Formoterol improves the deficits of mitochondrial homeostasis including dynamic and transport in human SH-SY5Y neuroblastoma cells induced by mitochondrial UQCRC1 mutation in human neuronal SH-SY5Y cells via a β2-adrenoreceptor activation

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Abstract

Formoterol, a β2-adrenergic receptor (β2AR) agonist, has been showed to support mitochondrial biogenesis in various diseases. However, its efficacy is controversial in Parkinson's disease (PD) and its regulatory mechanism of mitochondrial homeostasis remains unclear. This study used a cell model of human reductase core protein (UQCRC1) variants in familial parkinsonism, which expressed mitochondrial dysfunction and dynamic imbalance, to explore the therapeutic effects of formoterol and their underlying mechanism. The results indicated that formoterol treatment for 24 h improved cell proliferation and neural cell activity and afforded neuroprotection against oxidative-stress-induced cell death. Furthermore, mitochondrial function, including mitochondrial DNA copy number, respiratory rate, and complex III activation, was comprehensive recovered, as was the dynamic balance of fusion/fission events. Formoterol treatment properly induced mitochondrial fission and reduced the extensive hypertubulation observed. The underlying mechanism of action of the drug may proceed through the restoration of the ERK signal and the inhibition of Akt overaction in mutant cells, thus significantly upregulating the mitochondrial fission protein Drp-1, including its phosphorylation at Ser616 and dephosphorylation at Ser637, as well as Pink-1, in contrast with the fusion protein Mfn2. Moreover, formoterol contributed to the segregation of healthy mitochondria for distribution and mitochondrial transport, as our data revealed the facilitation of mitochondrial anterograde movement and mobility by the drug, to normalize mitochondrial distribution in mutant cells. This study provided preliminary evidence that formoterol offers neuroprotection and acts as a balance regulator of mitochondrial dynamic to improvement of mitochondrial homeostasis, which renders it a promising therapeutic candidate for PD.

Highlights

- Formoterol activates β2AR to protect UQCRC1-mutant neural cells.
- Formoterol improves neurite growth and mitochondrial distribution.
- Formoterol restores mitochondrial dysfunction and dynamic imbalances.
- Formoterol restores the ERK signal and reduces Akt overactivation without affecting PKA activity.
- Formoterol restores mitochondrial anterograde movement and mobility.

Introduction

Formoterol is a long-acting beta2-adrenergic receptor (β2AR) agonist and a type of medication that is used for treating asthma and chronic obstructive pulmonary disease (COPDA), as it relaxes the muscles of the airways, thus affording easier breathing[1]. It is also often used in combination with other medications to help control the symptoms of Parkinson's disease (PD), such as tremors, stiffness, and difficulty with movement[2]. The relationship between β2AR agonists and the risk of PD remains a subject of debate. Although some studies have found no significant association between β2AR medications and PD risk[3, 4], others have suggested that the chronic use of β2AR blockers has neuroprotective effects[5].
and reduces the risk of PD[6–8]. Moreover, their underlying mechanism has been suggested to be related with the regulation of α-synuclein transcription[9], which increases the activity of dopamine, a neurotransmitter, and decreases neuroinflammation in the brain[5]. Furthermore, formoterol stimulates the production of signaling molecules (such as cyclic AMP) that play a key role in many physiological processes, including the regulation of energy metabolism[10]. Recently, the effect of formoterol on mitochondria, the “powerhouses” of cells, was noted, which has been consistently correlated with the restoration of mitochondrial activity, biogenesis, and homeostasis in different cells and tissues, thus potentially contributing to its effects on metabolism and energy production[11, 12]. However, the exact molecular machinery via which formoterol affects mitochondrial function in PD is not fully understood.

Mitochondria are dynamic organelles that undergo continuous processes of fusion and fission. This phenomenon maintains the hemostasis of mitochondria and the integrity of mitochondrial DNA (mtDNA) in many ways. Mitochondrial fusion shares materials to sustain stress-induced damage. In contrast, mitochondrial fission helps to distribute mitochondria evenly within a cell through mitochondrial transport, together with microtubules, to ensure that all parts of the cell have access to the energy required by them[13]. This process can trigger cells to discard damaged mitochondria via mitophagy, to control mitochondrial quality[14]. Notably, mitochondrial dynamic dysfunction also implicates the regulation of cell signaling, the cell cycle, and apoptotic mechanisms in stresses caused by genetic and environmental factors, and beyond to cellular energy support[15]. Thus, disruptions in the balance of mitochondrial dynamics have been implicated in the development and progression of various diseases, including PD.

Mitochondrial ubiquinol-cytochrome c reductase core protein 1 (UQCRC1)-associated parkinsonism with polyneuropathy is a rare genetic disorder that is caused by a missense mutation (c.941A > C; p.Tyr314Ser) in the UQCRC1 gene[16, 17]. The exact underlying mechanisms involve energy deficits, oxidative stress, and loss of the engagement of cytochrome c to trigger neuronal death[16, 18, 19]; however, the impact on mitochondrial dynamics remains unclear. In particular, a marked dysregulation of mitochondrial dynamics, as revealed by abnormally elongated mitochondria and an irregular shape, is consistently observed in both UQCRC1-mutant neurons and animals[16, 18]. Therefore, in the present study, through the exploration of the machinery related to the maintenance of mitochondrial homeostasis (including mitochondrial fission, fusion, transport, and biogenesis) in a cellular model of UQCRC1 parkinsonism, the therapeutic potential of the targeting of mitochondrial dynamics by formoterol for the future therapy of PD was examined.

