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The mitochondria-targeted anti-oxidant MitoQ reduces aspects of mitochondrial fission in the 6-OHDA cell model of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder for which available treatments provide symptom relief but do not stop disease progression. Mitochondria, and in particular mitochondrial dynamics, have been postulated as plausible pharmacological targets. Mitochondria-targeted antioxidants have been developed to prevent mitochondrial oxidative damage, and to alter the involvement of reactive oxygen species (ROS) in signaling pathways. In this study, we have dissected the effect of MitoQ, which is produced by covalent attachment of ubiquinone to a triphenylphosphonium lipophilic cation by a ten carbon alkyl chain. MitoQ was tested in an in vitro PD model which involves addition of 6-hydroxydopamine (6-OHDA) to SH-SY5Y cell cultures. At sublethal concentrations of 50 μM, 6-OHDA did not induce increases in protein carbonyl, mitochondrial lipid peroxidation or mitochondrial DNA damage. However, after 3 h of treatment, 6-OHDA disrupts the mitochondrial morphology and activates the machinery of mitochondrial fission, but not fusion. Addition of 6-OHDA did not increase the levels of fission 1, mitofusins 1 and 2 or optic atrophy 1 proteins, but does lead to the translocation of dynamin related protein 1 from the cytosol to the mitochondria. Pre-treatment with MitoQ (50 nM, 30 min) results in the inhibition of the mitochondrial translocation of Drp1. Furthermore, MitoQ also inhibited the translocation of the pro-apoptotic protein Bax to the mitochondria. These findings provide mechanistic evidence for a role for redox events contributing to mitochondrial fission and suggest the potential of mitochondria-targeted therapeutics in diseases that involve mitochondrial fragmentation due to oxidative stress.

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1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder. At present, common medications provide some dramatic relief of the symptoms but fail to abrogate disease development. Unfortunately, efforts to find effective agents that provide protection against neurodegeneration have been rather unsuccessful [1]. Oxidative stress, resulting from an imbalance between generation of reactive oxygen species (ROS) and antioxidant mechanisms, is an important factor in the etiology and pathogenesis of degenerative diseases. Besides their importance for bioenergetics, mitochondria are involved in oxidative damage and cell signaling leading to cell death through the generation of ROS. Therefore, mitochondrial dysfunction is central to a broad range of pathologies [2–5].

There are a series of mitochondrial antioxidant defenses for intercepting ROS and minimizing oxidative damage. But, excessive production of ROS or disruption of the antioxidant defenses leads to extensive oxidative damage of the mitochondria [6]. Pathology, toxicology, and genetics suggest mitochondrial oxidative stress as an etiological cause in PD [7,8]. A plausible general therapeutic approach thus could be the reduction of mitochondrial oxidative damage [6,9]. A number of mitochondria-targeted therapies are being developed [10], in particular antioxidants conjugated to triphenylphosphonium cation (TPP) such as mitoquinone, mitovitamin E and mitophenyltertbutyline [11]. The best characterized mitochondria-targeted antioxidant is MitoQ ([10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl] triphenylphosphonium), which consists of a ubiquinone moiety that is linked to a TPP cation by a 10-carbon alkyl chain [12]. MitoQ accumulates within the mitochondria and, once it is there, is continually recycled to the active ubiquinol antioxidant by complex II of the respiratory chain [12,13]. MitoQ has been used to prevent mitochondrial oxidative damage and to block the involvement of ROS in signaling pathways [12,13]. MitoQ functions most likely through its direct antioxidant action,

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although concomitant antiinflammatory and antihypoxic properties have been shown [14–16]. MitoQ has been developed as a therapy for humans and has undergone clinical trials [17,18].

Different experimental models have proved effective in the search for clues to the underlying cause of Parkinson's Disease (PD) [19], for instance, the administration of 6-hydroxydopamine (6-OHDA) to experimental animals or cell cultures [20]. However the mechanisms by which 6-OHDA elicits its neurotoxic effects have yet to be fully elucidated, although a role for oxidative stress (reviewed in [2]) and the participation of mitochondrially-mediated cell death [21], including morphological alterations of mitochondria such as fragmentation [22] are likely.

Mitochondria undergo continuous fission, fusion and movement referred to as mitochondrial dynamics. Major neurodegenerative diseases, including PD, involve disruption of mitochondrial dynamics. These structural abnormalities are caused by an imbalance in highly conserved GTPase genes, which are essential for mitochondrial fission and fusion. GTPase dynamin-related protein-1 (Drp1), fission 1 (Fis1), mitofusins 1 and 2 (Mfn1, Mfn2), and optic atrophy 1 (Opa1) proteins —regulate, maintain and remodel mammalian mitochondria [23,24]. Remarkably, in several disease models, the manipulation of mitochondrial fusion or fission can partially rescue disease phenotypes [23,25]. Therefore, interference with those early processes may yield a neuroprotective approach. Recent studies reported that impaired mitochondrial dynamics involve the participation of Drp1 in different neurodegenerative disease, including Alzheimer's [25,26] and Huntington's [24,27]. In addition, in a previous study, we have shown that in an in vitro pharmacological model of Parkinson's disease, 6-OHDA disrupts mitochondrial dynamics by inducing mitochondrial fragmentation. In this case activation of the mitochondrial fission machinery was monitored by assaying the translocation of Drp1 from the cytosol to the mitochondria [22]. The translocation of Drp1 to the mitochondria took place very early in the process, before apoptotic hallmarks such as collapse of the mitochondrial membrane potential or cytochrome c release become apparent, and was accompanied by formation of Bax foci on the mitochondrial surface [21]. In the present study we found that MitoQ dramatically diminished 6-OHDA-induced mitochondrial fragmentation. Through its capacity to alter mitochondrial redox processes MitoQ seems to exert its effects by preventing the migration of Drp1 and Bax to mitochondria.

2. Material and methods

2.1. Reagents and plasmids

DMEM-F12, penicillin–streptomycin, gentamicin and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen. 6-OHDA from SIGMA. The BCA protein assay kit from PIERCE. The pDsRed2-mito vector was provided by Clontech, Drp1-GFP was provided by T. Wilson and Dr. S. Strack, Dept. Pharmacology, University of Iowa Carver College of Medicine. GFP-Bax was a gift from Dr. J.H.M. Prehn (Department of Physiology and RCSI Neuroscience Research Centre, Royal College of Surgeons, Ireland).

