Apoptotic cell death and altered calcium homeostasis caused by frataxin depletion in dorsal root ganglia neurons can be prevented by BH4 domain of Bcl-xL protein

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Friedreich ataxia (FRDA) is a neurodegenerative disease characterized by a decreased expression of the mitochondrial protein frataxin. Major neurological symptoms of the disease are due to degeneration of dorsal root ganglion (DRG) sensory neurons. In this study we have explored the neurodegenerative events occurring by frataxin depletion on primary cultures of neurons obtained from rat DRGs. Reduction of 80% of frataxin levels in these cells was achieved by transduction with lentivirus containing shRNA silencing sequences. Frataxin depletion caused mitochondrial membrane potential decrease, neurite degeneration and apoptotic cell death. A marked increase of free intracellular Ca\(^{2+}\) levels and alteration in Ca\(^{2+}\)-mediated signaling pathways was also observed, thus suggesting that altered calcium homeostasis can play a pivotal role in neurodegeneration caused by frataxin deficiency. These deleterious effects were reverted by the addition of a cell-penetrant TAT peptide coupled to the BH4, the anti-apoptotic domain of Bcl-xL. Treatment of cultured frataxin-depleted neurons with TAT-BH4 was able to restore the free intracellular Ca\(^{2+}\) levels and protect the neurons from degeneration. These observations open the possibility of new therapies of FRDA based on modulating the Ca\(^{2+}\) signaling and prevent apoptotic process to protect DRG neurons from neurodegeneration.

INTRODUCTION

Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative disease caused by decreased expression of the mitochondrial protein frataxin due to large expansions of GAA triplet repeats in the first intron of the gene (1), promoting the formation of altered DNA structures or heterochromatin and transcriptional silencing (2). As a result, low percentages of frataxin are produced, generally below 30% of the normal levels. Patients with FRDA suffer progressive limb and gait ataxia, dystarthritis, reduced tendon reflex, extensor plantar responses and loss of their sense of position (3). The first pathologic changes are the loss of large sensory neurons in dorsal root ganglia (DRG), followed by degeneration of the spino-cerebellar and cortico-spinal tracts (4).

DRG sensory neurons express the highest levels of frataxin and display a high vulnerability to frataxin down-regulation (5). In mouse models, introduction of expanded GAA repeats effectively reduced frataxin levels and progressive neuronal pathology was observed (6). These results agree with the deleterious effects of frataxin depletion reported in DRG neurons using conditional knockout mice (7). Although the specific vulnerability of DRGs in FRDA has been deeply studied (8), the mechanisms leading to pathological changes occurring in these particular neurons after frataxin reduction are poorly understood.

Calcium regulates several physiological processes acting as an intracellular messenger. However, disruption of its homeostasis, due to an increase of free cytoplasmic Ca\(^{2+}\), can induce cell death by apoptosis (9). The most common mechanisms through which altered Ca\(^{2+}\) homeostasis promotes cell death are activation of Ca\(^{2+}\)-dependent proteases, nitric oxide synthases (NOS) and the Ca\(^{2+}\)-dependent transcription factor cAMP response element-binding protein (CREB). It is also worth mentioning that neurodegenerative diseases such as

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Alzheimer, Parkinson and Motoneuron disease show Ca\(^{2+}\)-dependent cell death (10).

In response to intracellular free Ca\(^{2+}\) increase, caspase and calpain proteases are activated and, among others, they cleave \(\alpha\)-fodrin, a cytoskeletal protein (11). Specific fragments of cleaved \(\alpha\)-fodrin have been described in Alzheimer disease (12). Also, increased free Ca\(^{2+}\) levels in the cytoplasm induce neuronal NOS (nNOS). This enzyme catalyzes the production of nitric oxide (NO) from L-arginine. NO can react with reactive oxygen species (ROS), producing a high reactive nitrogen species such as peroxynitrite, which, in turn, contributes to neuronal degeneration. Moreover, nNOS is involved in cell death by the activation of CREB (13, 14). This factor positively regulates the expression of the main pro-apoptotic protein Bax. Increase of Bax levels in the mitochondrial membrane would, in turn, alter the mitochondrial membrane potential promoting apoptotic cell death. Alteration in nNOS levels has been detected in several models of Alzheimer’s and Parkinson’s diseases (15, 16).

Apoptotic cell death induced by alterations on Ca\(^{2+}\) homeostasis can be counteracted by Bcl-x\(_L\) since it has been described that over-expression of this protein modulates intracellular Ca\(^{2+}\) and apoptotic signaling pathways (17). Beneficial effects of Bcl-x\(_L\) protein in cell survival has been demonstrated in several peripheral and central nervous system pathologies (18). The anti-apoptotic BH4 domain of Bcl-x\(_L\) interacts with pro-apoptotic proteins like Bax on mitochondrial membrane. Moreover, it has been described that BH4 domain of the Bcl-x\(_L\) protein is required for prevention of apoptotic cell death by inhibiting the loss of \(\Delta \Psi_m\) (19).