Materials and Methods

Cell culture and treatment

The wild-type (WT) and mutant UQCRC1 knock-in human neuroblastoma SH-SY5Y cell lines were gifts from the laboratory of Dr. Chin-Hsien Lin[17]. The cells were individually transfected with CRISPR/Cas9 plasmids without site mutation (as a control in this experiment, WT) or carried the c.941A > C
(p.Tyr314Ser) heterozygous variant of the UQCRC1 gene identified in patients with PD (UQCRC1 mutation). All cells were cultured in DMEM/F12 (Life Technologies, GIBCO BRL, Rockville, MD) supplemented with 10% FBS (BIOSER, Buenos Aires, Argentina), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, GIBCO BRL), then grown in a humidified atmosphere containing 5% CO₂.

Mutant cells were treated with 1 µM formoterol alone or in combination with 100 µM propranolol, a β2AR antagonist, for 24 h in the presence or absence of 10 µM tert-butylhydroperoxide (tBH)-induced oxidative stress.

**GFP-labeled mitochondria**

Cells at 80% confluency were transfected with 40 µg of plasmid DNA encoding mitochondrial-matrix-localized AcGFP (import targeting sequence of cytochrome c oxidase subunit 8, COX8) (Clontech, Palo Alto, CA, USA) using electroporation (ECM; BTX Harvard Apparatus, Holliston, MA, USA) according to our previous study[20]. Thirty-six hours later, the cells were transferred to normal growth medium for 48 h, followed by G418 selection (500 mg/mL).

**Cell number, cell viability, and neurite outgrowth**

Cell morphology after the various treatments as assessed using a bright-field Olympus BX43 microscope with the CellSens software (Olympus, Tokyo, Japan). Subsequently, cell number and viability were analyzed using a Cell Scepter electronic cell counter (EMD Millipore Corporation, Billerica, MA) and the WST-1 assay (Roche Diagnostics, Taipei, Taiwan), respectively; moreover, the percentage of tBH-induced apoptotic cells was examined using 7-amino actinomycin D (7-AAD) (BD Pharmigen, Franklin Lakes, NJ, USA) staining and detected by a NucleoCounter ® NC-3000™ fluorescence image cytometer (ChemoMetec, Alleroed, Denmark). The neurite lengths of the treated cells was manually quantified using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

**Quantitative mitochondrial morphology and density**

To visualize mitochondria, stained cells were mounted onto a perfusion chamber in culture medium and imaged at 37°C using an Olympus FluoView FV 1200 confocal microscope. The subtypes of mitochondrial morphology were quantified using the automatic classification system developed by Peng et al.[21]. This system classified mitochondria into six subtypes based on their shape, as follows: single globe, swollen globe, straight tubule, twisting tubule, branched tubule, and loop. To calculate the proportion of globe mitochondria, the single globe and swollen globe shapes were summed and contrasted to the proportion of reticular mitochondria (i.e., the combined population of straight tubule, twisting tubule, and branched mitochondria). The analysis was performed on micrographs from three independent areas per group, and approximately 200–350 mitochondria from 6–8 cells in each image were analyzed semi-automatically. The mitochondrial counts were presented as the total number of mitochondria per cell according to data mentioned above.

**Mitochondrial function**
Mitochondrial function was comprehensively assessed by measuring the individual mitochondrial respiratory chain function, including basal respiration, ATP-linked production and complex III activity, mitochondrial DNA (mtDNA) copy number, and reactive oxygen species (ROS) generation (as a whole and in mitochondria (mtROS)).

Mitochondrial respiration within saponin (1.25 ng/mL)-permeabilized cells was analyzed via high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) and expressed as the respiratory oxygen flow (pmol/second/million cells). The analysis started with the measurement of basal respiration (BR), which was defined as that recorded in cells in MiR05 buffer (Oroboros Instruments) in the absence of additional substrates or effectors; in contrast to ATP-linked production, which was defined as the oligomycin (1 µM)-mediated reduction of respiration in the presence of additional substrates [glutamate(G, 10 mM), malate (M, 2 mM), and ADP (2.5 mM)] (Sigma Aldrich, St Louis, MO, USA); and complex III activity, which was defined as the difference in the respiration change between the addition of duroquinone (DuroQ) (0.5 mM) (Sigma Aldrich) and antimycin A (AA, 5 µM) (Sigma Aldrich). Finally, shutdown of the mitochondrial electron transport was executed by adding an inhibitor of the mitochondrial Complex IV, sodium azide (20 mM).

For the analysis of mtDNA copy number, DNA was extracted from cultured cells using a Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). Next, quantitative PCR was performed using the SYBR Green PCR Master Mix (Roche Applied Science) and an ABI Prism 7300 system (Applied Biosystems) with specific primer pairs to amplify the mtDNA-encoded nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene (forward primer: 5′–AACATACCCATGGCCAACCT–3′; and reverse primer: 5′–AGCGAAGGGTTGTAGTAGCCC–3′) and the nuclear DNA-encoded β-actin gene (as an internal control; forward primer: 5′–AGAAAATCTGGCACCACACC–3′; and reverse primer: 5′–CACCTTCTACAATGAGCTGCG–3′) from a total of 50 ng of DNA. The mtDNA copy number was determined based on the copy number ratio between these two genes (ND1/β-actin)[22].

ROS production was analyzed by measuring total ROS using the 2,7-dichlorofluorescein diacetate (DCFH-DA, Life Technologies) probe, whereas mitochondrial superoxide production was assessed using the MitoSox Red (Life Technologies) probe. The cells were incubated in medium containing 10 µM DCFH-DA and 50 nM MitoSox Red at 37°C for 15 min for staining in the dark. All stained cells were washed twice with PBS, resuspended in PBS, and kept on ice for immediate detection using a NucleoCounter® NC-3000™ fluorescence image cytometer.

