, and DYT1 patient fibroblast cell lines GM02551, GM03208, GM032011, GM02306 were purchased from Coriell Institute for Medical Research (Camden, NJ). The Coriell Cell Repository maintains the consent and privacy of the donor fibroblast samples. DYT1 patient fibroblast cells, FFF13111983 [43], were provided from Dr. Mirella Filocamo (Biobank from Patients affected by Genetic Diseases, L’Istituto Giannina Gaslini, Genoa, Italy), a member of the Telethon Network of Genetic Biobanks (project no. GTB12001). The primary fibroblast cells were grown in culture, as described [24]. For more information about the human fibroblast cell lines, see Table S1. Human fibroblast cells were seeded, and incubated overnight before the cAMP assay.

Western blot

SDS-PAGE electrophoresis and protein transfer were carried out as described [23]. Membranes were probed with antibodies against β-actin (1:5,000), and mouse torsinA (1:1000) (Abcam) diluted in TBST and visualized with HRP conjugated to secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Secondary antibodies for Western blots were sheep anti-mouse IgG-HRP (1:10,000) or donkey anti-rabbit...
IgG-HRP (1:10,000) (Amersham Pharmacia Biotech, Piscataway, NJ).

**Chemicals**

Forskolin (Sigma, St. Louis, MO, USA) was dissolved in DMSO at 10 mM concentration. Thapsigargin (Sigma, St. Louis, MO, USA) was suspended in DMSO at 3 mM. The phenylbutyric acid or 4-phenylbutyrate (4-PBA; Sigma, St. Louis, MO, USA) was dissolved in filtered sterile water at a 1 M stock concentration.

**Data analysis and statistics**

Statistical analysis was performed using GraphPad Prism software (version 3.0). Results are expressed as mean ± S.E.M. of at least three separate experiments unless otherwise indicated. Statistical significance of comparison between two groups was determined by two-tailed Student’s t-test where indicated. Multiple comparison analyses of variance between the groups were performed by a one-way ANOVA test, followed by a Tukey’s multiple comparison post hoc tests. Significant differences were considered at p values of less than 0.05.

**Results**

Abnormal torsinA function downregulates cAMP production in dystonia mice and human cell models

We first observed a potential relationship between mutant torsinA and adenylate cyclase during our studies using cholera toxin to examine the role torsinA might play in ERAD [24]. We found that cAMP levels in cell lines expressing mutant torsinA were lower after treatment with cholera toxin, a well-known cAMP agonist, when compared to control cell lines.

Thus, to test the hypothesis that torsinA status might affect agonist-induced adenylate cyclase activities, we examined cAMP production in several dystonia models using forskolin, which is a general and direct activator of the adenylate cyclases. We first demonstrated complete lack of torsinA expression in torsinA knockout model (Figure 1A). Then, we investigated the forskolin-induced cAMP response in wild-type (torsinA+/+), and homozygous (torsinA−/−) MEFs (Figure 1B), as well as from torsinA+/+, torsinA−/+; and torsinA−/− neurons from knockout dystonia mice (Figure 1C). Results showed a strong loss of cAMP signal in torsinA−/− MEFs (n = 3; p<0.001; Figure 1B), and in torsinA−/+; neurons (n = 3; p<0.01; Figure 1C) compared to wild-type controls upon stimulation of the cells with forskolin. Similar results were obtained in primary neuron cultures from torsinA knock-in mice (Figure 1D). The presence of torsinAΔE in heterozygous and homozygous neurons led to lower levels of cAMP (n = 3; p<0.05; Figure 1D), when compared to controls. Furthermore, we confirmed that the inhibition of forskolin-induced cAMP production in cells lacking torsinA or expressing mutant torsinA, torsinAΔE, were not due to a reduction of intracellular levels of ATP in our DYT1 dystonia models, which could have confounded our results (Figure 1E, and 1F). There were no significant differences in the ATP levels measured in this study between wild type cells and cells from knockout (Figure 1E) or knock-in mice (Figure 1F).