To analyze the molecular and cellular consequences of frataxin depletion, we used cultured DRG neurons as a cell model of FRDA. In these cells, frataxin expression was repressed by shRNAs using lentivirus as vectors. Analyses of these frataxin-deficient neurons indicate significant increase of neurite degeneration, decrease of mitochondrial membrane potential and apoptotic cell death. We also observed alterations of nNOS, CREB and calpain and caspase-mediated \(\alpha\)-fodrin fragmentation. This phenotype could be explained by the marked increase of free intracellular Ca\(^{2+}\) observed in these cells. Recovering from these pathological changes has been achieved by TAT-mediated delivery of anti-apoptotic domain BH4 of Bcl-x\(_L\) (TAT-BH4). Systemic administration of this peptide was able to prevent these neurons from neurite degeneration and apoptotic cell death, recovering Ca\(^{2+}\) homeostasis and all the downstream effects. These results offer a rationale for a potential new design for FRDA therapy based on controlling apoptosis and regulation of Ca\(^{2+}\) homeostasis in the DRG neurons.

**MATERIALS AND METHODS**

**Primary DRG sensory neurons culture**

DRGs were extracted from neonatal rats (P3-P4) and washed three times with GHEBs (137 mM NaCl, 2.6 mM KCl, 25 mM glucose, 25 mM HEPES, 100 \(\mu\)g/ml penicillin/streptomycin) before being dissociated with 0.25% Trypsin in GHEBs media for 40 min. Ganglia were mechanically disrupted with a pipette tip until obtaining a single cell suspension in culture media, which was centrifuged at 1000 rpm through 10% BSA solution (Sigma) for 5 min, followed by re-suspension in enriched Neurobasal culture media consisting of 2% horse serum, 125 nm L-glutamine (Invitrogen), 100 U/ml penicillin plus 100 ng/ml Streptomycin (NBMc) and supplemented with nerve growth factor (NGF 50 ng/ml). The cells were then plated in a four-well tissue dish (Nunc, Thermo Fisher Scientific) pre-treated with collagen (Sigma), at a cell density of 10 000 cells/well for survival experiments and 30 000 cells for western blots. To prevent the growth of non-neuronal cells, culture media were supplemented with the anti-mitotic agent Aphidicoline at a final concentration 3 \(\mu\)g/\(\mu\)l (Sigma).

**Plasmids and production of lentiviral particles**

Lentiviral vectors encoding short-hairpin RNA-interfering sequences shFxn1, shFxn2 for Frataxin or scrambled sequence (used as control) were purchased from Sigma (Mission\textsuperscript{®} shRNA, Gene Bank accession number NM_000144.3-796s1c1, NM-008044.1-600s1c1 and SHC002, respectively). Plasmid over-expressing human Bcl-x\(_L\) was provided by Dr J. Comella (20). Lentiviruses were propagated in HEK293 T cells using the polyethyleneimine cell transfection method (21). Lentiviruses were titrated with QuickTiter\textsuperscript{TM} HIV Lentivirus Quantitation Kit (ref VPK 108H, Cell Biolabs). For lentiviral transduction, DRG neurons were plated in four-well dishes and 24 h later the medium containing lentivirus particles (20 \(\mu\)g/cell) was added. Transduction was allowed to proceed for 20 h. After that, media containing lentivirus was substituted by fresh culture media.

**TAT-BH4 fusion-peptide experiments**

TAT-BH4 peptide was obtained from Calbiochem (La Jolla, CA) and control HIV-TAT peptide from Sigma. TAT-BH4 peptide was dissolved in DMSO and TAT control peptide in distilled water, both to a final concentration of 1.3 mM. For survival analysis, both peptides were diluted in culture media to final concentrations of 1 or 2 \(\mu\)M. Media containing TAT peptide were added with a DMSO volume equivalent to that used when adding TAT-BH4 peptide.

**Calcium chelator BAPTA experiments**

1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraaceticacid tetraacetate (BAPTA-AM) was obtained from Invitrogen and dissolved in DMSO to a final concentration of 5 mM. For survival analyses, BAPTA was diluted in culture media to a final concentration of 5 \(\mu\)M or 12 \(\mu\)M (see Results). A volume of DMSO equivalent to that used for BAPTA supplementation was added to control cultures.