**Mitochondrial motility**

Mitochondrial transport imaging was carried out according to a previous study, with some modifications[23]. The live time-lapse imaging of cells treated with GFP-labeled mitochondria was performed using an Olympus FV1200 confocal laser scanning microscope at 37°C with 5% CO₂. The cells were imaged for 10 min with continuous recording. The acquired images were analyzed for mitochondrial motility using the CellSens (Olympus) software. Briefly, based on morphological criteria, for the analysis of the axonal initial segment, including the proximal and hillock regions, a 10–20-µm segment located at
least 10 µm away from the soma was selected. The mitochondrial velocity duration time and the
directionality of the movement, as obtained by measuring the angle of the slope, were analyzed in the
kymographs generated by CellSens. The proportion of motile mitochondria was manually calculated by
dividing the number of mitochondria moving faster than average by the total number of mitochondria,
based on the image sequences of the kymographs. At least 7–10 axons from 3–5 different neural cells
were analyzed in each group. Considering that the axon extension in mutant cells was not obvious
compared with that observed in WT cells, the mobility parameter was analyzed in the proximal region of
axons exclusively.

**Western blotting and anti-phosphorylated-Akt-1 antibody array**

Treated cells were rinsed with PBS and then suspended in RIPA buffer (Thermo Pierce, Rockford, IL, USA)
supplemented with a complete protease and phosphatase inhibitor cocktail (EMD Millipore Corporation).
The cells were kept on ice for 30 min and then homogenized. The extracts were centrifuged at 14,000 × g
for 20 min at 4°C, and the supernatants were analyzed using the Bradford assay. Total proteins (20–30
µg) were separated by SDS–PAGE and transferred onto Immobilon-P membranes (EMD Millipore
Corporation). The membranes were then incubated with the primary antibodies, as follows: anti-OPA1
(1:1000, BD Biosciences, Franklin Lakes, NJ, USA), anti-mitofusin 2 (Mfn2) (1:1000, NOVUS Biologicals),
anti-dynamin-related protein 1 (Drp-1) (1:500, EMD Millipore Corporation), anti-phospho-Drp-1-S616
(1:1000, Cell Signaling Technology), anti-phospho-Drp-1-S637 (1:1000, Thermo Fisher Scientific, Inc.,
Waltham, MA, USA), anti-PTEN-induced kinase 1 (Pink-1) (1:1000, NOVUS Biologicals), anti-parkin
(1:1000, Abcam), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000, Cell Signaling
Technology), anti-phospho-Akt (Ser473) (1:1000, Cell Signaling Technology), and anti-β-actin (1:1000,
NOVUS Biologicals). The membranes were subsequently incubated with horseradish peroxidase (HRP)-
conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA) at a dilution of 1:10000. The
signals were detected using an image-acquisition system (FUSION SL; Viber Lourmat, Marne-la-Vallee,
France) and quantified using the Gel-Pro analyzer software (Version 3.0) (Media Cybernetics, Silver
Spring, MD, USA).

The Human/Mouse Akt Pathway Phosphorylation Array C1 includes key proteins of interest in the
relevant pathways (ERK1, ERK2, PRAS40, GSK3A, PTEN, GSK3B, RAF-1, mTOR, RPS6, Akt, p27, RSK1,
AMPKa, p53, RSK2, BAD, P70S6K, 4E-BP1, and PDK1) and was performed according to the
manufacturer's instructions (RayBiotech, Norcross, GA, USA)[24]. Briefly, total protein preparation and
HRP signal detection were carried out as described above. A total of 100 µg of the protein extracts from
three independent cell lysates was added to each well and incubated for 24 h at 4°C. Subsequently, the
antibody array membranes were washed and incubated with the HRP-conjugated anti-rabbit IgG antibody
included in the kit at room temperature for 2 h. The membranes were washed extensively before detection
using chemiluminescence.

**Protein kinase A activity**
Protein kinase A (PKA) activity was examined using a PKA Colorimetric Activity Kit (Thermo Fisher Scientific), including a PKA substrate-coated 96-well plate(s), a PKA standard, and an anti-phospho-PKA substrate antibody. Briefly, cell lysis was carried out in the presence of the supplied ATP and phosphorylation was achieved by the immobilized PKA substrate. After a 90-min incubation followed by a wash, a rabbit antibody specific for the phospho-PKA substrate was bound to the modified immobilized substrate. Then, an antibody specific for rabbit IgG labeled with peroxidase was also added to the plate, to bind to the rabbit anti-phospho-PKA substrate. After short incubation and wash steps, the substrate was added and the absorbance was read at 450 nm using a CLARIOstar microplate reader (BMG LabTech, Ortenberg, Germany). The intensity of the color that developed was directly proportional to the amount of PKA in the samples and standards. All samples were read off the standard curve.

Statistics

The data are presented in the form of the mean ± standard deviation (SD) and were obtained from a minimum of three independent experiments. Associations were assessed with paired Student’s t-test. Significance was set at \( P < 0.05 \).

Results

**Formoterol increased the viability of UQCRC1 mutant neurons and protected against tBH-induced cell damage in a β2-adrenoceptor-dependent manner**

The mutant cells displayed a decreased neurite length and an impaired neurite outgrowth compared with to WT cells (Fig. 1A, B); moreover, the levels of cell viability and proliferation in the mutant cells were lower than those observed in WT cells (Fig. 1C). Those parameters were significantly improved after treatment with formoterol for 24 h (Fig. 1A–C). Compared with the disease control group (treated with DMSO), the formoterol-treated group exhibited a remarkable increase in cell activity and cell number, by approximately 1.4-fold (left axis of Fig. 1B) and 1.6-fold (right axis of Fig. 1B), respectively. Furthermore, this treatment elicited a significant increase in neurite outgrowth, of up to 1.7-fold (Fig. 1C). In contrast, when propranolol was used in combination with formoterol, the aforementioned regulations were completely abolished (Fig. 1A–C). Notably, there were no significant differences in cell viability, growth, and neurite outgrowth between the groups treated with formoterol plus propranolol and the DMSO group. These findings indicate that the response induced by formoterol is mediated through the activation of β2-adrenergic receptors (β2ARs).