2.2. Cell culture and drug treatment procedures

SH-SY5Y cell lines were obtained from the American Type Culture Collection (ATCC). Cell cultures were grown as described previously [28] in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 2 mM L-glutamine, 20 units·mL^{-1} penicillin-streptomycin, 5 mg·mL^{-1} gentamicin and 15% (v/v) FBS. Cells were grown in a humidified cell incubator at 37 °C under a 5% CO_2 atmosphere. Cells were plated 24 h before transfection at a density of 5.3×10^4 cells cm^{-2}, on μ-dish-35 mm Ibidi GmbH (Munich, Germany). 6-OHDA was added to culture medium at a final concentration of 50 μM. Duration of pre-treatment with MitoQ and TPP was 30 min and were maintained during the whole experiments.

2.3. Transfections

Twenty-four hours before transfection, cells were plated at a density of 5.3×10^4 cells cm^{-2} on μ-dish-35 mm Ibidi GmbH (Munich, Germany). Transfection was achieved using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were transfected with plasmids encoding pDsRed2, Drp1-GFP, and Bax-GFP. After 4 h of incubation the transfection mixture was removed and replaced with fresh complete medium. To allow protein expression, further experiments were performed 24 h after transfection.

2.4. Image acquisition and processing

Micrographs were processed with Huygens Deconvolution Software (Scientific Volume Imaging) and Adobe Photoshop. For quantitative analysis of mitochondrial morphology, the three patterns of mitochondrial morphology (filamentous, punctuate or intermediate) were recorded in at least 100 cells per coverslip observed on adjacent fields at magnification 63×. We assessed the robustness of this classification by comparing data obtained with separate cover slips from the same experiment and from successive passages. In addition, the monitoring of the mitochondrial morphology was performed by two independent examiners on three different cultures. The proportions observed were similar in all these experiments demonstrating that mitochondrial morphology could be reliably analyzed and did not vary within and between experiments under basal culture conditions. We performed the experiments using a “blind” counter.

2.5. Analysis of mitochondrial morphology

Cells were transfected with pDsRed2-Mito, which leads to the expression of fluorescent DsRed2 in mitochondria, thereby labeling the organelles. The transfected cells are then subjected to experimental treatments to evaluate mitochondrial morphology by fluorescence microscopy. For quantification, the percentage of cells with abnormal mitochondrial morphologies was determined and taken as a measure of the proportion of cells with fragmented mitochondria. Most of the cells had either fragmented or filamentous mitochondria, whereas a small percentage (<10%) of the cells contained both fragmented and filamentous mitochondria. In case of the latter, the mitochondrial morphology was classified according to the majority (>70%) of the mitochondria.

2.6. Protein carbonyl determination

SH-SY5Y cells were cultured in 150 mm Dish plates until they were confluent. Then, cells were scraped off, resuspended in cold PBS, and centrifuged in Eppendorf tubes for 5 min at 12,000 rpm. After this, supernatants were discarded and the pellets were resuspended in 300 μL of cold PBS and sonicated. Protein Carbonyls were assayed by ELISA, using a commercial kit (BioCell Corp., New Zealand) and following the manufacturer's instructions. Protein was quantified with the BCA Protein Assay Reagent (Pierce). Samples were assayed in triplicate using protein carbonyl and BSA protein standards that are supplied with the kit.

2.7. Measurement of mtDNA damage and copy number

SH-SY5Y cells were grown in 6-well plates at a confluence of 700,000 cells well^{-1}. The following day, cells were exposed to drugs for 3 h after which the spent medium was removed and cells were scraped off, resuspended in 0.5 mL of cold PBS, and transferred to Eppendorf tubes. The cell suspensions were centrifugated for 3 min at 12,000 rpm, and the pellet was resuspended in 200 μL of cold PBS. Total DNA was isolated from these cells using the Qiagen DNeasy

Blood and Tissue kit. For DNA quantification we used the PicoGreen dsDNA assay kit (Invitrogen) and a SpectraMax fluorimetric plate reader (excitation wavelength 488 nm, emission wavelength 515 nm). DNA was then diluted to 3 ng/μL in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at −20 °C.

Damage to mtDNA was assayed using a PCR method which measures the amplification of a very long (~10 kb) section of mtDNA. Damage to the mtDNA (e.g. strand breaks, abasic sites, and certain types of oxidative lesions) blocks the progression of the DNA polymerase, thus decreasing amplification. To control for mtDNA copy number, amplification of the long DNA target was normalized to that of a short (126 bp) mtDNA region. Amplification of a short mtDNA target is ~100-fold less likely to be affected by random damage, allowing for comparison of DNA damage between different samples.

PCR primers were modified from published ones. The long mtDNA target encompasses nt 3278 to nt 13343 (10,065 bp; ND1 to ND5). The forward primer was 5′-TCTAAGCCTCCTTATTCGAGCCGA-3′ and the reverse primer was 5′-TTTCATCATGCGGAGATGTTGGATGG-3′. The short DNA target for amplification spans the region from nt 3278 to nt 3404 (126 bp; ND1). The reverse primer was the same as for the long mtDNA target and the forward primer was 5′-CCCCACAAACCCCATTACTAAACCCA-3′. Primers were synthesized by Sigma-Genosys, diluted to 10 μM in TE buffer, and stored as aliquots at −20 °C. PCRs were performed using the GeneAmp XL PCR kit (Applied Biosystems) in a total volume of 50 μL, consisting of 15 ng DNA template, 1 U r*Tth* DNA polymerase XL, and 35 μL PCR master mix containing 5 μg BSA, 200 μM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.0 mM magnesium acetate (0.9 mM for the short mtDNA target). The PCR was initiated with a manual hot start by heating the mixture to 75 °C before adding the DNA polymerase. The PCR parameters were, for the short mtDNA target, 18 cycles of 30 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C followed by 10 min at 75 °C. For the long mtDNA target the conditions were: 1 min at 94 °C, followed by 19 cycles of 15 s at 74 °C and 12 min at 64 °C followed by a final extension step of 10 min at 72 °C. PCR products were quantified by the PicoGreen assay. Amplification linearity was tested by adding controls with 25% or 50% of the amount of DNA template. The reaction was considered linear if the amplification obtained in the controls was between 40% and 60%. All samples were amplified in duplicate and corrected for the control without template.

