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Activation of mitochondrial fusion provides a new treatment for mitochondria-related diseases

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ABSTRACT

Mitochondria fragmentation destabilizes mitochondrial membranes, promotes oxidative stress and facilitates cell death, thereby contributing to the development and the progression of several mitochondria-related diseases. Accordingly, compounds that reverse mitochondrial fragmentation could have therapeutic potential in treating such diseases. BGP-15, a hydroxylamine derivative, prevents insulin resistance in humans and protects against several oxidative stress-related diseases in animal models. Here we show that BGP-15 promotes mitochondrial fusion by activating optic atrophy 1 (OPA1), a GTPase dynamin protein that assist fusion of the inner mitochondrial membranes. Suppression of Mfn1, Mfn2 or OPA1 prevents BGP-15-induced mitochondrial fusion. BGP-15 activates Akt, S6K, mTOR, ERK1/2 and AS160, and reduces JNK phosphorylation which can contribute to its protective effects. Furthermore, BGP-15 protects lung structure, activates mitochondrial fusion, and stabilizes cristae membranes *in vivo* determined by electron microscopy in a model of pulmonary arterial hypertension. These data provide the first evidence that a drug promoting mitochondrial fusion in *in vitro* and *in vivo* systems can reduce or prevent the progression of mitochondria-related disorders.

1. Introduction

Mitochondria are the major energy-producing organelles, and play a significant role in determining cell survival and death [1–4]. They are dynamic organelles undergoing frequent fission and fusion cycles that result in major morphological changes. Membrane bound dynamin GTPases, dynamin-related protein 1 (Drp1), mitochondrial fission protein 1 (Fis1), mitofusin 1/2 (Mfn1/2) and optic atrophy 1 (OPA1) drive these changes [5–7]. Mitochondrial fragmentation plays an important role in the development and progression of several diseases, including diabetes [4,8–12], neurodegenerative diseases [5,13,14], muscular dystrophies [15,16], nonalcoholic fatty liver disease and other types of hepatotoxicity [17–19], and pulmonary arterial hypertension (PAH) [20,21] and several other diseases related to oxidative stress [2,22].

Reactive oxygen species (ROS) deriving from different sources induce activated mitochondrial fission catalyzed by Drp1 and Fis1, and lead to fragmented mitochondria [1–4,8]. These mitochondria have lower membrane potential, produce less ATP, generate significantly more ROS, and facilitate the release of proapoptotic mitochondrial proteins [7,16,23]. Therefore, modulation of mitochondrial fission or fusion pathways by synthetic chemicals could provide novel molecular mechanism to protect cells in oxidative stress, and could be a new way to design novel mitochondrial drugs.

BGP-15 (O-[3-piperidino-2-hydroxy-1-propyl]-nicotinic amidoxime) possesses a wide range of cytoprotective effects [24–33] but lacks a clear intracellular molecular target. BGP-15 protects the mitochondrial membrane system, decreases oxidative stress [27,28], inhibits the nuclear translocation of apoptosis-inducing factor (AIF) from mitochondria [27], and inhibits mitogen-activated protein kinase (MAPK) activation [26,29]. BGP-15 is effective in ameliorating obesity induced

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insulin resistance [29,33], improves insulin action in humans [30], slows the progression of severe muscular dystrophy [24], and also increases mitochondrial biogenesis [33] in rodents.

Based on the observations above, we raise the possibility that BGP-15 can influence mitochondrial fragmentation, and provide evidence in cultured cells that BGP-15-activated mitochondrial fusion requires active OPA1, Mfn1/2 and Akt (also called protein kinase B). In addition, we show that BGP-15 activates OPA1 GTPase and promotes its polymerization *in vitro*. Since oxidative stress and mitochondrial fragmentation play important role in the development of PAH [20,21], we used this model to provide evidence for the BGP-15-induced mitochondrial fusion in an *in vivo* system.

2. Materials and methods

2.1. Materials

All chemicals for cell culture studies were from PAA Laboratories (Cölbe, Germany) and Gibco/Invitrogen (Life Technologies, Carlsbad, CA, USA). Hydrogen peroxide (H_2O_2), protease inhibitor mixture and all remaining chemicals were purchased from Sigma–Aldrich Co. (Budapest, Hungary). Fluorescent dye MitoTracker Red was obtained from Molecular Probes (Life Technologies, Carlsbad, CA, USA). Hematoxylin and eosin were purchased from Sigma–Aldrich Co. BGP-15 was a gift from N-Gene (New York, NY, USA). The following primer antibodies were used: anti-Akt, anti-phospho-Akt, anti-phospho-Akt substrate of 160 kDa (AS160), anti-phospho-mammalian target of rapamycin (mTOR), anti-phospho-p70 S6 kinase (S6K), anti-phospho-p44/42 MAPK (ERK1/2), anti-phospho-p38 MAPK (p38), anti-phospho-JNK MAPK (JNK1/2) (Cell Signaling Technology, Danvers, MA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EMD Millipore, Billerica, MA, USA), anti-Mfn1, anti-Mfn2, anti-Drp1, anti-Fis1 (Santa Cruz Biotechnology, Heidelberg, Germany) and anti-OPA1 (Invitrogen; Life Technologies, Carlsbad, CA, USA). The horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Sigma–Aldrich Co., XF Calibrant, XF Base Medium and XFp Cell Mito Stress Test Kit including oligomycin, FCCP, rotenone/antimycin A were purchased from Agilent Technologies (Kromat Ltd., Budapest, Hungary). All reagents were of the highest purity commercially available.