Similar findings were observed in mutant cells with tBH-induced oxidative damage (Fig. 1D–E). Compared with the DMSO control, formoterol afforded a significant (1.9-fold) increase in neurite length under oxidative stress conditions by activating β2AR (Fig. 1E). This particular dosage of tBH did not induce a significant level of cell apoptosis in wild-type (WT) cells, in contrast to mutant cells, which exhibited a substantial 2.6-fold increase in the death rate compared with their non-treated counterparts (Fig. 1F–G). Moreover, after a 24-h treatment, formoterol effectively reduced the tBH-induced cell death by approximately 55% compared with the DMSO control (Fig. 1G). Finally, the addition of propranolol
nullified this effect, further supporting the effects of formoterol's action on various cellular responses in a β2AR-dependent manner.

**Formoterol enhanced mtDNA copy number, reduced ROS levels, and restored mitochondrial respiration and complex III activity, but not ATP production**

To evaluate mitochondrial function comprehensively, we analyzed various parameters, including mtDNA copy number, ROS generation in total cells or mitochondria, and the mitochondrial respiration rates using different substrates and inhibitors (Fig. 2). Compared with WT cells, mutant cells exhibited a significantly lower number of mtDNA copies (Fig. 2A), higher levels of both total and mtROS generation (Fig. 2B, C), and a reduced cellular respiratory performance (Fig. 1D, left panel), which included BR, electron-transport-linked ATP production (oligomycin-mediated reduction), and CIII-linked respiration (respiratory difference between duroquinone, a CIII substrate, and antimycin A, a CIII inhibitor), as illustrated in Fig. 1E. In addition, formoterol uniformly and significantly ameliorated the aforementioned functional loss in mutant cells and increased the availability of the mitochondrial substrates glutamate and malate compared with the DMSO control, with the exception of ATP production (Fig. 2D, E). However, the beneficial effects of formoterol were nullified upon the addition of propranolol, with the exception of the expression of mtROS (Fig. 2C). This indicated the direct regulation of mitochondrial function via a formoterol-mediated β2AR action.

**Formoterol corrected the mitochondrial morphology and increased mitochondrial counts**

Compared with WT cells, mutant cells exhibited abnormalities in mitochondrial distribution and morphology. There was a noticeable deficiency in mitochondrial distribution along neurites, with abundant reticular mitochondria [including branched (purple color), twisting (oranges color), and loop (red color) subtypes], and a reduced number in contrast with tubular (green color), single (blue color), and swollen (yellow color) globe mitochondria. This resulted in a disorganized mitochondrial network with irregular shapes and sizes that spread throughout the soma (Fig. 3A, B). In turn, formoterol effectively corrected the imbalance in mitochondrial morphology. In fact, it facilitated a shift in mitochondrial distribution toward neurites and axons in mutant cells, similar to that observed in WT cells (Fig. 3A). Notably, formoterol reduced the fraction of the reticular mitochondria subtype by approximately 50% compared with the DMSO control while increasing the fraction of the tubular and globe mitochondria subtypes. The addition of propranolol abolished this induction, thus indicating that the actions of formoterol regarding the modification of mitochondrial morphology were specifically mediated by the activation of β2AR (Fig. 3B). Furthermore, the mitochondrial count in mutant cells was significantly lower than that in WT cells (Fig. 3C). Among all treatments, only formoterol significantly increased the mitochondrial count relative to the DMSO control (Fig. 3C). This finding further validated the effect of formoterol in adjusting mitochondrial function and dynamics.
Formoterol modulated the dynamic balance between mitochondrial fusion and fission via p-ERK activation and Akt inhibition

Compared with WT cells, mutant cells exhibited a stronger tendency toward mitochondrial fusion vs. division, as depicted in Fig. 4. An analysis of protein levels (Fig. 4A) and western blotting quantification (Fig. 4A, right panel) revealed the significant upregulation of the mitochondrial fusion protein Mfn2 (but not OPA1), and the notable downregulation of the mitochondrial fission protein Drp-1, including its activated form at two phosphorylation sites—serine residue 616 [Drp-1 S616], which is responsible for promoting mitochondrial fission; and serine residue 637 [Drp-1 S637]—, indicating opposing regulation of mitochondrial fission in mutant cells (Fig. 4A). Formoterol treatment effectively corrected the expression pattern of mitochondrial dynamic proteins in mutant cells, aligning it closely with that observed in WT cells (Fig. 4A). Notably, there was a significant downregulation of Mfn2 and Drp-1 S637, accompanied by the upregulation of Drp-1 and Drp-1 S616, in the formoterol group compared with the DMSO control (Fig. 4A). These effects were mediated by β2AR activation, as evidenced by the reversal of performance upon the addition of propranolol (Fig. 4A). Furthermore, formoterol significantly downregulated the mitophagy-related protein parkin while simultaneously upregulating both the full-length Pink-1 (L-form) and the cleaved Pink-1 (S-form) proteins. This pattern of Pink-1 regulation was distinct from the inhibition of full-length Pink-1 observed in mutant cells without any impact on the parkin protein (Fig. 4A). Interestingly, the inhibition of parkin by formoterol did not depend on the activation of the upstream β2AR, as evidenced by the lack of differences between the formoterol treatments with and without propranolol (Fig. 4A). This indicates that the effect of formoterol on parkin is independent of β2AR activation.