**Measurement of DRG neuronal survival and neurite degeneration**

Neuronal survival was measured as described (21) and expressed as the percentage of cells counted at 5 days with respect to the initial value. Morphometric analyses of neurite degeneration were performed as described (22), with some modifications. Specifically, at the desired days after lentivirus transduction of DRG neurons, phase-contrast images were taken using a \(\times20\) lens. A grid was created over each image with NIH Image J.
software, using the grid plugin (line area = 100 000 pixels × 2). Healthy and degenerated neurites (displaying swelling and/or blebbing) were counted at three power-fields per image, three images for each well. Three different wells were counted for each condition and the experiments were repeated at least three times.

**Immunofluorescence analysis**

Cultures were fixed in 4% paraformaldehyde and incubated overnight at 4°C with specific antibodies at indicated dilutions: SM132 (1:1000) from Covance, SM131 (1:1500); βIII-Tubulin (1:400) and anti-TAT (1:100) from Sigma or PGP 9.5 (1:500) from Chemicon. Anti-rabbit or anti-mouse secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen), respectively, were used at 1:1000 dilutions and incubated for 1 h at room temperature, protected from light. For cell nucleus staining, the fixed cells were incubated for 1 h with Hoechst33258 (0.05 μg/ml). Micrographs were taken using an Olympus FluoView IX71.

**Assessment of mitochondrial membrane potential (ΔΨ) m**

The ΔΨ m was assessed using the dual-emission, mitochondrion-specific, lipophilic, cationic dye 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetrachrylpyridyl-1-carcobayanide iodide (JC-1) (Gibco-Invitrogen). Cultures of DRG neurons were first incubated with 5 mg/ml JC-1 at 37°C for 30 min and then washed 3 times with culture media. Images were taken using an Olympus FliuorView IX71 microscope (excitation λ = 460–490 nm and emission higher than λ≥10 nm). Fluorescence is green/yellow in mitochondria with low ΔΨ m and red/orange in those with high ΔΨ m.

**Free intracellular Ca2+ measurement**

Free intracellular Ca2+ was detected using a Fluo-8 no wash Ca2+ assay kit. DRG neurons were cultured in 96-well microplate with black wall and clear bottom (20 000 cell per well). The cells were transduced with lentivirus containing Scr, shFxn1 or shFxn2. After 3 days, transduced DRG neurons were washed with Ca2+-free media and incubated with Fluo8 for 30 min at 37°C. Calcium2+ flux (F) was measured by monitoring the fluorescence intensity at Ex/Em = 490/525. The fluorescence of Ca2+ -saturated probe (Fmax) was measured after the addition of 50 μM of ionomycin. The absence of free Ca2+ content (Fmin) into the cells was detected after the addition of 50 mM of EGTA. To determine the arbitrary fluorescence units (AUF) the following equation was used: AUF = [(F - Fmin)/(Fmax - F)] × 100. The AUF in frataxin-depleted neurons (shFxn1 and shFxn2) were represented as the percent of the control AUF values (100%).

**Western blot analysis**

To obtain crude extracts, cells were first rinsed in ice-cold PBS, pH 7.2, and lysed with 2% SDS and 125 mM Tris. Protein concentration was determined with BioRad protein assay. After SDS-polyacrylamide gels electrophoresis, proteins were transferred to Immobilon-P or Nitrocellulose membranes (Millipore). The membranes were probed with specific antibodies at the indicated dilutions: anti-Frataxin (1:500) from Santa Cruz Biotechnology, anti-Bax (1:1000), anti-Cleaved Caspase-3 (1:1000) and anti-Phospho-CREB (1:1000) and CREB (1:1000) from Cell Signaling, Bel-xL (1:1000) from BD Transduction Laboratories, anti-α-Tubulin (1:1000) from Biomol International, Exeter, UK, anti-3-nitrotyrosine (1:1000) from Abcam and anti-α-Tubulin (1:50 000) from Sigma. Chemoluminescence was developed using the ECL Advance western blotting detection kit (GE Healthcare).

**Statistical analysis**

The data obtained from at least three independent experiments were used for statistical analyses. Values were expressed as mean ± SEM (error bars). One-way ANOVA was used to assess survival, neurite degeneration and SM131 differences between groups for variable treatment. If the ANOVA test was statistically significant, we performed post hoc pair wise comparisons using the Bonferroni test. P-values lower than 0.05(*), 0.01(**) or 0.001 (***)) were considered significant.