2.8. Preparation of mitochondrial fractions

Cells were washed with ice-cold PBS, left on ice for 10 min, and then resuspended in isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium-EDTA, 1 mM sodium-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris–HCl, pH 7.4) containing a proteinase inhibitor mixture (Roche, Basel, Switzerland). After 40 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 *g* for 5 min. The mitochondrial fractions were fractionated at 750 *g* for 10 min and 14,000 *g* for 20 min, respectively, and separated from the supernatant (cytosolic fraction).

2.9. Western blotting

For measurements of cellular concentrations of Mfn1, Mfn2, Opa1, Fis1, Drp1 and Bax, cells were washed with PBS and lysed for 5 min in 30 μL office-cold lysis buffer, consisting of 80 mM KCl, 250 mM sucrose, 500 μg/mL digitonin, and 1 μg/mL each of the protease inhibitors leupeptin, aprotinin, pepstatin, and 0.1 mM PMSF in PBS. Cell lysates were centrifuged for 5 min at 10,000 *g*. Protein concentrations were quantified spectrophotometrically (Micro BCA Protein Reagent Kit; Pierce, Rockford, IL), and equal amounts of protein (30 μg) were loaded

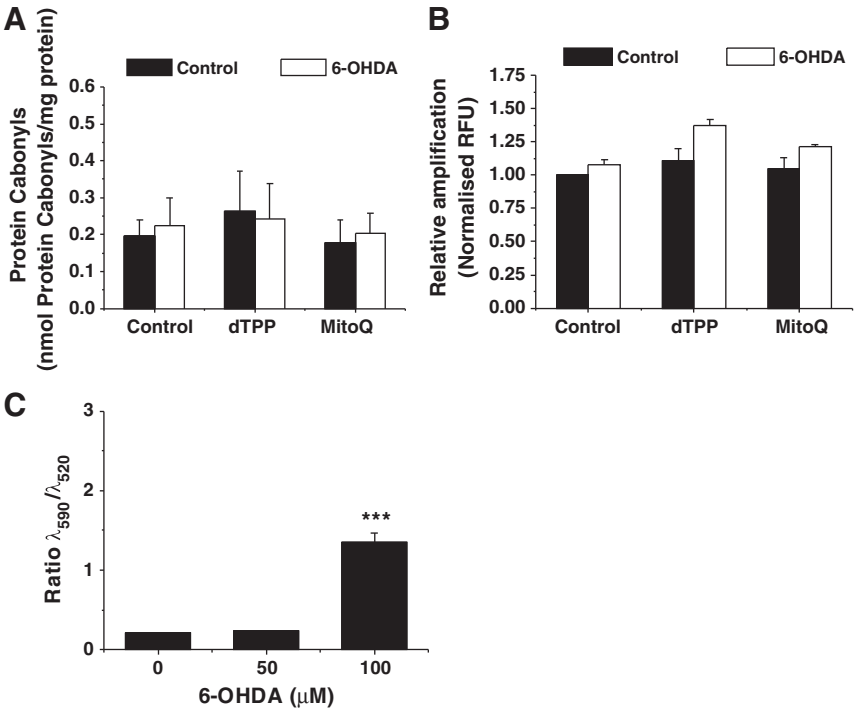

Fig. 1. 6-OHDA does not induce large scale mitochondrial oxidative damage. A. Protein carbonyl levels in total cellular extracts of SH-SY5Y upon a 3 h treatment with 50 μM 6-OHDA. B. MtDNA integrity following a 3 h treatment with 6-OHDA was determined by semi-quantitative PCR. C. After 3 h of 6-OHDA treatment the levels of mitochondrial phospholipid peroxidation was determined by calculating the oxidation of MitoPerOx within cells. The concentration of 100 μM 6-OHDA was used as positive control. The ratio of emission at 520 nm to 590 nm is shown in the bar graph. Given values are the mean ± SEM of five independent experiments that were performed in quadruplicate. (*$p<0.05$; ** $p<0.001$; one-way ANOVA post hoc Tukey).

onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Billerica, MA). Nonspecific protein binding was blocked with Blotto (4% w/v nonfat dried milk, 4% bovine serum albumin (Sigma), and 0.1% Tween 20 (Sigma)) in PBS for 1 h. The membranes were incubated with anti-Fis1 (Atlas Antibodies 1:500), anti-Mfn1(Santa Cruz; 1:1000 dilution), anti-; Mfn2 (abcam;1:1000 dilution), anti-Opa1(BD; 1:1000 dilution), anti-Bax (Cell Signaling 1:1000 dilution), anti-COX IV (Cell signaling; 1:1000dilution), anti-α-Tubuline (Santa Cruz; 1:1000) and anti-Drp1 (BD Biosciences 1:1,000 dilution) overnight at 4 °C. After washing with Blotto, the membranes were incubated with a secondary antibody (1:5000 dilution of a peroxidase-conjugated from Promega, Madison, WI) in Blotto. The signal was detected using an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.10. Mitochondrial viability

Mitochondrial viability was assessed in the SH-SY5y cells from control and experimental treatments (n = 3) the mitochondrial-dependent reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetraz9olium bromide (MTT) to formazan. Briefly, in this reduction SH-SY5Y cells were seeded in 48-well plates at a density of 10^5 cells per well. After treatments, MTT (5 mg/mL in PBS) was added to the plates, and the cells (control and experimental) were incubated for 1 h. The medium was then replaced with DMSO, and MTT absorption was measured at