To elucidate further the pathway linking mitochondrial dynamic proteins with downstream β2AR activation, an intercellular signaling array with MAPK phosphorylation antibodies was employed (Fig. 4B). Mutant cells displayed elevated levels of Akt phosphorylation at S473, in contrast with the phosphorylation status of ERK1/2 at T202/Y204 and Y185/Y187, compared with WT cells (Fig. 4B, left panel). Formoterol effectively counter-regulated the expression of Akt and ERK phosphorylation, and this specific regulation was demonstrated to be dependent on β2AR activation, as evidenced by the abolished regulation upon co-treatment with propranolol (Fig. 4B, left panel). Furthermore, a comparison of pathway-related phosphorylated proteins integrated into the array was conducted for each set of groups (UQCRC1mut-DMSO vs. WT; UQCRC1mut-F vs. UQCRC1mut-DMSO; UQCRC1mut-F + P vs. UQCRC1mut-F). The differences in the expression of the corresponding proteins are listed individually in the right panel of the bar graph depicted in Fig. 4B (for the raw data, see Supplementary information, Table S1). Among the molecules that were involved in the upregulation triggered by formoterol, 4E-BP1 exhibited the highest level of induction (Fig. 4B). A western blot analysis further confirmed the restoration of ERK signaling and the inhibition of Akt activation by formoterol treatment in mutant cells (Fig. 4C). Furthermore, formoterol treatment had no impact on the already high level of PKA activity in mutant cells compared with WT cells (Fig. 4D).

Figure 4E depicts the pathway diagram corresponding to the regulatory relevance of mitochondrial dynamics through the formoterol-induced activation of β2AR. The improvement in the dynamic balance
afforded by formoterol was attributed to its simultaneous promotion of the ERK pathway and inhibition of the Akt pathway. This led to an increase in the ratio of Drp-1 S616/Drp-1 S637, resulting in the favoring of mitochondrial fission over fusion. Simultaneously, the inhibition of Akt activation downregulated Mfn2, thus further enhancing the manifestation of the aforementioned effects.

**Formoterol improved mitochondrial transportation and mobility**

Time-lapse recordings of MitoGFP-labeled mitochondria, together with corresponding kymographs, were utilized to analyze mitochondrial transport in proximal axons between adjacent cells (Fig. 5). The representative kymographs provided in Fig. 5A illustrated the bidirectional movement of signals as diagonal lines, revealing more active and abrupt movements of mitochondria in WT cells (Supplementary Movie 1) compared with mutant cells (Supplementary Movie 2). In addition, it had a higher velocity for anterograde transport (mean velocity: WT cells, 51.2 ± 0.013 nm/s; vs. UQCRC1 mutant cells, 7.2 ± 0.002 nm/s; Fig. 5B). However, the rate of mitochondrial retrograde transport did not differ between WT and diseased cells (Fig. 5B). Furthermore, a significant reduction in motile mitochondria was observed in both the anterograde (52.6% reduction) and retrograde (48.6% reduction) transport in mutant cells (Fig. 5C). DMSO treatment did not affect the aforementioned performances in diseased cells (Fig. 5A-C). Formoterol treatment significantly improved mitochondrial mobility (Supplementary Movie 3), increasing the anterograde movement velocity by 2.1-fold (mean velocity, 25.6 ± 0.009 nm/s; Fig. 5B) and the motile ratio by 1.5-fold (Fig. 5C) compared with the DMSO control. Nevertheless, formoterol did not exert significant effects on the frequency and rate of mitochondrial retrograde movement (Figs. 5B, C). The induction of mitochondrial velocity and mobility by formoterol was consistently abolished addition of propranolol (Figs. 5A–C) (Supplementary Movie 4). Moreover, individual frames from the time-lapse images presented in Fig. 5D further demonstrated that the mitochondria observed in the WT and formoterol-treated groups exhibited significant forward displacement in axons during the time-lapse sequence (the anterograde direction is indicated by the arrow in Fig. 5D). In contrast, stationary mitochondria were observed in the mutant cell groups and groups treated with formoterol plus propranolol or propranolol alone (Fig. 5D). These findings indicate that the improvement in mitochondrial velocity and mobility afforded by formoterol occurred via β2-adrenoceptor activation, as the effects were abolished by the addition of propranolol.

**Discussion**

The effect of formoterol on mitochondrial function has been validated in various diseases, including traumatic brain injury[25], spinal cord injury[26], diabetic kidney disease[27], and acute kidney injury, with those studies providing compelling evidence in support of the use of β2AR ligands for therapeutic mitochondrial biogenesis; however, the machinery underlying the mitochondrial dynamics of fusion/fission and movement remains unclear in PD, and their therapeutic efficacy is controversial. In particular, abnormalities in mitochondrial dynamic processes, specifically in fusion and fission, play a crucial role in the pathogenesis of PD[13]. This study initially revealed that formoterol treatment improved
cell proliferation and neural cell activity and had a neuroprotective effect against ROS-induced cell death. Mitochondrial dysfunction was also comprehensively restored, including the dynamic imbalance of fusion/fission, mitochondrial movement, and mobility. The related machinery may involve the activation of the ERK and the inhibition of the Akt signaling cascades downstream of B2AR, thus restoring mitochondrial fission and decreasing persistent hyperfusion. This contributes to the segregation of healthy mitochondria for proper distribution during cell division and mitochondrial transport, as our findings showed that the facilitation of mitochondrial anterograde movement by formoterol normalized mitochondrial distribution in UQCR-C-mutant neural cells.