**RESULTS**

**Frataxin depletion induces neurite degeneration in DRG neurons**

Since many of the major neurologic problems in FRDA patients are attributable to DRG degeneration, we set up a cell model to study FRDA by silencing frataxin expression in primary cultures of DRG neurons. To this end, two shRNA constructs (here referred as shFxn1 and shFxn2) targeting specific sites of rat frataxin sequence were used. DRG neurons were transduced with lentiviruses carrying either shFxn1 or shFxn2, as described in Materials and Methods. Cells transduced with scrambled shRNA were used as controls. Frataxin content was evaluated 5 days post-transduction showing an 80% decrease compared with cells transduced with scrambled shRNA (Fig. 1A). This reduction is similar to that observed in FRDA patients (23). Several studies have described that axonal degeneration is a hallmark of many neurologic disorders (22, 24). Morphometric analyses of neurite morphology 5 days after transduction indicated that frataxin-deficient DRG neurons show a marked axonal degeneration compared with control cells (Fig. 1B). Immunofluorescence images obtained using SM132 (recognizing NF-200) and β-III tubulin antibodies (a specific neuronal marker) showed an accumulation of NF-200 in neurites of frataxin-deficient DRG neurons (Fig. 1C), which is in agreement with the neurite morphological changes observed in Figure 1B.

It has also been described that axonal swelling in DRG sensory neurons is sufficient to elicit aberrant neurofilament phosphorylation in neuronal perikarya (25, 26). To detect the presence of this form in frataxin-depleted DRG neurons, we used SMI-31, which recognizes phosphorylated NF-200, and PGP 9.5 as a neuronal marker. These neurons exhibit a significant increase in the number of SMI-31 positive cell bodies compared with control cells (Fig. 1D).
Figure 1. Frataxin depletion induces neurite degeneration of DRG neurons. Rat DRG neurons were transduced with lentivirus vectors containing shFxn1, shFxn2 or scrambled shRNA. (A) Protein extracts from 5-day-transduced cultures were probed with an anti-Frataxin antibody by western blot analysis and re-probed with an antibody against α-Tubulin, used as a loading control. Histogram represents the quantification of the relative expression of Frataxin normalized by α-Tubulin content. Data from three independent experiments are presented as mean ± SEM (error bars). (B) Axons visualized by phase-contrast microscopy. Arrows indicate neurite swelling present only in degenerated neurons. Histogram shows the percentage of degenerated neurites after frataxin depletion. (C) Immunofluorescence analysis after double staining with SMI32 and βIII-Tubulin antibodies. Arrows indicate neurofilament accumulation. Scale bar in B and C is 12 μm. (D) Immunofluorescence images obtained after double staining with phosphorylated neurofilament (SMI31) and PGP 9.5 antibodies. Scale bar is 20 μm. The histogram represents the number of SMI31 positive cell bodies relative to total cell number for each condition analyzed. Data are the mean ± SEM obtained from nine wells in three independent experiments. Asterisks in A, B and C indicate significant differences (**P < 0.01 or ***P < 0.001 compared with Scrambled transduced neurons).
Frataxin depletion induces apoptotic cell death in DRG neurons

Apoptosis is an important mechanism of cell death in many peripheral nervous system disorders including FRDA (27, 28). To test whether this process occurs in our model we first analyzed the viability of DRG neurons after frataxin depletion. As shown in Figure 2A, 5 days after lentivirus transduction, frataxin-deficient neurons showed a significant decrease of cell viability compared with control cultures (Fig. 2A). To test whether apoptosis is playing a role in cell death observed in our model, we analyzed the presence of caspase 3 cleaved fragment. Compared with control cells, levels of this apoptotic marker increased ~4-fold in cells silenced with shFxn1 and shFxn2 (Fig. 2B). Regarding the characterization of the events upstream from caspase activation, the levels of Bax, a pro-apoptotic protein, showed a 3-fold increase compared with control cells (Fig. 2B). In agreement with these results, we have also observed nuclei with apoptotic morphology in frataxin-deficient cells (Fig. 2C). It is known that most of the mitochondrial-dependent apoptotic processes are associated with the loss of $\Delta \Psi_m$. As shown in Figure 2D, analysis of DRG neurons 5 days after frataxin depletion using the JC-1 probe as an indicator of mitochondrial potential indicator revealed a clear decrease of $\Psi_m$. Overall, these results show that in DRG neurons, frataxin deficiency induces apoptotic cell death.

Alterations of Ca$^{2+}$ homeostasis and its signaling components are involved in the apoptotic cell death of frataxin-deficient DRG neurons

The Ca$^{2+}$ ion is one of the main messengers involved in the neuronal physiology. However, the Ca$^{2+}$ homeostasis can be found altered in pathological conditions (29). Free Ca$^{2+}$ levels were measured in DRG neurons at Day 4 after frataxin depletion. Neurons transduced with shFxn1 and shFxn2 show up to 3–4-fold increase (403 ± 86.26 and 355.8 ± 87.65%, respectively) in free Ca$^{2+}$ levels compared with the cells transduced with scrambled shRNA control (100%). A representative image of increased cellular fluorescence induced by free calcium is shown in Figure 3A.