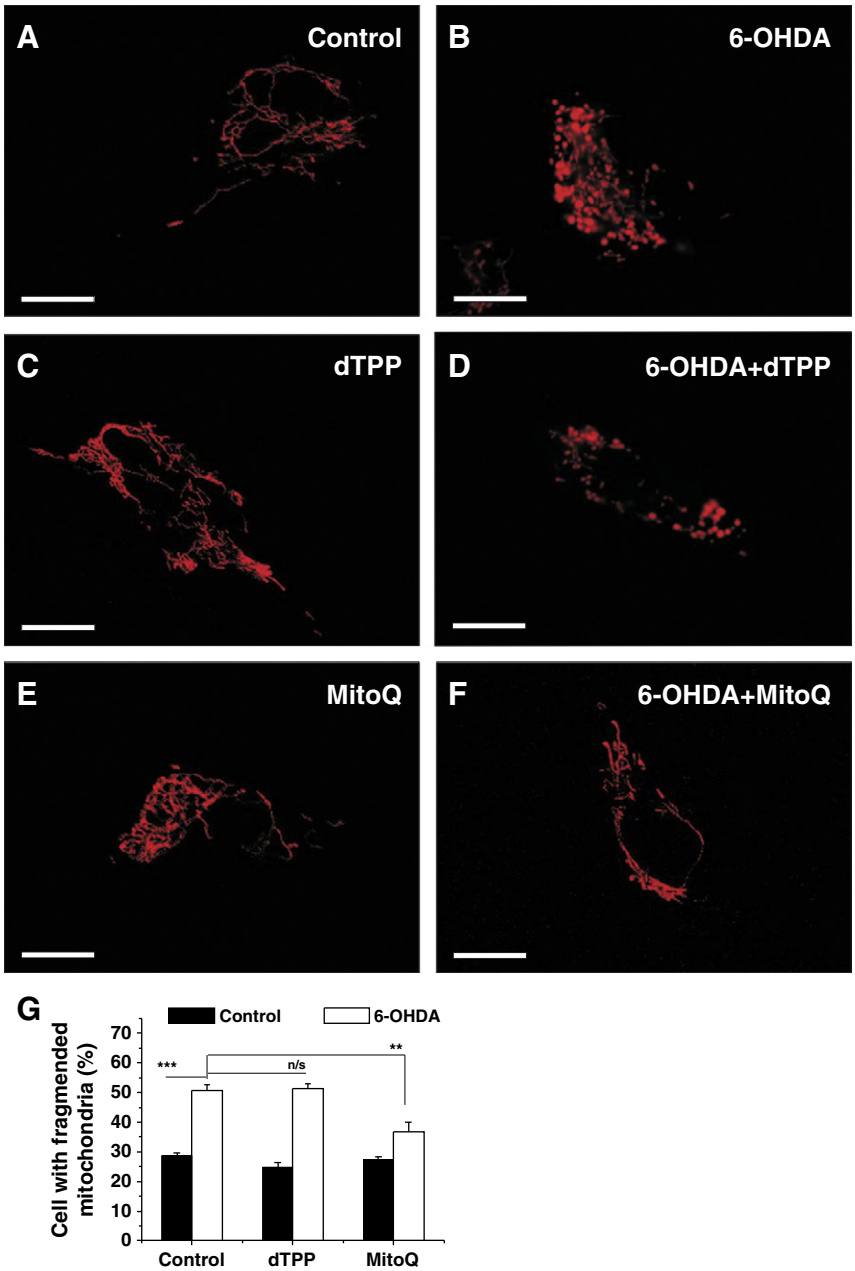

Fig. 2. MitoQ reduces 6-OHDA-induced alterations of mitochondrial morphology. SH-SY5Y cells were transfected with the pDsRed2-mito vector. Twenty-four hours later cells were pre-treated (or not) with 5 nM of dTPP or MitoQ for 30 min, followed by a 3 h treatment with 50 μM 6-OHDA. Representative confocal images of mitochondrial morphology under the different conditions are shown in A–F. Scale bar, 10 μm. G. Proportions of cells with fragmented mitochondrial patterns were determined after a 3 h treatment with 6-OHDA. Data in histograms are the mean ± SEM of four independent experiments. Statistical significance was determined by a two-tailed Student's test: n/s, $p > 0.05$; **$p < 0.01$; ***$p < 0.001$.

570 nm. Results were expressed as the percentage of MTT reduction, assuming that the absorbance of the control SH-SY5Y cells was 100%.

2.11. Intracellular lipid peroxidation measurement

SH-SY5Y cells were plated at a density of 5.3 10^4 cells/cm^2 on μ-dish 35 mm plates obtained from IBIDI GmbH, (Munich, Germany). Twenty-four hours later, the cells were treated with 6-OHDA, (100 or 50 μM) for 2.5 h after which the medium was refreshed by adding fresh medium with the same concentration of 6-OHDA plus 100 nM MitoPerOx [29], and incubating the plates for 30 min at 37 °C in the dark. Then, plates were washed three times with 1× PBS and 1 mL HEPES was added. Cells were visualized with a Zeiss Confocal Microscope, using a 63× oil-immersion objective. In each field, two different photos were taken: one at 520 nm and one at 590 nm. We analyzed significant regions of interest from all the photos, using ImageJ, and calculated the 520/590 nm ratio, which is a good measure of lipid peroxidation production. In the presence of intracellular lipid peroxidation, there is an increment in the 520 nm emission while 590 nm decreases.

3. Results

Addition of 6-OHDA to SH-SY5Y cell cultures is an in vitro experimental model of PD. In this study, we have determined the effect of the mitochondria-targeted antioxidant MitoQ on mitochondrial fragmentation in this model. We chose 50 μM 6-OHDA because 3 h of treatment with this concentration is non-lethal but does alter mitochondrial morphology [22].

3.1. 6-OHDA has minimal effects on mitochondrial oxidative damage

Oxidative stress is a condition in which ROS production overwhelms antioxidant defenses. Its occurrence can be detected by determining the levels of protein carbonyl groups or the presence of oxidative mitochondrial DNA damage. The levels of protein carbonyl groups were determined in SH-SY5Y cells that had been challenged with 6-OHDA (50 μM, 3 h). 6-OHDA did not increase the total cellular levels of protein carbonyl groups (Fig. 1A). In addition, we determined the effect on mtDNA damage and deletion. Again, 6-OHDA did not disrupt mtDNA (Fig. 1B). Taken together, we conclude that SH-SY5Y cells that were challenged for 3 h with 50 μM 6-OHDA present do not undergo extensive oxidative damage. In a third approach we used the ratiometric fluorescent probe MitoPerOx for assessing mitochondrial phospholipid peroxidation within living cells [29]. Three hours after 6-OHDA addition, no significant differences were found between cell cultures challenged or not with 50 μM 6-OHDA (Fig. 1C). Of note, when we performed similar experiments with a higher concentration of 100 μM 6-OHDA, increases in all the oxidative hallmarks mentioned above were found. Taken together, we conclude that SH-SY5Y cells that were challenged for 3 h with 50 μM 6-OHDA present do not undergo extensive oxidative damage and suggest that a relatively mild redox signaling process can initiate mitochondrial fission. In addition, 3 h after treatment, 50 μM 6-OHDA did not compromise the mitochondrial viability of SH-SY5Y cells, as measured using the MTT assay (data not shown).