Although mitochondrial function and dynamics are generally interrelated, in certain cases the interconnection between mitochondrial dynamics and vital function can be dissociated[28]. The inhibition of mitochondrial respiration and mitochondrial ATP production using sublethal concentrations of mitochondrial complex I (CI) and CIII inhibitors did not trigger mitochondrial fragmentation, in contrast to CV inhibitors[29]. This revealed that CIII may play an energy-independent role in the regulation of the mitochondrial dynamic balance. A recent study showed that, in UQCR2-mutant (c.665G > C; p.Gly222Ala) fibroblasts derived from patients with severe encephalomyopathy, a significant mitochondrial fragment was accompanying by a high degree of mitochondrial branching[30]. It is well known that UQRC1 and UQRC2 are both subunits of CIII that play essential roles in the structure and function of CIII. Mutations or disruptions in these subunits can lead to mitochondrial dysfunction and various related disorders[16, 30]. We also found a notably increased reticular network of mitochondria in UQRC1-mutant neural cells; however, unlike that observed in UQRC2-mutant cells[30], the proportion of fragmented mitochondria was relatively decreased. Moreover, a notable increase in Mfn2, but not OPA1, and a decrease in Drp1 were observed in UQRC1-mutant cells, whereas Mfn2 and OPA1 remained unaffected and Drp1 was significantly upregulated[30]. This suggests that UQRC1 mutation has a more significant impact on disrupting the mitochondrial dynamics balance toward fusion, to adapt to stressful conditions. This regulation could be reversed by reducing mitochondrial stress through formoterol treatment, thereby restoring a balance between mitochondrial fusion and fission and preventing excessive mitochondrial fusion and aggregation. In fact, excessive mitochondrial hyperfusion impairs mitochondrial distribution and turnover, thus inducing locomotor defects in Drosophila models of Charcot–Marie–Tooth disease type 2A neuropathy[31]. Prolonged elongation of mitochondria has been demonstrated to impair mitophagy, which consequently affects the clearance of dysfunctional mitochondria and diminishes mitochondrial function[32, 33]. Moreover, the maintenance of elongated mitochondria leads to the disruption of its distribution and transport in cells[28, 34], as evidenced in the present study. In addition, abnormally elongated mitochondria also interfere with the interactions with other cellular structures, thus potentially affecting cellular signaling and responses[28].

Formoterol treatment led to the comprehensive restoration of mitochondrial function, including mitochondrial respiration and CIII activity and biogenesis, with the exception of ATP production. Based on our findings, we suggest that this could be related to the increase in mitochondrial biogenesis, for the generation of new mitochondria, which can increase the overall respiratory capacity of cells. Furthermore, this study further found that increasing the availability of certain metabolic substrates via cellular
signaling and regulatory mechanisms, such as the ERK pathway, may lead to an increase in BR without necessarily a direct impact on ATP synthesis[35, 36]. This supports our finding that the increase in the availability of mitochondrial substrates, i.e., glutamate and malate, can be considered as an influencing factor. Conversely, formoterol activates the activated cyclic AMP-PKA pathway, which is a β2AR downstream effector pathway that has been shown to modulate the regulation of mitochondrial metabolism and function[37]. Interestingly, we found that formoterol treatment did not further enhance the already high performance of PKA activity in mutant cells compared with WT cells. This suggests that the effects of formoterol on mitochondrial function may vary according to various factors, such as cell type, dosage, treatment duration, and specific experimental conditions[27].

Drp1 is a key regulator of mitochondrial fission that can be phosphorylated at different sites by distinct signaling transduction guided in context, to adjust the process of mitochondrial fission. For instance, phosphorylation of Drp1 S616 enhances its GTPase activity, leading to increased mitochondrial fission. Conversely, phosphorylation of Drp1 Ser637 inhibits this process[38, 39]. We found that formoterol treatment increased Drp-1 activity via phosphorylated Drp1 S616 and dephosphorylated Drp-1 S637, to provide a fine-tuned control mechanism for mitochondrial fission. Consequently, extensive elongation of the mitochondrial network was decreased, restoring a balanced pattern between mitochondrial fission and fusion. Recently, a study reported the contribution of the Akt-1 pathway to the inhibition of mitochondrial fission through the parallel regulation of dephosphorylated Drp1 S616 and phosphorylated Drp1 S637. Conversely, the MEK1–ERK pathway was involved in promoting mitochondrial fission through the phosphorylation of Drp1 S616 and dephosphorylation of Drp1S637[38]. The two axes of the Akt1–Drp1 and MEK1–ERK-Drp1 pathways can be switched to remodel the mitochondrial dynamics in somatic cell reprogramming[38]. This reflects our finding that UQCRCS1-mutant cells, which have a deficiency in mitochondrial fission, displayed a lower ERK signaling and a higher Akt activity compared with the WT cells. Formoterol certainly adjusted the dynamic toward fission through the parallel regulation of activating ERK signaling for the phosphorylation of Drp1 S616 and inhibiting Akt signaling for the phosphorylation of Drp-1 S637. Furthermore, mutant cells exhibited increased levels of Mfn2 proteins, in contrast to Drp-1. The physiological levels of Mfn2 expression are strongly correlated with the Akt signaling pathway, to promote mitochondrial fusion[40]. Therefore, the literature mentioned above consistently emphasizes that the overactivation of the Akt pathway, as opposed to ERK signals, causes an imbalance in mitochondrial dynamics, thus favoring fusion over fission. This disruption in the dynamic balance of mitochondria can have significant implications for cellular functions and may contribute to various cellular processes and diseases.

Several of the known genes associated with the familial forms of PD are involved in the regulation of mitochondrial function, including parkin and Pink-1[41]. The downregulation of Pink-1 affects the mitochondrial fusion–fission machinery and sensitizes mitochondria to neurotoxins in dopaminergic cells[42]. Pink-1 not only phosphorylates Drp-1 S616 to activate mitochondrial fission[43], but also phosphorylates Mfn2 to promote parkin recruitment for mitophagy, which is a selective process that removes damaged mitochondria[44]. Loss of Pink-1 impairs mitochondrial function, leading to mitochondrial dysfunction, increases in oxidative stress, and a compromised cellular energy
production[45]. This is in line with our observation of reduced Pink-1 expression in mutant cells. Interestingly, in cells treated with formoterol, both full-length Pink-1 and its 52-kDa cleaved form[46] were upregulated, whereas the levels of parkin and Mfn2 were significantly decreased. Thus, we suggest that the formoterol-induced upregulation of Pink-1, with consequent mitochondrial fission, does not active mitophagy. In fact, Pink-1 regulates mitophagy-independent mitochondrial fission by phosphorylating Drp-1 S616[43]. Moreover, increasing the levels of cytosolic cleaved Pink-1 and ablating Mfn2 also repressed parkin translocation to mitochondria for mitophagy[44, 47]. Conversely, although the role of cleaved Pink-1 in neuronal functions remains poorly understood, in healthy mitochondria, Pink-1 is cleaved at the inner mitochondrial membrane and is retro-translocated to the cytosol, where it exerts extra-mitochondrial functions that are important for neuronal development, neuronal survival, synaptogenesis, and plasticity; these are crucial implications for PD[48, 49]. Thus, we suggest that restoration of Pink-1 could play a multifaceted role in supporting neuronal function in the context of the benefits of formoterol.