2.2. Animals

Wistar male rats were purchased from Innovo Ltd. (Gödöllő, Hungary). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and was approved by the Animal Research Review Committee of the University of Pécs, Hungary. All animals were housed one or two per cage, under optimal laboratory conditions (controlled temperature, humidity and 12:12 h light-dark cycles) with free access to water and standard rodent chow. Starting at 8 weeks of age, the animals were assigned into four groups. Eight animals were allocated to control group receiving subcutaneous injection of isotonic saline (0.1 ml/kg) on day 0. Eight animals were allocated to BGP-15 group receiving subcutaneous injection of isotonic saline (0.1 ml/kg) on day 0 and BGP-15 (20 mg/kg per day, per os, in the drinking water) from day 0 to day 28. Eight animals were allocated to PAH group receiving 60 mg/kg subcutaneous injection of monocrotaline (MCT) on day 0. Eight animals were allocated to PAH + BGP-15 group receiving 60 mg/kg subcutaneous injection of MCT on day 0 and BGP-15 (20 mg/kg per day, per os, in the drinking water) from day 0 to day 28.

2.3. Cell cultures

WRL-68 (HeLa derivative), C2C12 (mouse C3H muscle myoblast), A549 (human lung carcinoma) and Sf9 (derived from pupal ovarian tissue of *Spodoptera frugiperda*) cell lines were obtained from the European Collection of Cell Cultures (Salisbury, UK). The WRL-68, C2C12 and A549 cell lines were maintained in a humidified 5% CO_2 atmosphere at 37 °C while Sf9 cells were cultured in a 100% air atmosphere at 27 °C. WRL-68 cells were cultured in Eagle's minimum essential medium (MEM), C2C12 and A549 cells in Dubelcco modified Eagle's medium (DMEM) (PAA Laboratories) while Sf9 cells in TC-100 insect medium (Sigma–Aldrich Co.). All media contained 10% bovine serum and antibiotic solution (1% penicillin and streptomycin mixture) (Gibco/Invitrogen). Cells were passaged at 3-day intervals.

Cells were seeded at a starting density of 5×10^5 cells/well in a 6-well plate for immunoblotting and at a density of 1×10^5 cells/well for fluorescent or confocal laser scanning microscopy.

2.4. Immunoblot analysis

The WRL-68 cells were seeded into a 6-well plate and cultured overnight. After subjecting the cells to 50 μM BGP-15 for 3 h, the cells were harvested in ice-cold lysis buffer containing 0.5 mM sodium metavanadate, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor mixture in phosphate-buffered saline (PBS). The proteins were precipitated by trichloroacetic acid, washed three times with −20 °C acetone, and subjected to sodium-dodecyl sulphate polyacrylamide gel electrophoresis. Proteins (20 μg/lane) were separated on 12% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low-fat milk for 1 h at room temperature and then exposed to primary antibodies at 4 °C overnight at the manufacturer's proposed dilution in blocking solution. Appropriate horseradish peroxidase-conjugated secondary antibodies were used for 2 h at room temperature in 1:5000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Life Technologies).

2.5. Construction of mitochondria-directed red and green fluorescent proteins

Mitochondria-directed enhanced red fluorescent protein (mERFP) expressing plasmid was constructed as follows: The mitochondrial targeting sequence (MTS) was amplified by polymerase chain reaction (PCR) from cytochrome *c* oxidase subunit VIIIa (COX8A) gene (RZPD), and the amplified sequence was inserted into pDsRed-Monomer-N1 mammalian expression plasmid (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France) between XhoI and HindIII restriction sites.

Construction of plasmid for the expression of mitochondria-directed enhanced green fluorescent protein (mEGFP) are described below: MTS was amplified from cytochrome *c* oxidase subunit VIIIa coding sequence (from RZPD) by PCR and ligated into pEGFP-N3 mammalian expression plasmid (Clontech) between BglII and SalI restriction sites.

2.6. Polyethylene glycol (PEG) fusion assay

The PEG fusion assay was performed as described previously [34]. Briefly, WRL-68 cells were transiently transfected with mERFP or mEGFP fluorescent protein. The next day, the PEG fusion assays were then performed. WRL-68 cells expressing mERFP were co-plated with WRL-68 cells expressing mEGFP on glass coverslips. Cycloheximide (20 μg/ml) was added 30 min before fusion. The 70–100% confluent cells in a 35-mm culture dish were then washed with MEM without serum and incubated for exactly 1 min with 750 μl of a prewarmed (37 °C) solution of PEG 1500 (50% [wt/vol] in MEM, Sigma–Aldrich Co.). The cultures were fixed in 4% formalin, and the cells were visualized by Nikon Eclipse Ti-U fluorescent microscope equipped with a Spot RT3 camera using a 60× objective and epifluorescent illumination.