3.2. MitoQ abrogates 6-OHDA-induced mitochondrial fission

Our previous work has shown that 6-OHDA induces mitochondrial fission in SH-SY5Y cell cultures [22]. In the current study, to be able to determine mitochondrial morphology, we have used the pDsRed2-mito plasmid to express the mitochondrial protein DsRed2 (Fig. 2). As expected, control cells contained elongated mitochondria. Addition of 50 μM 6-OHDA resulted in morphologically altered mitochondria, with apparently shorter filaments (Fig. 2B, also see [22]). Quantitative analysis revealed that 6-OHDA resulted in an increase in the proportion of cells with mitochondrial morphology alterations, being 24% ($p<0.001$) after 3 h of treatment (Fig. 2B, G).

In order to ascertain the role of mitochondrial oxidative events in this process, we used the mitochondria-targeted antioxidant MitoQ. First, we tested if the presence of the control compound dTPP, which has the same mitochondria-targeting motif as MitoQ but which lacks its antioxidant properties, has any effect on mitochondrial morphology. Control cell cultures expressing DsRed2 did not show mitochondrial alterations after 3 h of dTPP (50 nM) treatment (Fig. 2C, G). In addition, a 30 min pre-treatment with 50 nM of dTPP did not decrease the percentage of cells showing fragmented mitochondria after 3 h of treatment with 6-OHDA (Fig. 2C, G). Furthermore, the mitochondrial morphology of cells under control conditions was unaffected by the addition of 50 nM MitoQ (Fig. 2E, G). Subsequently, cell cultures that were pre-treated for 30 min with 50 nM MitoQ were challenged with 50 μM 6-OHDA. Upon 3 h of 6-OHDA treatment, MitoQ significantly decreased the number of cells with fragmented mitochondria (Fig. 2F, G).

3.3. 6-OHDA activates fission but not fusion of mitochondria

Mitochondrial fusion is controlled by three dynamin-related GTPases: Mfn1 and Mfn2, in the outer mitochondrial membrane, and Opa1 in the inner mitochondrial membrane. To determine whether the change in mitochondrial morphology was due to an increase in mitochondrial fusion, we explored the protein expression levels of these three GTPases. Lysates from SH-SY5Y cells that had been challenged with 50 μM 6-OHDA were compared with control cells analyzed by immunoblotting. After 3 h of treatment, all the GTPase proteins assayed had similar expression levels under both conditions (Fig. 3A–C).

In the next set of experiments, we ascertained the role of Fis1 and Drp1, two proteins that are involved in mitochondrial fission, in this model. Levels of the outer mitochondrial membrane protein Fis1 were unaltered upon a 3 h treatment with 50 μM 6-OHDA (Fig. 3D). Drp1 is a cytosolic protein that migrates to mitochondria to induce mitochondrial fission [30]. SH-SY5Y cells were transfected with the fluorescent chimeric protein GFP-Drp1, which enables us to monitor the intracellular localization of Drp1 using confocal microscopy. Untreated cells showed cytosolic localization of GFP-Drp1 (Fig. 4A). Upon a 3 h treatment with 50 μM 6-OHDA, a punctuated distribution

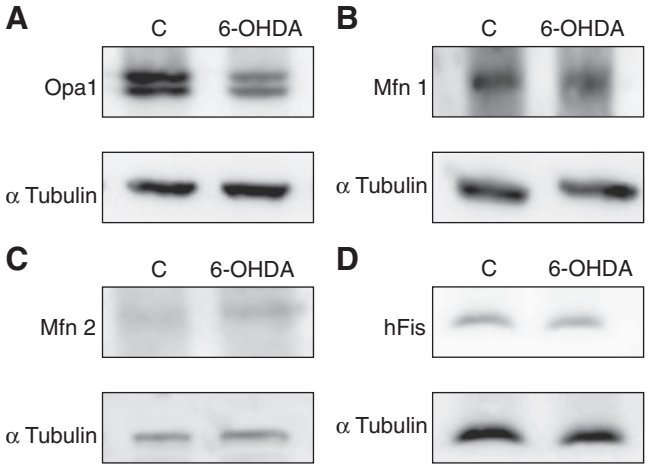

Fig. 3. 6-OHDA does not induce mitochondrial fusion. Protein expression levels of Opa1 (A) Mfn1 (B), Mfn2 (C), and Fish1 (D) in lysates prepared from total cellular extracts were subjected to immunoblotting. Equal amounts of protein (30 μg/lane) were loaded on the gels. α-Tubulin levels were used as an index of total cell load. The immunoblots shown are representative of three independent experiments.