It is known that increase of free Ca$^{2+}$ levels promote the activation neuronal nitric oxide synthase (nNOS). Alteration of nNOS protein amounts has been reported in several neurodegenerative disorders (30). In our model, analyses of nNOS amounts in frataxin-depleted neurons 5 days post-transduction showed a 4-fold increase compared with that of scrambled control (Fig. 3B). The combination of NO and superoxide anion give peroxynitrite which, in turn, can modify tyrosine residues producing protein bound 3-nitrotyrosine (3-NT) (31). In our model, frataxin-depleted DRG neurons display a marked increase of 3-NT compared with the control (Fig. 3C). The elevated cytosolic Ca$^{2+}$ levels are the signals to phosphorylate Ca$^{2+}$-dependent transcription factor-CREB causing its activation. Western blot analysis of cell extracts at 5 days after transducing DRG neurons with shFxn1 or shFxn2 showed a 2-fold increase of phosphorylation of CREB levels compared with the control (100%) (Fig. 3D). This result also explains the increased Bax levels shown in Figure 2B, since it is well known that the activation of CREB leads to transcription of Bax gene (32).

Moreover, increase of free Ca$^{2+}$ induces the cleavage of cytoskeleton protein α-fodrin by calpain and/or by caspase rendering fragments of 150 and 120 kDa, respectively. In our model, at 5 days after frataxin depletion, a 3-fold increase in levels of both fragments (150 and 120 kDa) were detected in the shFxn1 and shFxn2 transduced cells compared with the scrambled control (Fig. 3E).

In order to confirm that alterations observed in our model after frataxin depletion were due to the interruption of calcium homeostasis we used a highly selective Ca$^{2+}$ chelator BAPTA-AM (Fig. 4). DRG neurons were transduced with shFxn1, shFxn2 or scrambled shRNA for 20 h. The lentivirus-containing media were then replaced with fresh culture media containing 5 or 12 μM BAPTA or DMSO (used in control cultures). Survival analysis of frataxin-deficient DRG neurons at Day 5 after treatment demonstrated a dose-dependent neuroprotective effect of BAPTA treatment (Fig. 4A). Moreover, western blot analysis of the cells treated with 12 μM BAPTA showed a reduction of the fodrin-cleaved fragments of 120 and 150 kDa in shFxn1 and shFxn2 cells compared with their respective controls (cells treated with DMSO) (Fig. 4B).

Effects of Bcl-xL on cell viability of frataxin-depleted DRGs

In the nervous system, Bcl-xL is one of the proteins that strongly controls the apoptotic processes by interaction with pro-apoptotic protein Bax. Beneficial effects of Bcl-xL over-expression in cell survival have been demonstrated in several peripheral and central nervous system pathologies (18, 24). In this study we have shown that frataxin depletion leads to increase of the apoptotic markers in DRG neurons; for this reason, we evaluated the role of Bcl-xL over-expression to prevent the observed neuronal degeneration. To that purpose, DRG neurons were co-transduced with both, human Bcl-xL (hBcl-xL) and shFxn1 or shFxn2. Over-expression of Bcl-xL was confirmed by western blot 5 days post-transduction (Fig. 5A). Of note, hBcl-xL over-expression did not affect frataxin interference. As shown in Figure 5B, cell death was about 50% in DRG neurons 5 days after frataxin depletion; in contrast, over-expression of hBcl-xL maintained cell viability at around 90%. We also confirmed that Bcl-xL suppressed cytoskeleton alterations detected after frataxin depletion (Fig. 5C and D).

TAT-BH4 peptide maintains Ca$^{2+}$ homeostasis and protects frataxin-depleted DRGs from degeneration

The neuroprotective effects obtained in our model by over-expression of hBcl-xL provide clues for a potential FRDA therapy. In the last 10 years a new technology has been developed to introduce proteins or peptides into cells using transactivation domain TAT. Based on the fact that BH4 domain of Bcl-xL is crucial for prevention of apoptotic mitochondrial changes (33), cultures of frataxin-depleted DRG neurons were added with pharmacologic cell-permeable peptide TAT carrying the BH4 domain. DRG neurons were transduced with shFxn1, shFxn2 or scrambled shRNA for 20 h. The lentivirus-containing media were then replaced with fresh culture media containing TAT-BH4 or TAT peptide (used in control cultures). Cultures were supplemented with each peptide every 24 h until the end.
of the experiment (Day 5). The accumulation of TAT protein after its administration in cultures of lentivirus-transduced cells was confirmed by immunofluorescence using an anti-TAT antibody (Fig. 6A). Survival analysis of frataxin-deficient DRG neurons at Day 5 of the treatment with 1 or 2 μM TAT-BH4 showed a dose-dependent neuroprotective effect (Fig. 6B). No significant changes were detected in the survival values of control cells after TAT or TAT-BH4 treatment.