To test whether these results translate to human cell models, we investigated whether cAMP levels were also altered in human fibroblast lines prepared from various dystonia DYT1 patients and healthy controls. At rest, there was no overall difference between control and DYT1 fibroblasts in basal levels of intracellular cAMP (Figure 2A). After forskolin stimulation, however, the induction of cAMP levels in DYT1 cells was significantly lower compared to control cells (n = 3; p<0.05; Figure 2A). One of the control cell lines, GM00023B, presented the lowest cAMP response to forskolin among control cell lines, and comparable in magnitude to the signals observed in DYT1 fibroblasts. However, we also observed that the GM00023B cells also presented the lowest basal level of cAMP, suggesting a general lower activity of adenylate cyclase in this specific line. Thus, to control for this, the cAMP levels induced by forskolin were normalized to the basal level of cAMP for each cell line and represented again by fold change after forskolin treatment. When the data was normalized to cell basal levels, all fibroblasts from healthy patients had uniformly increased responses to forskolin stimulation compared to all DYT1 fibroblast lines (n = 3; p<0.001; Figure 2B).

4-PBA, an ER stress inhibitor, rescues ER stress and cAMP defects in DYT1 patient fibroblasts

We previously reported that the absence of torsinA or the presence of malfunctioned torsinAΔE induces ER stress [23,24].
Here, we confirmed the activation of ER stress in DYT1 patient fibroblast cells by quantitative real-time PCR (qPCR) of the spliced form of the transcription factor X-box binding protein 1 (sXBP1). Our results show that DYT1 fibroblasts induced more XBP1 splicing (Figure 3) in both unstimulated (DMSO treatment), and stimulated (Thapsigargin treatment) conditions compared to control cells. Also we confirmed that neurons expressing torsinAΔE have higher levels of sXBP1 compared to control neurons (Figure S1) in unstimulated conditions. This suggests that the presence of torsinAΔE enhances ER stress.

Furthermore, we investigated whether 4-PBA, a small molecule with chaperone-like activity, may decrease ER stress in our dystonia cell models. 4-PBA major function is to scavenge ammonia and glutamine [44], however, recent studies have described 4-PBA as a potent inhibitor of HDAC [45], and function as a chemical chaperone during ER stress [46]. Rubenstein et al. first discovered the chaperone-like activity of 4-PBA when examining its effect on the trafficking of CFTRΔ508 to the cell surface [47]. In ER stress, 4-PBA may improve mislocalization,
and/or aggregation, and trafficking of proteins associated with human disease [48].

In parallel experiments, gradual titrations of 4-PBA (2.5, 5, 10, 15 mM, 20 mM) were performed to assess the effects of PBA on ER stress in health fibroblast cells, and in DYT1 fibroblast cells. We observed, however, that treatment with concentrations 2.5 and 5 mM did not affect ER stress in health fibroblast cells, or in DYT1 fibroblast cells (Figure 3A). However, the treatment with 10 mM 4-PBA abrogated the thapsigargin response in DYT1 patient fibroblast cells (n = 3; p<0.001; Figure 3B), but had no effect on the response to thapsigargin in healthy fibroblast cells. In contrast, higher concentrations 15 and 20 mM 4-PBA were toxic for both cell types (data not shown). This result suggests that reduced chaperone function in cells expressing torsinA may be responsible for increased ER stress. Since impaired expression of G protein coupled receptors (GPCRs) may be responsible for deficiencies in the cAMP/PKA signaling, we assessed whether mitigation of ER stress with 4-PBA could rescue the cAMP defects of DYT1 fibroblast cells (Figure 4A). We observed that the treatment with 4-PBA enhanced the cAMP response to forskolin in DYT1 patient fibroblast cells, with signals comparable to control cell lines (n = 3; p<0.05; Figure 4A). We note that pretreatment of 4-PBA reducing ER stress did not affect the forskolin-stimulated cAMP level in the control cell lines (Figure 4A), suggesting that 4-PBA had effects only on cells expressing mutant torsinA. Thus, 4-PBA appears to rescue both ER stress and cAMP accumulation in DYT1 fibroblast cells. In addition, we observed no significant changes in ATP levels measured in this study between healthy fibroblast cells and DYT1 patient fibroblast cells (Figure 4B).