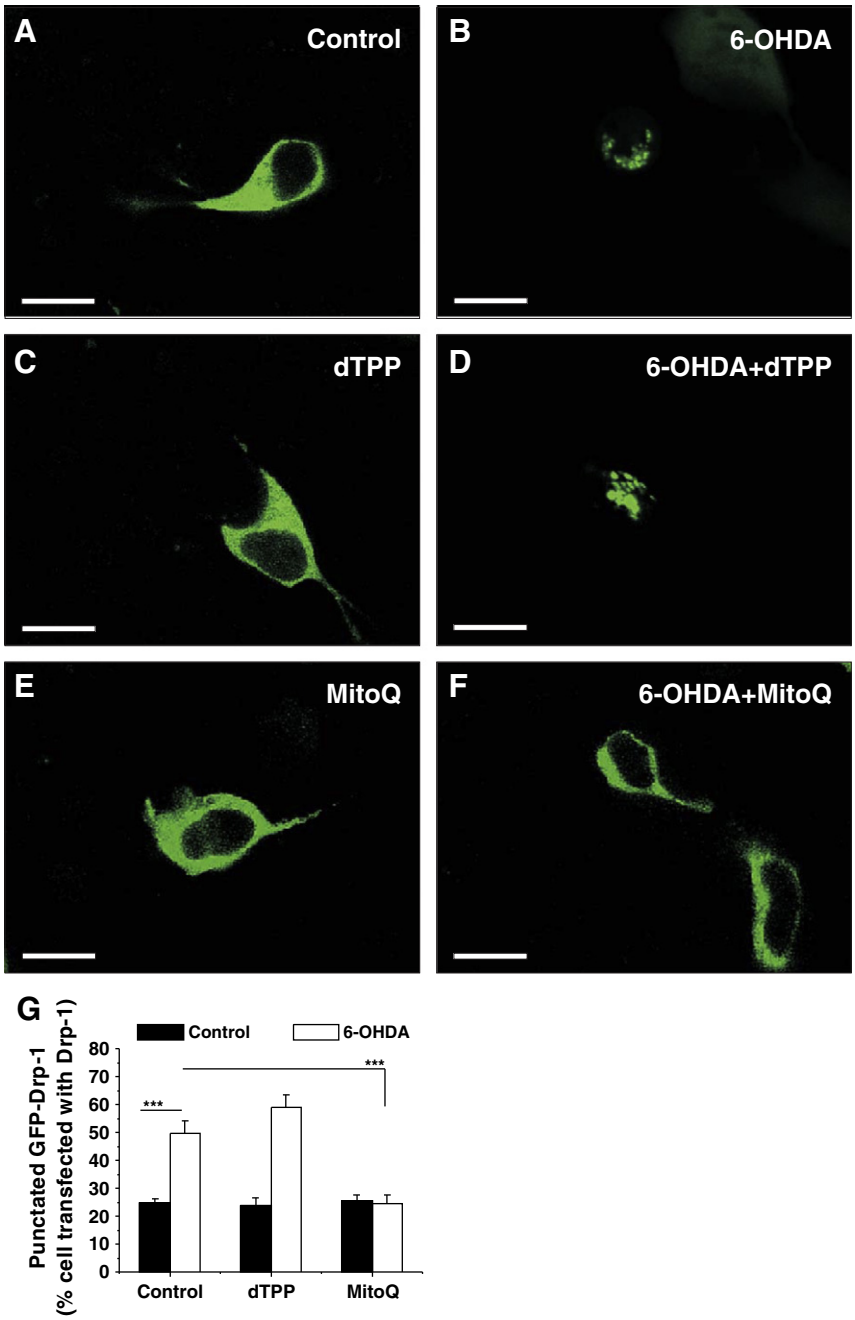

Fig. 4. MitoQ reduces the 6-OHDA-induced mitochondrial translocation of Drp1. SH-SY5Y cells were transfected with GFP-Drp1 vector for 24 h, and pre-treated (or not) with 50 nM of dTPP or MitoQ for 30 min, followed by a 3 h treatment with 50 μM 6-OHDA. Representative confocal images of mitochondrial morphology under the different conditions are shown in A–F. Scale bar, 10 μm. G. Percentages of cells with punctuate Drp1 distribution were determined 3 h after addition of 6-OHDA. Results shown are the mean ± SEM of at least three experiments, each performed in triplicate. Statistical significance was determined by a two-tailed Student's test: ***$p<0.001$.

of GFP-Drp1 was observed, indicating that Drp1 was efficiently translocated to the mitochondria (Fig. 4B, G).

3.4. MitoQ reduces 6-OHDA-induced mitochondrial translocation of Drp1

Next, we determined the percentage of cells with a punctate distribution of Drp1 following pre-treatment for 30 min with 50 nM MitoQ before challenge with 50 μM 6-OHDA. MitoQ reduced the number of cells with a punctate appearance of Drp1 by about 74% in 6-OHDA-challenged SH-SY5Y cell cultures (Fig. 4F, G). In addition, 50 nM dTPP failed to induce Drp1 translocation in control cultures, and did not prevent the 6-OHDA-induced effect on Drp1 localization (Fig. 4D, G).

3.5. MitoQ reduces 6-OHDA-induced mitochondrial translocation of Bax

Previously, we have reported that the intracellular localization of the pro-apoptotic protein Bax changes after 12 h treatment with 6-OHDA [21]. In the next set of experiments, we investigated if this effect is modulated by MitoQ. Cell cultures were transfected with GFP-Bax, and 24 h later they were pre-treated with either 50 nM dTPP or MitoQ for 30 min, followed by addition of 50 μM 6-OHDA (Fig. 5). After 12 h of 6-OHDA treatment, we determined the percentage of cells with punctate GFP-Bax distribution (Fig. 5G). The addition of dTPP did not modify the cellular localization of Bax in control cultures, and did not abrogate the effects of 6-OHDA (Fig. 5C–D, G). However, MitoQ reduced the number of cells with a punctate

appearance of Bax in cell cultures that were challenged with 50 μM 6-OHDA (Fig. 5F,G). In addition, we corroborated the inhibitory effect of MitoQ on 6-OHDA-induced translocation from mitochondrial fractions by immunoblotting (Fig. 5H).

4. Discussion

In the present study, we have focused on assessing the effect of MitoQ on cellular pathways that contribute to 6-OHDA-induced mitochondrial fission in SH-SY5Y cells. We utilized a sublethal 6-OHDA concentration of 50 μM, which is able to induce mitochondrial morphological alterations. We did not observe an increase in total protein carbonyls or in oxidative damage to mtDNA, suggesting that these levels of 6-OHDA did not act by extensive oxidative damage but rather by relative mild redox effects. Notably, MitoQ reduced the mitochondrial morphology alterations induced by 6-OHDA. This suggests that a mild mitochondrial redox process induced by 6-OHDA that can be prevented by MitoQ is an early event in the change in mitochondrial morphology caused by 6HD. In addition, MitoQ prevented the 6-OHDA-induced translocation of both Drp1 and Bax to mitochondria.