To test whether TAT-BH4 is able not only to prevent cell death in frataxin-depleted neurons but also to rescue them once degeneration starts, TAT-BH4 was added to cultures 2 days after the transduction procedure. Once lentivirus media was replaced with fresh media, cells were cultured for 2 days and then 2 μM of TAT-BH4 or TAT alone (used as a control), were added to the cultures. Peptide treatment was repeated daily until the end of the experiment (Day 5). As shown in Figure 6C, addition of TAT-BH4 rescued DRG neurons from cell death compared with control cultures in which only the TAT peptide was added. In agreement with these improved survival values, frataxin-deficient cells treated with TAT-BH4 exhibited a significant decrease in degenerated neurites compared with those treated only with the TAT peptide (Fig. 6D).

Based on the fact that BH4 domain of Bcl-xL is able to protect cells from the increase of intracellular Ca^{2+} levels (34), we checked this fact in our model. As shown in Figure 7A, TAT-BH4 (2 μM) treatment was able to reduce free intracellular Ca^{2+} levels close to those observed in control cells. Moreover, TAT-BH4 treatment was able to restore Ca-signaling pathway in DRG neurons affected after frataxin depletion (Fig. 7B). Decreased levels of p-CREB as well as Bax levels in frataxin-depleted neurons treated with TAT-BH4 were also evident when compared with their respective controls (cells treated with TAT + DMSO).

We also tested whether α-fodrin breakdown pattern was reduced by TAT-BH4 treatment. Western blot analysis indicated a decrease of the 120 and 150 kDa fragments of α-fodrin in TAT-BH4-treated frataxin-depleted neurons...
compared with their respective controls (cells transduced with shFxnl and shFxn2 treated with TAT + DMSO alone) (Fig. 7C).

Moreover, we analyzed the effects of TAT-BH4 treatment on mitochondrial membrane potential of frataxin-depleted neurons. Using JC-1 dye we observed a recovery of mitochondrial membrane potential in TAT-BH4-treated shFxnl and shFxn2 neurons in contrast to those treated with TAT peptide, used as a control (Fig. 7D).

**DISCUSSION**

Many models of FRDA exist, in organisms from yeast to mouse (35, 36). These models are important for understanding frataxin function and for testing potential therapeutic agents. Several studies have reported the loss of neuronal cells after frataxin depletion, suggesting that some of the phenotypes observed in patients with FRDA could be the consequence of these events (27). Although the consequences of frataxin depletion...
in neuronal cells have been investigated, the mechanisms by which frataxin depletion induces these alterations are still under intense research. In this context, it has been recently described that frataxin depletion in DRG neurons is associated with defects on antioxidant defense mechanisms which would be the consequence of a decreased Nrf2 expression (37).

In this study we propose the alterations of Ca\(^{2+}\) homeostasis and signaling as a pathway by which decreased frataxin levels lead to apoptotic cell death. To analyze the mechanisms through which frataxin depletion promotes neurodegeneration, we have set up a cellular model to study FRDA using DRG sensory neurons, the most affected in the disease. Frataxin protein amounts in this model were reduced to <30% of normal values resembling those found in patients with FRDA (23). Frataxin depletion in these cells promotes neurite degeneration, as indicated by swelling and accumulation of neurofilaments. Neurofilament aggregates are common pathological markers for diverse neurodegenerative diseases including FRDA (38). We have also detected accumulation of phosphorylated neurofilament in the neuronal cell bodies of frataxin-deficient cells. This result is in agreement to that reported in neurons from anterior horns of FRDA patients (39). In this context, it is worth mentioning that in neurodegenerative disorders, accumulation of phosphorylated NFs also occurs in the perikaryon although under normal conditions phosphorylation of NF-200 is only present in the neurites (40, 41).