**Discussion**

Here, we addressed the involvement of torsinA in the response to ER stress and to the cAMP pathways. How these two pathways are linked mechanistically remains unclear, but it is possible though that the ER stress induced by mutant torsinA in patients with DYT1 dystonia contributes to neuronal dysfunction through a cAMP defect (Figure 5). In our previous studies on the torsinA-related AAA-ATPase p97, which also operates in ERAD and induces ER stress upon dysfunction, we found an inhibitory effect on adenylate cyclase when p97 was mutated [49]. Remarkably, we...
also observed that 4-PBA treatments can attenuate the ER stress caused by torsinA mutants and this is associated with rescue of the forskolin-induced cAMP-response. We propose that 4-PBA might have therapeutic benefits in patients with dystonia.

Defective proteasome function and ER stress have a contributing role in various neurologic diseases [50]. In response to stress, cells temporarily decrease protein synthesis, delay membrane trafficking, increase movement of unfolded proteins out of the ER into the cytoplasm via ERAD, and up-regulate transcription of some chaperone proteins [51]. Recently, our group has shown an aberrant regulation of proteasome activity in dystonia [24]. Protein components of the ERAD pathway, for example, p97 and Derlin-1, as well as torsinA, have been found in inclusion bodies in brain and peripheral nerves from patients with a variety of neurodegenerative diseases, as well as dystonia [52–55].

Additionally, complementary evidence indicates that torsinA can reduce ER stress caused by ER protein overload in nematodes, and that torsinA deficient MEFs have increased levels of ER stress, as compared with wild-type MEFs [23,24]. Interestingly, it has been proposed that ER stress can cause down-regulation of cAMP production in diabetes [56], and that a cAMP elevating reagent (CGS21680, an adenosine A2 receptor agonist) enhances proteasome activity in synapses of the striatum of R6/2 Huntington mouse model [57]. Interestingly, it has been shown that the proteasome activity can be regulated by PKA [58]. Future studies are needed to determine whether the impairment of proteasome activity in cells expressing torsinAΔE may cause accumulation of PKA regulatory subunits and/or suppression of PKA activity.

Our findings linking ER stress and cAMP signaling in dystonia models, also highlights that there is much to consider about striatal...
signaling to cAMP and PKA. Benzodiazepines are frequently used in the treatment of dystonia. This drug has muscle-relaxant properties and alleviates pain caused by muscle spasms and various dystonias [59,60]. Interestingly, the administration of diazepam, a benzodiazepine anxiolytic, in rhesus monkeys, is known to increase cAMP by inhibiting PDE cAMP hydrolysis [61].

Figure 4. cAMP defects in dystonia models are rescued by 4-PBA treatments. (A) Healthy control fibroblast cells (3 lines represented in white 1–3: HF6, GM01651, and GM02912) and DYT1 patient fibroblast cells (3 lines represented in black 4–5: GM02306, GM02551, and GM03211) were pre-treated with or without 4-PBA for 18 hours, followed by 10 μM forskolin stimulation for 30 minutes. As observed previously, DYT1 fibroblast lines have significantly lower levels of cAMP accumulation after forskolin stimulation compared to healthy control fibroblast lines (**P<0.01; one-way ANOVA and Tukey’s post hoc comparison, n = 3 experiments). However, the 4-PBA treatments also significantly rescued cAMP accumulation deficiency in DYT1 patient fibroblast lines comparable to control fibroblasts cAMP levels (*P<0.5; one-way ANOVA and Tukey’s post hoc comparison, n = 3 experiments). (B) No significant changes were observed in the intracellular ATP levels from healthy control cells (HF6, GM01651, GM02912) and DYT1 fibroblast cells (GM2306, GM02551, GM3211).