Pathology, toxicology, and genetics suggest that mitochondrial oxidative stress is an etiological factor in PD [7,8]. Under physiological conditions, 6-OHDA is rapidly and non-enzymatically oxidized to form ROS. Previous work in our group and elsewhere has shown an

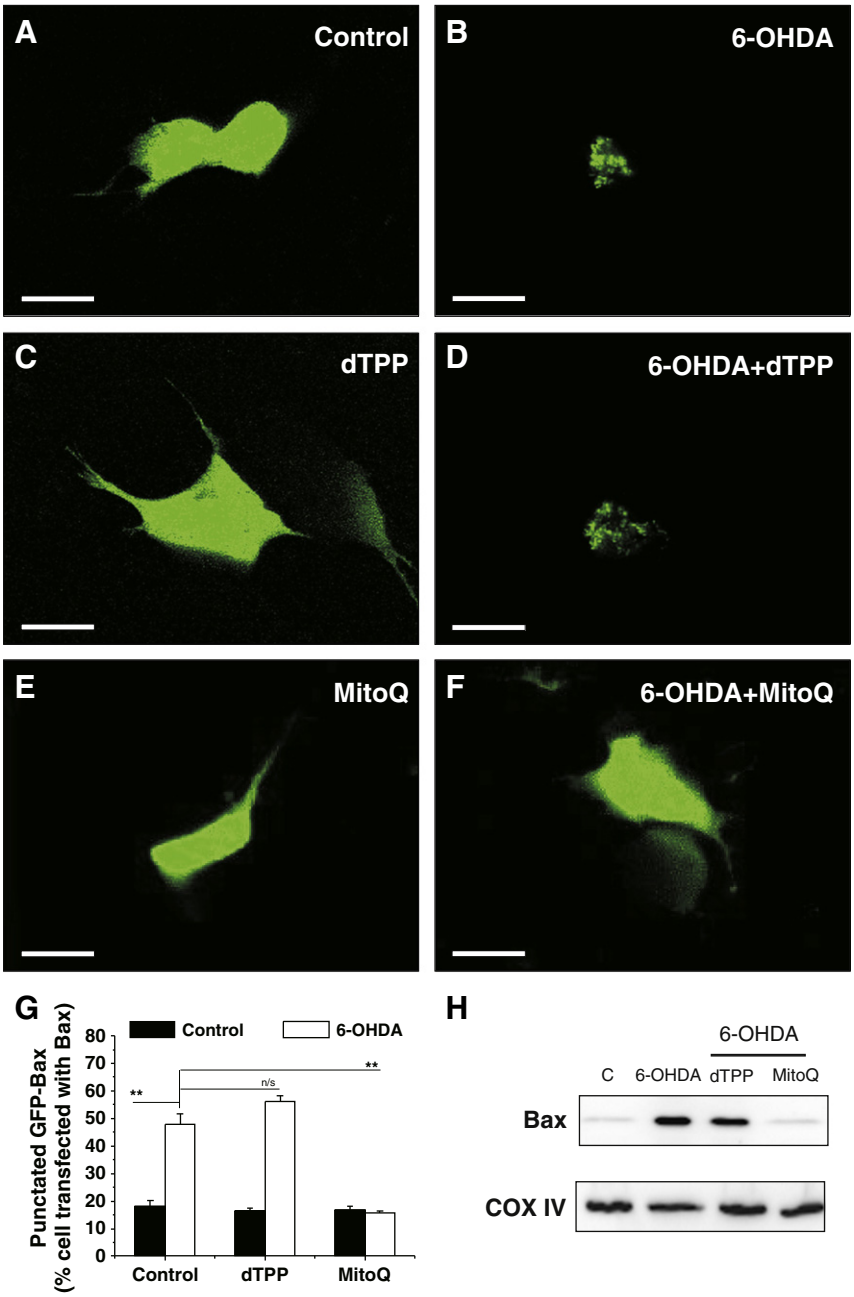

Fig. 5. MitoQ reduces the 6-OHDA-induced mitochondrial translocation of Bax. SH-SY5Y cells were transfected for 24 h with GFP-Bax vector, and were pre-treated (or not) with 50 nM dTPP or MitoQ for 30 min, followed by a 12 h treatment with 50 μM 6-OHDA. Representative confocal images of mitochondrial morphology under the different conditions are shown in A–F. Scale bar, 10 μm. G Percentages of cells with punctuate Bax distribution were determined 12 h after addition of 6-OHDA. Results shown are the mean ± SEM of at least three experiments, each performed in triplicate. Statistical significance was determined by a two-tailed Student's test: n/s, $p > 0.05$; **$p < 0.01$. H. Representative immunoblot analysis of Bax levels in mitochondrial fractions obtained from SH-SY5Y cells. Cell cultures were pre-treated with 50 nM dTPP or MitoQ for 30 min, followed by a 12 h treatment with 50 μM 6-OHDA. Equal amounts of protein (30 μg/lane) were loaded on the gels. COX-IV was used as an index of mitochondrial load. The immunoblots shown are representative of three independent experiments.

increase in H_2O_2 in cell cultures challenged with 6-OHDA [31–34]. As the 6-OHDA concentration used in our study was sublethal, and we chose an early time point, SH-SY5Y cells challenged with 6-OHDA did not present hallmarks of oxidative stress. Neither total protein carbonyl group levels nor oxidative mtDNA damage levels were significantly modified by 6-OHDA. This suggests that the effects on the cell were relatively mild redox signals probably originating in the mitochondrial matrix in response to 6-OHDA oxidative stress, and this was abrogated by pretreatment with MitoQ. In addition, we corroborated the lack of excessive oxidative stress in these conditions using the ratiometric fluorescent probe MitoPerOx for assessing mitochondrial phospholipid peroxidation.

Several classes of small molecular antioxidant mimetics have been shown to protect against central nervous system injuries such as dopaminergic neuron degeneration [25,35–38]. Neurons are metabolically active cells with high energy demands at locations distant from the cell body. Because of this, these cells are particularly dependent on mitochondrial function, as reflected by the observation that mitochondrial-dysfunction diseases often have a neurodegenerative component [23].