Our results also show that decreased frataxin levels in DRG neurons leads to loss of mitochondrial membrane potential, cleaved caspase 3 and apoptotic cell death. These alterations have also been reported in other neuronal models of FRDA (27, 28). Several studies demonstrated that mitochondrial membrane potential alteration and caspase 3 activation observed in neurodegenerative diseases are due to the disruption of Ca\(^{2+}\) homeostasis (42). Ca\(^{2+}\) is one of the most important molecules involved in neuronal signaling; however its excessive release into cytoplasm leads to neuronal death. In normal conditions the Ca\(^{2+}\) homeostasis is regulated by mitochondria and endoplasmic reticulum. Upon oxidative stress produced by mitochondrial ROS generation, the presence of free Ca\(^{2+}\) in the cytoplasm triggers the activation of calcium-dependent proteins (43). One of them is a nNOS. In DRG neurons, nNOS is the main enzyme involved in endogenous NO production (44) and changes in its expression may regulate the pathophysiological functions of NO (45). In our model, as in models of several neurodegenerative diseases (30), increased levels of nNOS and 3-nitrotyrosine-protein levels have been detected. Some authors suggest that nNOS contributes to apoptotic cell death by CREB-mediated increase of Bax protein levels (13, 14). CREB is the transcription factor which is activated after the increase of intracellular Ca\(^{2+}\) (14). In our model, increase of p-CREB levels together with enhanced of Bax levels has been shown. Bax is a main pro-apoptotic protein of Bcl-2 family which is involved in the activation of the mechanisms leading to apoptotic cell death by collapsing the mitochondrial membrane potential (46). In this context, decrease of mitochondrial membrane potential and apoptotic markers (caspase 3 active fragment and nuclear fragmentation) has been observed in our model.

Alteration of Ca\(^{2+}\) homeostasis also leads to activation of a protease calpain which, in turn, triggers apoptotic cell death by the activation of caspase 3 pathways in a Bax-dependent manner (43). One of the substrates cleaved after calpain activation is the cytoskeleton protein α-fodrin. In addition, α-fodrin can be cleaved also by caspase. In our model, calpain-dependent fragment, as well as caspase-dependent fragment of α-fodrin has been detected. Moreover, using calcium chelator BAPTA, we have observed a decrease of fodrin-cleaved fragments and reduction of cell death due to frataxin depletion. Taking together, these observations suggest that increase of intracellular Ca\(^{2+}\) is involved in the apoptotic cell death observed in frataxin-depleted DRG neurons. It is worth mentioning that these results are in agreement to results obtained with fibroblasts from FRDA patients in which treatment with BAPTA (a Ca\(^{2+}\) chelator) or with apoptosis inhibitors rescued fibroblasts from deleterious effects of frataxin deficiency (47).