Formoterol has been shown to decrease the glucose-induced imbalance in mitochondrial dynamics and restore mitochondrial homeostasis in the context of renal proximal tubule cells under diabetic conditions[50]. A similar regulatory role of formoterol was found by us in the treatment of UQCRCT1-mutant cells resulting in PD, although the specific mechanisms underlying the regulation of mitochondrial homeostasis appear to be dependent on various cellular contexts. Moreover, our findings provide further evidence of the beneficial effects of formoterol, as it significantly promoted anterograde mitochondrial transport and increased mitochondrial mobility. Several studies have explored strategies aimed at enhancing anterograde mitochondrial transport as a means of neuroprotection, to manage mitochondrial-dysfunction-related neurodegenerative disorders[51–53]. Because the promotion of mitochondrial anterograde movement facilitates the transport of mitochondria toward axon terminals, to support the energy demands required for synaptic transmission and neuronal signaling[52, 53], maintain mitochondrial health via the overall quality control of mitochondria within neurons[53], and ensure proper neuronal functions, such as calcium regulation, excitability, and neurotransmission[51, 52]. However, the causes of the reduction in anterograde mitochondrial axonal transport and mitochondrial mobility caused by UQCRCT1 mutations, whereas retrograde movement is unaffected, remain unclear. Based on limited findings, we suggest that sustained mitochondrial hyperfusion could hinder mitochondrial transportation by modifying the size, shape, and distribution of mitochondria. Mitochondrial hyperfusion has been indicated to disrupt the interactions between mitochondria and motor proteins, affecting their proper engagement and transport along the cytoskeleton during stress promotion[54]. The entanglement and close proximity of fused mitochondria could restrict their movement along microtubules, thereby reducing their mobility to distribute and reach specific cellular regions[54]. In contrast, studies have shown that Pink1 forms a multiprotein complex with Miro and Milton, thus linking Pink1 function to regulate mitochondrial movement in axons[55, 56], and its effect on mitochondrial motility is direct, rather than a secondary effect of changes in mitochondrial length[56]. Moreover, the localization of cleaved Pink-1 to the cytosol has been shown to enhance the anterograde-mediated trafficking of mitochondria in neurites
(both in dendrites and axons) via the phosphorylation of Miro2[48, 57]. Therefore, the regulatory role of Pink-1 in mitochondrial transportation is worthy of further investigation.

**Conclusion**

In summary, formoterol not only improved mitochondrial function, but also restored mitochondrial dynamics and transportation, which contributed to the improvement of the perturbation of mitochondrial homeostasis triggered by UQRC1 mutation, although further investigation is required to understand how UQRC1 variation leads to abnormal mitochondria dynamics. Furthermore, through the activation of B2AR, formoterol effectively corrected the mitochondrial dynamics imbalance, thus potentially aiding in the treatment of familial PD caused by genetic defects. Finally, formoterol may hold potential for treating other neurodegenerative diseases with a similar etiology.

**Abbreviations**

Parkinson's disease (PD); reductase core protein (UQRC1); β2-adrenergic receptor (B2AR)

**Declarations**

**Headings**

Restoration of mitochondrial function, dynamics, and transport by formoterol in familial Parkinsonism induced by UQRC1 mutation

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**Declaration of competing interest**

All authors have no conflict of interest, as well as a financial or personal relationship, with other organizations.

**Authorship contribution**

**Jui-Chih Chang**: Investigation, Methodology, Writing-original draft, Writing-review & editing, Project administration. **Huei-Shin Chang**: Investigation, Methodology, Validation. **Yi-Chun Chao**: Investigation, Validation. **Ching-Shan Huang**: Investigation, Validation. **Yong-Shiou Lin**: Investigation.

Data Availability

The datasets generated during and/or analysed during the current study are not publicly available as consent for publication of raw data was not obtained from study participants, but are available from the corresponding author on reasonable request.

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Figures
Figure 1

Cell viability and neurite outgrowth in the absence or presence of tBH stimulation. Cell morphology (A, D) and neurite length (B, E) were observed after treatments with or without tBH stimulation for 24 h (10 μM) (A, D). Cell viability was determined based on a WST-1 analysis and cell proliferation (C). The tBH-induced cell death was measured using 7-AAD staining and flow cytometry analysis (F), then quantified (G). *P < 0.05 vs. the WT group or the treated Ctrl group. **P < 0.05 vs. the DMSO group. #P < 0.05 vs. the F+P group. Data are presented as the mean ± SD. WT: wild-type; Ctrl: control; DMSO, dimethylsulfoxide; F, formoterol; F+P: formoterol plus propranolol; P: propranolol; UQRC1\textsuperscript{mut}: mutation of the ubiquinol-cytochrome c reductase core protein 1 gene; 7AAD; 7-aminoactinomycin D; tBH: tertiary-butyl hydroperoxide. N = 6
Figure 2