In vivo, MitoQ is taken up rapidly and extensively by mitochondria, where it is reduced to the active antioxidant ubiquinol form [10]. MitoQ is nontoxic and orally bioavailable in animal models and humans [14,18]. MitoQ protects mitochondria by decreasing oxidative damage to mitochondrial proteins, lipids, and DNA [11,12]. It also has been shown to decrease mitochondrial damage and to lower the levels of mitochondrial ROS in a range of animal models (reviewed in [13]).

MitoQ blocks 6-OHDA induced mitochondrial fission. By intervening in this process, MitoQ modulates mitochondrial function. It enables mitochondrial recruitment to critical subcellular compartments, content exchange between mitochondria, mitochondrial shape control, communication with the cytosol, and mitochondrial quality control. When mitochondrial dynamics is disrupted, cellular dysfunction ensues. Mitochondrial fission is a highly regulated process, and is mediated by a defined set of proteins [39–43]. Our data support the notion that MitoQ blocks the activation of the mitochondrial fission machinery by preventing mitochondrial translocation of Drp1, a large GTPase of the dynamin family. Drp1 is cytosolic, but a subpopulation of the protein migrates to mitochondria and accumulates at sites of future fission, forming discrete spots [44,45]. According to in vitro studies, Drp1 assembles into rings around mitochondria and constricts their membranes. This results in degradation of the organelles in a GTP hydrolysis-dependent manner [40]. In our study, 6-OHDA recruited Drp1 to mitochondria. Confocal microscopy analysis of cell cultures overexpressing Drp1-GFP revealed that addition of 6-OHDA modified the cytosolic and diffuse distribution of Drp1, leading to a punctate mitochondrial distribution. Administration of 50 nM MitoQ was sufficient to inhibit 6-OHDA-induced Drp1 mitochondrial translocation in SH-SY5Y cells. This observation can be explained by the fact that MitoQ is preventing posttranscriptional changes in Drp1. Accordingly, redox modification of Drp1 may link excessive mitochondrial fission to neuronal injury in neurodegeneration [24,26]. In line with these observations, using a neurodegenerative model that monitors amyloid-β toxicity to N2a cells, Manczak et al. have reported how MitoQ appears to protect mitochondrial structure by preventing mitochondrial genes from abnormal expression [25].

In addition, MitoQ abrogates the 6-OHDA-induced mitochondrial translocation of Bax in SH-SY5Y cells. We and other groups have described that Bax actively participates in neurodegenerative processes [21,46]. Bax co-localized with Drp1 and Mfn2 at mitochondrial fission sites in apoptotic cells [47], and has been suggested to participate directly in apoptotic mitochondrial fission. Previously, in a different neurodegenerative model using MEF cell cultures from mice which lack Bax, we have shown that this protein is not required for 6-OHDA-induced mitochondrial fragmentation [22]. Interestingly, MitoQ also mediates protection against cardiac ischemia–reperfusion injury, diabetic nephropathy, adriamycin-induced cardiotoxicity, and hepatitis C-induced liver injury [17,36–38] and whether prevention of mitochondrial fission contributes to these processes will be an area of future activity (Fig. 6).

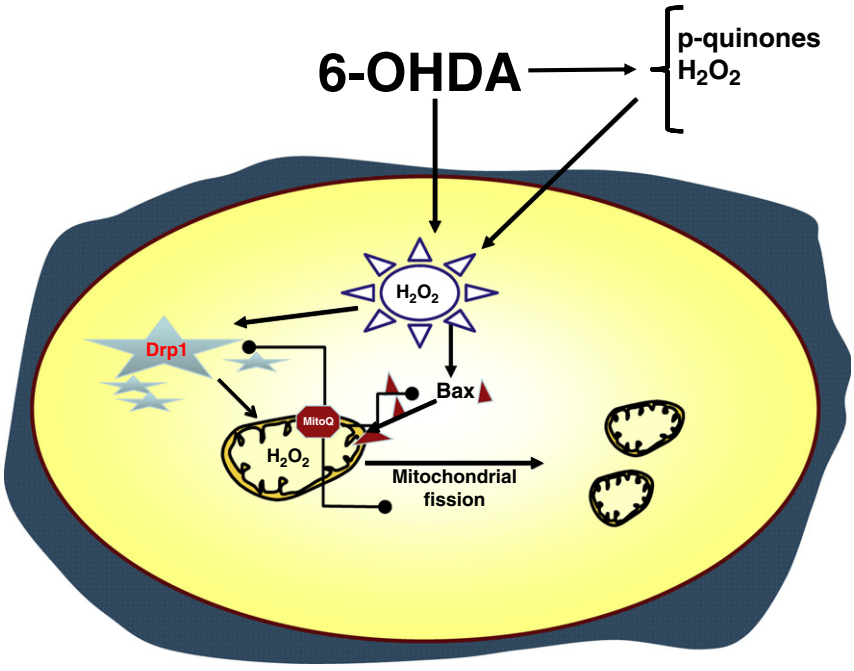

Fig. 6. Schematic diagram of the MitoQ-modulated abrogation of mitochondrial morphology alterations in the 6-OHDA PD model. Auto-oxidation of 6-OHDA leads to the formation of H_2O_2, either extracellularly or within the cell, and this is able to reach the mitochondrial matrix. Mitochondrial fission induced by 6-OHDA-mediated stress is an early event. H_2O_2 may activate the mitochondrial fission machinery, including the mitochondrial translocation of either Drp1 or Bax. MitoQ accumulated in the mitochondrial membrane may block H_2O_2 increases in the mitochondrial matrix. MitoQ is effective at concentrations that do not lead to induction of detectable oxidative stress hallmarks. MitoQ seems to prevent the activation of the mitochondrial fission machinery by preventing Drp1 translocation to the mitochondria. In addition, MitoQ also blocked the formation of mitochondrial Bax foci in response to 6-OHDA in SH-SY5Y cell cultures.

In conclusion, our data suggest that 6-OHDA treatment leads to mitochondrial fragmentation by inducing a mild redox stress on mitochondria that is sufficient to induce a translocation of Drp1 to the mitochondria and thereby increase mitochondrial fission. This fragmentation can be largely prevented by the mitochondria-targeted antioxidant MitoQ, consistent with a mitochondrial redox signaling event leading to Drp1 recruitment. Furthermore, these data suggest that mitochondria-targeted antioxidants may be worth exploring as potential therapies in pathologies involving mitochondrial fragmentation.

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