One of the key proteins involved in the control of the apoptotic processes induced by Ca\(^{2+}\) is Bcl-x\(_L\). It has been described that over-expression of this protein is able to regulate Ca\(^{2+}\) homeostasis. Moreover, Bcl-x\(_L\) has demonstrated a wide range of cell survival activities in diverse pathological events in the nervous system (18). In our model, lentivirus-mediated over-expression of human Bcl-x\(_L\) was able to prevent neurite swelling and cell death without altering low frataxin levels. The encouraging results obtained with Bcl-x\(_L\) led us to test the effect of BH4...
Figure 5. Over-expression of Bcl-xL blocks neurite degeneration and cell death induced by frataxin depletion. DRG neurons were co-transduced with lentivirus carrying human Bcl-xL and with lentivirus carrying either shFxn1, shFxn2 or scrambled (control) sequences. Images and analyses were performed 5 days after transduction. (A) Phase contrast and Green Fluorescent Protein (GFP) microscopy images of co-transduced DRGs to show the expression of the hBcl-xL protein in Scr, shFxn1 or shFxn2 (scale bar is 12 μm). Protein extracts from cells co-transduced with hBcl-xL (shFxn1 + hBcl-xL, shFxn2 + hBcl-xL or Scr + hBcl-xL) were analyzed by western blot with anti-Bcl-xL or anti-Frataxin antibodies and antibody against α-Tubulin as a loading control. (B) Percentage of cell survival after 5 days of transduction with the indicated lentiviruses. Asterisks indicate significant differences (***, P < 0.001) compared with shFxn1 or shFxn2 transduced neurons. (C) Representative phase-contrast microscopy images showing neurite morphology of the cells co-transduced with the corresponding shRNA and hBcl-xL. (D) Immunofluorescence analyses of the cells as in (C) with SMI32 antibody to test neurofilament accumulation. Scale bar in (C) and (D) is 20 μm.
Figure 6. Addition of TAT-BH4 decreases neuronal death and neurite degeneration observed in frataxin-depleted DRG neurons. Cultures of DRG neurons were transduced with lentivirus carrying shFxn1, shFxn2 or scrambled sequences. After 20 h, lentivirus-containing media was withdrawn and fresh media containing TAT-BH4, was added to each transduced culture (control cultures were treated with TAT + DMSO). (A) Representative immunofluorescence microscopy images of frataxin-defective neurons immunostained with anti-TAT and SMI32 antibodies, after 5 days treatment with TAT + DMSO or TAT-BH4. (B) Histogram indicates percentage of cell survival of frataxin-depleted neurons treated daily with 1 μM TAT-BH4, 2 μM TAT-BH4 or TAT + DMSO, respectively. Asterisks indicate significant differences (***P < 0.001) compared with TAT-DMSO treated neurons. (C) Images of representative same microscopic fields at 0 and 5 days after lentivirus transduction with the indicated shRNAs. Cultured DRG neurons were transduced with lentivirus vectors containing shFxn1, shFxn2 or scrambled sequences. The lentivirus-containing media were replaced (after 20 h) with fresh medium. After 48 h culture media were replaced every 24 h with media containing 2 μM of TAT + DMSO or TAT-BH4, respectively. Arrows indicate dead cells (Day 5). Scale bar is 20 μm. Histogram values in (C) represent the percentage of cell survival 5 days after lentivirus transduction. Asterisks in (B) and (C) indicate significant differences (***P < 0.001) compared with TAT-DMSO treated neurons. (D) Representative images showing immunostaining with SMI32, indicating neurofilament accumulation in the cells described above. Scale bar is 20 μm. Quantitation of neurites with neurofilament aggregates is shown in the histogram.
Figure 7. TAT-BH4 restores of Ca\(^{2+}\) homeostasis and mitochondrial membrane potential, and decrease of calpain and caspase-mediated α-fodrin cleavage in frataxin-deficient DRG neurons. Cultured DRG neurons were transduced with lentivirus vectors containing shFxn1, shFxn2 or scrambled shRNA. The lentivirus-containing media were replaced (after 20 h) with fresh medium. After 48 h culture media were replaced every 24 h with media containing 2 μM of TAT + DMSO or TAT-BH4, respectively. The histogram in (A) represents the intracellular fluorescence of free Ca\(^{2+}\) in DRG neurons 5 days after lentivirus transduction with scrambled, shFxn1 and shFxn2 supplemented or not with 2 μM TAT-BH4. The values in the histogram were calculated as indicated in Materials and Methods. The asterisks indicate significant differences (\(^*P < 0.05\)) compared with TAT-DMSO treated neurons. Protein extracts from DRG neurons transduced with shFxn1, shFxn2 or Scrambled and treated daily with 2 μM TAT-BH4, or TAT + DMSO were probed with p-CREB, CREB and BAX antibodies in (B) and with anti-α-fodrin in (C). Loading controls were obtained by reprobing, membranes with antibody against α-Tubulin. (D) Microscopy images of cells incubated with JC-1 probe to test mitochondrial membrane potential. Cells were cultured as described above. Inserts represent a magnified area for better clarity. Scale bar is 10 μm. Mitochondria with normal ΔΨ\(_m\) have orange-reddish color; cells with decreased ΔΨ\(_m\) have a green-yellowish color.
domain in our cell model. This was achieved by a using protein or peptide fusion to a cell-penetrating peptide (TAT) technology (48). TAT fusion proteins have been successfully used to alleviate neurodegenerative processes observed after spinal cord and brain injuries (49). In addition, a TAT-frataxin protein was able to protect dopaminergic neurons from oxidative stress in a mouse model of Parkinson disease (50) and increase lifespan and cardiac function in a conditional FRDA mouse model (51). TAT-BH4 has also demonstrated promising results in a mouse model of amyotrophic lateral sclerosis, improving animal lifespan (34). In this work we demonstrated that TAT-mediated delivery of BH4 domain of Bel-xL is able to protect DRG neurons from the pathological changes observed after frataxin depletion. We chose BH4 domain of the Bel-xL protein because it is a main domain required for prevention of apoptotic cell death. Moreover, BH4 domain blocks the Ca\(^{2+}\) release from endoplasmic reticulum (52) and closes voltage-dependent anion channels. Blocking Ca\(^{2+}\) entry into mitochondria (53) protects mitochondria from the loss of ΔΨ\(_m\) in the presence of Bax (19, 33). In our model, TAT-BH4 restores mitochondria ΔΨ\(_m\) and decrease p-CREB and Bax levels and reduces calpain and caspase-mediated α-fodrin cleavage. These events suggest that improved viability and protective effects on neurite morphology observed in our model after TAT-BH4 treatment would be the consequence of restoring Ca\(^{2+}\) homeostasis and mitochondrial membrane potential. A summary of these events is shown in Figure 8.

In conclusion, the present work shows that systematic exogenous administration of TAT-BH4, either initially or 2 days after lentivirus transduction, is able to protect DRG neurons from the cellular alterations induced by increased free Ca\(^{2+}\) levels which occur after frataxin depletion. Taken together, these results open the possibility of a new therapeutic strategy for FRDA based on regulation of Ca\(^{2+}\) homeostasis and apoptosis in these neurons.

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