Mitochondrial function and generation of reactive oxygen species. Mitochondrial function was comprehensively assessed after 24 h of treatment by analyzing the mtDNA content (DNA copy number) (A), the level of reactive oxygen species in total (total ROS) (B) and in mitochondria (mtROS) (C), and mitochondrial respiration (D, E). Mitochondrial respiration was measured in permeabilized cells using different substrates and inhibitors of the respiratory chain complexes; the comparison between WT and mutant cells (left panel) or between mutant cells with different treatments (right panel) is shown (D). The induced oxygen consumption (oxygen flux) was integrated and quantified according to different approaches, to provide an indirect measurement of mitochondrial activity, including basal respiration, ATP-linked production, and complex III activity (E). *P < 0.05 vs. the WT group. **P < 0.05 vs. the DMSO group. #P < 0.05 vs. the F+P group. Data are presented as the mean ± SD. WT: wild-type; Ctrl: control; DMSO, dimethylsulfoxide; F, formoterol; F+P: formoterol plus propranolol; P: propranolol; UQCRC1mut: mutation of the ubiquinol-cytochrome c reductase core protein 1 gene; BR: basal respiration, G: glutamate, M: malate, Oligo: oligomycin; DuroQ: duroquinone, AA: antimycin A, SA: sodium azide. N = 3
Figure 3

**Mitochondrial morphology and distribution.** The morphology of GFP-labeled mitochondria was observed after 24 h of treatment (A, upper panel). The classification of mitochondrial morphology was further analyzed using an automatic system and is presented by the labeling of different color-coded subtypes of mitochondria, to show the morphological composition of each group of representative cells (lower panel in A). The six distinct mitochondrial subtypes were classified as class three, as follows: globe, including single (blue) and swollen (yellow); tubule, including straight tubule (green) and reticular networks (including branched tubule (purple), twisting tubule (orange), and loop (red)) (A). The percentage of each mitochondrial subtype among the total mitochondrial population was calculated and integrated into the three categories, as described above (B). The mitochondrial counts are presented as the total number of mitochondria per cell (C). *P < 0.05 vs. the WT group. **P < 0.05 vs. the DMSO group. #P < 0.05 vs. the F+P group. Data are presented as the mean ± SD. WT: wild-type; Ctrl: control; DMSO, dimethylsulfoxide; F, formoterol; F+P: formoterol plus propranolol; P: propranolol; UQCRC1mut: mutation of the ubiquinol-cytochrome c reductase core protein 1 gene
Figure 4

Expression of mitochondrial dynamic proteins and the upstream related signaling activation mechanism of the b2 adrenergic receptor. The level of mitochondrial fusion-relate (OPA1 and Mfn2), fission-related [total and phosphorylated form of Drp-1 at serine 616 (S616) and 637 (S637)], and mitophagy-related [Long (L) and short form (S) of Pink-1 and parkin)] proteins was analyzed in both WT cells and mutant cells with or without 24-h treatments (A), as was the related regulatory signaling of ERK and Akt.
phosphorylation (B, C). Target proteins were analyzed by western blotting, quantified by normalizing to β-actin, and are presented as the fold change relative to the WT group (A, C). Representative arrays and densitometry of ERK and Akt pathway-related phosphorylated proteins were applied to the analysis of the pools of cell lysates from three independent experiments (B). The protein expression differences between the groups (UQCRC1mut-DMSO vs. WT; UQCRC1mut-F vs. UQCRC1mut-DMSO; UQCRC1mut-F+P vs. UQCRC1mut-F) were compared and the reverse regulation direction (up- or down regulation) between inter-groups, UQCRC1mut-DMSO vs. WT; UQCRC1mut-F vs. UQCRC1mut-DMSO, was indicated in the box (B, right panel). Among the Raf–ERK signaling molecules involved in the positive regulation of formoterol, the induction of 4E-BP1 was even higher than that of ERK1/2. In contrast, formoterol negatively regulated the Akt–RSK–S6-kinase signaling-related molecule (B). The simultaneous detection and quantification of protein kinase A (PKA) activity were achieved using a colorimetric activity assay (D). A path diagram was constructed based on the above findings to illustrate the regulatory relationship between the mitochondrial dynamic machinery and the activation of the β2 adrenergic receptor by formoterol (E). This showed the formoterol-mediated balance regulation via the facilitation of mitochondrial fission and the inhibition of abnormal mitochondrial hyperfusion in mutant cells after formoterol treatment. *P < 0.05 vs. the WT group. **P < 0.05 vs. the DMSO group. #P < 0.05 vs. the F+P group. Data are presented as the mean ± SD. WT: wild-type; Ctrl: control; DMSO, dimethylsulfoxide; F, formoterol; F+P: formoterol plus propranolol; P: propranolol; UQCRC1mut: mutation of ubiquinol-cytochrome c reductase core protein 1 gene. N = 3
Figure 5

Mitochondrial movement and mobility. Representative kymographs showing the movement of the mitochondria-labeling GFP signal in WT cells or mutant cells with or without 24-h treatments. Vertical lines were observed for stationary mitochondria, whereas moving mitochondria displayed diagonal lines, indicating their motion in either the anterograde or retrograde direction (A). Mitochondrial transport was quantified by measuring mitochondrial velocity (B) and calculating the proportion of motile mitochondria (C).
exceeding the average velocity in relation to the total mitochondrial population (C). Representative time-lapse images specifically showed the mitochondria undergoing anterograde movement (direction indicated by arrows) from their initial position (dashed line). \( *P < 0.05 \) vs. the WT group. \( **P < 0.05 \) vs. the DMSO group. \( #P < 0.05 \) vs. the F+P group. Data are presented as the mean ± SD. WT: wild-type; Ctrl: control; DMSO, dimethylsulfoxide; F, formoterol; F+P: formoterol plus propranolol; P: propranolol; UQCRC1\(^{\text{mut}}\): mutation of ubiquinol-cytochrome c reductase core protein 1 gene

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