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Resveratrol stimulates mitochondrial fusion by a mechanism requiring mitofusin-2

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ABSTRACT

Resveratrol (RES) is a plant-derived stilbene associated with a wide range of health benefits. Mitochondria are a key downstream target of RES, and in some cell types RES promotes mitochondrial biogenesis, altered cellular redox status, and a shift toward oxidative metabolism. Mitochondria exist as a dynamic network that continually remodels via fusion and fission processes, and the extent of fusion is related to cellular redox status and metabolism. We investigated RES's effects on mitochondrial network morphology in several cell lines using a quantitative approach to measure the extent of network fusion. 48 h continuous treatment with 10–20 μM RES stimulated mitochondrial fusion in C2C12 myoblasts, PC3 cancer cells, and mouse embryonic fibroblasts stimulated significant increases in fusion in all instances, resulting in larger and more highly branched mitochondrial networks. Mitofusin-2 (Mfn2) is a key protein facilitating mitochondrial fusion, and its expression was also stimulated by RES. Using Mfn2-null cells we demonstrated that RES's effects on mitochondrial fusion, cellular respiration rates, and cell growth are all dependent upon the presence of Mfn2. Taken together, these results demonstrate that Mfn2 and mitochondrial fusion are affected by RES in ways that appear to relate to RES's known effects on cellular metabolism and growth.

1. Introduction

Resveratrol (*trans*-3,4,5′ trihydroxystilbene; RES) is a plant-derived stilbene associated with protection from a battery of seemingly unrelated diseases [reviewed in Ref. [1]]. Aberrant cellular metabolism (either acute or chronic) is implicated in the aetiology of many disorders ameliorated by RES. RES exerts metabolic effects in many cell types, promoting mitochondrial biogenesis and a shift toward a more oxidative phenotype [2–5]. Mitochondria are thus an important target of RES [reviewed in Ref. [5]], which has been shown to stimulate mitochondrial biogenesis and altered redox status in skeletal muscle, cardiac and brain tissue, and in many cultured cell lines [2,6–8]. These RES-induced effects on mitochondrial metabolism may be central to its many cellular effects.

Mitochondrial dynamics are an important aspect of mitochondrial and cell physiology that has not been thoroughly explored in the context of RES. Within cells mitochondria exist in a dynamic equilibrium between fusion and fission of mitochondria to form highly connected and independent mitochondrial structures, respectively. This equilibrium is highly malleable, shifting in response to changes in nutrient availability, redox state and the activities of intracellular signalling cascades [reviewed in Ref. [9]]. Architectural changes in the mitochondrial network are thought to contribute to the 'metabolic efficiency' of the cell, with a more fused, interconnected network associated with increased oxidative metabolism. In contrast, a fragmented network is commonly observed in primarily glycolytic cells [10,11].

Mitochondrial network dynamics are also related to cell division. Mitochondria typically elongate and fuse during G1/S, and fragment during G2/M in a process known as mitotic fission. Interestingly, a highly fused mitochondrial network achieved by either the stimulation of fusion or inhibition of fission [12] can impede progression through the cell cycle, while fragmentation of the mitochondrial network appears to be permissive of rapid proliferation [7,12–14]. In support of the central role of mitochondrial

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morphology in cell physiology, pharmacological manipulation of the mitochondrial fusion state can directly affect proliferation. Stimulation of network hyperfusion by inhibition of the fission enzyme dynamin-related protein 1 (Drp1) with the compound mdivi-1 is sufficient to slow the growth of cancer cells [12,15]. Gene knockout of the fusion enzyme mitofusin2 (Mfn2) to produce a fragmented mitochondrial population significantly increases proliferative growth rate in B-cell lymphocytes and mouse embryonic fibroblasts (MEFs), while Mfn2 overexpression slows growth [12,16].

The slowed cell growth associated with mitochondrial hyperfusion is strikingly similar to the slowed cell growth elicited by RES in multiple cell lines and in vivo [17–19]. Given that changes in both mitochondrial turnover and redox state impact upon the morphology of the mitochondrial network [9,20] it is plausible that RES alters mitochondrial dynamics to affect cell proliferation. Two recent studies have shown that RES treatment is associated with changes in the levels of mitochondrial fusion/fission proteins under conditions of metabolic stress [21,22]. However, there are no reports of RES effects on mitochondrial dynamics under non-stressed conditions and nor has the fusion state of mitochondrial networks been quantitatively analysed in RES-treated cells. Here we use MiNA, a mitochondrial network morphology analysis tool [23] to determine features of the mitochondrial network in cells following treatment with RES. We demonstrate that RES stimulates mitochondrial fusion that is associated with increased Mfn2 expression and, using Mfn2-null cells, further show that Mfn2 is required for this increased fusion. We further show that RES's effects on cell growth are dependent on Mfn2.