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Figure 5. Model of torsinA modulation of cAMP. In healthy fibroblast cells (left panel), forskolin stimulates adenylate cyclase (AC) to synthesis cAMP, and there is no presence of Endoplasmic Reticulum (ER) stress in normal conditions. In absence of torsinA or in presence of mutant torsinA, torsinAΔE (right panel), cells present increased ER stress and lower cAMP accumulation. However, 4-PBA treatment rescues both ER stress and cAMP accumulation defects in DYT1 dystonia cell models by unoknow mechanism (s).

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Numerous lines of evidence have linked dopaminergic dysfunction to dystonia [4,3]. The physiological actions of dopamine are mediated by five distinct but closely related G-protein-coupled receptors (GPCRs) that are divided into two major groups: the D1 (D1, D5) and D2 (D2, D3, D4) classes of dopamine receptors [62]. The D2 class is coupled to inhibitory G-proteins (G0/i) that decrease adenylate cyclase activity and cAMP levels, in contrast to the D1 class which acts through stimulatory G-proteins (e.g. Gsz) to increase activity and cAMP levels [63]. Dystonia has been associated with mutations in genes encoding proteins critical for dopamine (DA) signaling, such as GTP-cyclohydrolase [34], tyrosine hydroxylase [35], as well as polymorphisms in the DA receptor subtype, D5R [64], and GNAL dystonia gene, which encodes G α olf, a central component for striatal responses to dopamine (DA) [36]. Furthermore, several imaging studies showed various DA-related abnormalities, such as altered receptor binding in basal ganglia, in patients with different forms of dystonia [55–67]. Interestingly, it has been shown that adenylate cyclase activation by forskolin is highly potentiated in the presence of stimulatory G-proteins, such as G α olf [68]. Mutations in GNAL remove essential functional regions of Gzolf, responsible for activation of the G protein receptor D1 and Adenosine Receptor A2 (A2R), affecting cAMP accumulation. Thus, it is possible that mutations in GNAL and TOR1A genes may lead to dystonia via a common mechanism related to cAMP signaling. Perhaps torsinA participates in the proper folding/oligomerization of GPCRs, or stimulatory G-proteins, reducing the function of these proteins.

Our results suggest that 4-PBA may have clinical utility in the treatment of dystonia. 4-PBA is a low molecular weight fatty acid already approved for clinical use as an ammonia scavenger in children with urea cycle disorders [69]. Several groups revealed that 4-PBA reverses the mislocalization and/or aggregation of the protein associated with human disease, including the 4-PBA reverses the mislocalization and/or aggregation of the fibrosis transmembrane conductance regulator [47], mutant torsinA E knock-in dystonia mice (*P<0.001; Student t-test, 2 independent experiments, with average of 2–3 embryos per genotype). Relative expression of sliced sXBP1 mRNA levels was measured by quantitative PCR. Results might indicate the presence of torsinA increases ER stress in neurons. (PDF)

Table S1  Human fibroblast cells used in this study. (PDF)

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Author Contributions
Conceived and designed the experiments: FCN JAC GMM WIL. Performed the experiments: FCN JAC XL. Analyzed the data: FCN JAC XZ. Contributed reagents/materials/analysis tools: FCN JAC XL. Wrote the paper: FCN JAC GMM WIL.

Supporting Information
Figure S1  Neurons expressing torsinAΔE has higher ER stress compared to control neurons. Total mRNA was prepared from a mixed neuronal culture of dissociated primary cortical and striatal neurons from a common cellular dysfunction. International journal of cell biology 2012: 634214.

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