2. Experimental procedures

2.1. Materials

Modified Eagle Medium with Earl salts, L-glutamine and sodium bicarbonate, Dulbecco's Modified Eagle Medium with high glucose, L-glutamine and sodium bicarbonate and Dulbecco's Modified Eagle Medium were obtained from Sigma–Aldrich (St. Louis, MO). Penicillin/streptomycin, non-essential amino acids, and fetal bovine serum were obtained from Hyclone (Logan, UTtah). RES was obtained from A.G. Scientific (San Diego, CA). BioRad protein dye was purchased from BioRad laboratories (Hercules,CA). Prestained broad range protein marker was purchased from BioLabs (New England, MA). Pierce Memcode Reversible Protein Stain Kit™ was obtained from Thermo Fisher Scientific (Mississauga, Canada). Mfn2 antibody was purchased from Abnova (Walnut, CA). Infrared dye-conjugated secondary antibody to mouse was purchased from Rockland Immunochemicals (Gilbertsville, PA). Renilla luciferase kit was purchased from Promega (Madison, WI). CellLight mtGFP and MitoTracker Red CMXRos dye were purchased from Life Technologies (Burlington, ON). All other chemicals and purified enzymes were obtained either from Sigma–Aldrich (St. Louis, MO), BioShop (Burlington, Canada) or Fisher Scientific (Mississauga, Canada) unless otherwise stated.

2.2. Cell lines and culture conditions

C2C12 (Sigma) and PC3 (ATCC) cell lines were cultured in accordance with the distributor's protocol. HeLa cell lines expressing N or C Mito venus-zipper-luciferase (mitoVZL) were a gift from Dr. Heidi McBride at McGill University. They were generated and cultured as described in Ref. [24]. Mfn2-null and wt MEFs were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). All cell lines were cultured at 37 °C in humidified 5% CO_2, 18% O_2 atmosphere. Cell density and population doubling time were determined by direct counting. Treatments were added directly to culture media and refreshed daily until cells were harvested.

2.3. Microscopy

Mitochondria were imaged following transfection with the mitochondrial targeted GFP probe CellLight mtGFP, as per the manufacturer's protocol, or following a 15 min incubation with 50 nM MitoTracker Red in a 37 °C, 5% CO_2 humidified incubator, as indicated in Results section. Cells were washed with warmed PBS, and imaged immediately with either an Olympus Fluoview 300 confocal microscope or Zeiss Axio Observer. Z1 inverted epifluorescence microscope equipped with ApoTome.2 optical sectioning, with a 63 × 1.4NA oil immersion lens.

2.4. Analysis of mitochondrial morphology

Mitochondrial morphology was analysed using the Mitochondrial Network Analysis tool (MiNA) a macro tool developed for use with the FIJI distribution of ImageJ [23]. Briefly, fluorescence images were loaded into the program in their native format using Bio-Formats plugin [25]. Images were prepared for analysis through the application of an unsharp mask with a 2 pixel radius to sharpen the image. A median filter was then applied using a 2 pixel radius to remove spurious features without causing a significant loss to high frequency information. The image was then skeletonized and the skeleton analysed using the Analyse Skeleton plugin included in the FIJI distribution [26]. Structures containing at least one branch point were considered networks [27].

2.5. Cell free mitochondrial fusion assay

Cells (including N and C mitoVZL expressing HeLa) were harvested by trypsinization, and mitochondrial isolation was performed at described in Ref. [21]. Briefly, 50 μg of C and N mitoVZL mitochondria were incubated in a reaction containing 10 mM HEPES pH7.4, 110 mM mannitol, 68 mM sucrose, 80 mM KCl, 0.5 mM EGTA, 2 mM $Mg(CH_3COO)_2$, 0.5 mM GTP, 2 mM K_2HPO_4, 1 mM ATP, 0.08 mM ADP, 5 mM Na succinate, 1 mM DTT and 5 mg/mL cytosol. The mitochondria were concentrated by centrifugation, then incubated on ice for 30 min. The mitochondria were resuspended and incubated in a 37 °C water bath for 30 min. Signal arising from ruptured mitochondria was quenched by incubation with 25 μg trypsin for 15 min on ice, after which trypsin activity was inhibited by incubation with 500 μg soybean trypsin inhibitor for 15 min on ice. Mitochondria were concentrated by centrifugation at 9000*g* for 1 min, and solubilized by 1 h incubation with 50 μL of lysis buffer (Promega) on ice. Luciferase activity was detected using a commercially available Renilla luciferase kit (Promega) and fluorescence was detected using a Varian Cary Eclipse fluorescence spectrometer.

2.6. Cell cycle analysis

5×10^5 cells were harvested via trypsinization and centrifugation (5 min at 240×*g*), washed once with PBS and then fixed via drop-wise addition of ice-cold ethanol (75% *v/v*) with routine vortexing. The cell suspensions were then kept at −20 °C for 2 h before undergoing centrifugation (5 min at 240×*g*) and two washes with ice-cold PBS. Fixed cells were incubated with 0.5 mL Propidium Iodide (PI)/RNase Staining Buffer (BD Pharmingen, USA) in darkness at room temperature for 15 min. The DNA content was measured using a BD Accuri C6 flow cytometer (BD Biosciences, USA). PI signal was detected in the orange range of the spectrum using a

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