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NARP mutation and mtDNA depletion trigger mitochondrial biogenesis which can be modulated by selenite supplementation

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ABSTRACT

The importance of mitochondrial biogenesis in the pathogenesis of mitochondrial diseases has been widely recognised but little is known about it with regard to NARP (**N**europathy, **A**taxia and **R**etinitis **P**igmentosa) syndrome. Since such knowledge would contribute to the understanding of the pathogenesis of this disease, we designed a study to provide comprehensive overview of mitochondrial biogenesis in cybrid cells harboring NARP mutation (8993T > G). We also used Rho0 cells with the same nuclear background to show that distinct mtDNA defects lead to distinct cellular responses irrespective of nuclear genome.

Mitochondrial biogenesis is regulated by mitochondria-to-nucleus (retrograde) communication which depends on intracellular signaling pathways sensitive to ROS. Since we previously found that selenite lowered ROS in NARP cybrids, we hypothesised that selenite could also modulate mitochondrial biogenesis in these cells.

Although the mitochondrial mass was not changed in NARP cybrids, we showed the compensatory upregulation of respiratory chain subunits which prompted us to investigate the transcription factors that regulate their expression such as PGC-1α, NRFs, and TFAM. Selenite supplementation increased the level of NRF1 and nuclear accumulation of NRF2, but we did not detect any major changes in the levels of investigated respiratory chain proteins. These subtle changes in mitochondrial biogenesis in response to selenite treatment support the hypothesis that selenite could be considered as a potential therapeutic agent of NARP syndrome due to its antioxidant properties. Moreover, it could also be tested with regard to other mitochondrial disorders associated with ROS overproduction.

1. Introduction

Mitochondrial biogenesis is tightly regulated to meet cellular energy requirements. The main regulatory role is played by **n**uclear **r**espiratory **f**actors 1 (NRF1), 2 (NRF2) and **m**itochondrial **t**ranscription **f**actors **A** (TFAM) and B. However, these transcription factors alone cannot account for the coordinated expression of about 1500 mitochondrial proteins. A growing body of evidence points to the integrative role of coactivators from the PGC-1 (**P**PAR-**g**amma **c**oactivator 1) family that precisely orchestrate these transcription factors and their target genes into a program of mitochondrial biogenesis (Scarpulla, 2006, 2008).

The importance of mitochondrial biogenesis with respect to pathogenesis of mitochondrial diseases has been widely recognised (Collombet et al., 1997; Heddi et al., 1993; Marusich et al., 1997; Reinecke et al., 2009). Although there is some data pointing to increased expression of genes related to oxidative phosphorylation and glycolysis in NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) patient cells (Heddi et al., 1999), the comprehensive overview of mitochondrial biogenesis and its regulatory factors with regard to this mitochondrial disease is missing. To address this issue, we investigated the total mitochondrial mass and the level of representative subunits of respiratory chain complexes II (CII-30), III (CIII-core 2), IV (CIV-II) and ATP synthase (CV-α) in NARP cybrid cells. We also extended our investigation to non-respiratory mitochondrial proteins such as mitochondrial chaperone mtHsp70 that facilitates protein import into the mitochondrial matrix (Deocaris et al., 2008), uncoupling protein UCP3 that can attenuate mitochondrial ROS production (Brand and Esteves, 2005) and electron carrier cytochrome c (Cyt C). Last but not least, we also investigated the levels of transcription factors NRF1, NRF2, TFAM and PGC-1α that regulate expression of genes encoding mitochondrial proteins. Since (García et al., 2000) used MRC5 Rho0 fibroblasts with the same nuclear background as NARP cybrids to compare the structure, functioning and assembly of ATP synthase, we also compared

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mitochondrial biogenesis in 143B Rho0 cells and NARP cybrids with the same nuclear background to show adaptive changes triggered by distinct mtDNA defects irrespective of nuclear genome.

Overproduction of ROS is known to contribute to the pathogenesis of mitochondrial diseases (Kirkinezos and Moraes, 2001; Wu et al., 2010). Previously, we found that selenite, an inorganic selenium compound, acted as an antioxidant in NARP cells (Wojewoda et al., 2010). Since it was shown that antioxidant spin-trap molecule inhibited apoptosis of NARP patient fibroblasts (Geromel et al., 2001) and other antioxidants rescued respiration and ATP synthesis in cybrids harboring NARP mutation (Mattiazzi et al., 2004), we suggested selenite as a potential therapeutic agent of NARP syndrome. This conclusion was additionally supported by the findings that selenite lowered Ca^{2+} and increased ATP levels in NARP cybrid cells (unpublished data). Therefore, we decided to investigate how selenite supplementation affected mitochondrial biogenesis in these cells.

We observed an increase in the levels of respiratory chain subunits in NARP cybrids which suggested that mitochondrial biogenesis was induced in these cells. Although the levels of these subunits were very low in Rho0 cells, elevated level of mtHsp70 that is implicated in the mitochondrial protein import also suggested the induction of mitochondrial biogenesis. Selenite only slightly lowered the level of some mitochondrial proteins in NARP cybrids as well as WT control cells. These subtle effects support the idea of selenite as a potential therapeutic antioxidant agent with regard to NARP syndrome and, possibly, other mitochondrial diseases.

2. Materials and methods

2.1. Chemicals and antibodies

High glucose Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). Trypsin, uridine (50 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) were from Sigma Aldrich (St. Louis, MO, USA). MitoTracker Green FM and MitoTracker CMXRos were from Invitrogen (Eugene, OR, USA). All other chemicals were of analytical grade.

Primary antibodies directed against PGC-1α (Western Blotting (WB): 1:1000) were from Calbiochem-Merck4Biosciences (Darmstadt, Germany), against NRF1 (WB: 1:200) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), against NRF2 phosphorylated at serine 40 (immunofluorescence (IF: 1:50) from Epitomics (Burlingame, CA, USA), against TFAM (WB: 8 μg/ml; IF: 1:100) from ProSci Incorporated (Poway, CA, USA), against cytochrome c (WB: 1:500; IF: 1:100) from BD Pharmingen (San Diego, CA, USA), against UCP3 (WB: 1:500) from Sigma Aldrich (St. Louis, MO, USA), against mtHsp70 (WB: 1:500) from AbCam (Cambridge, UK, USA), and those against Akt protein kinase phosphorylated at serine 473 (WB: 1:4000) were from Cell Signaling (Danvers, MA, USA). Premixed cocktail of primary antibodies directed against complex I subunit NDUFB8 (CI-20), complex II subunit 30 kDa (CII-30), complex III subunit core 2 (CIII-core2), complex IV subunit II (CIV-II) and ATP synthase subunit alpha (CV-α) (0.6 μg/ml) were from MitoSciences (Eugene, OR, USA). Secondary antibodies labelled with horseradish peroxidise (WB: 1:5000) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), those used for immunofluorescence staining were from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK) and Invitrogen (Eugene, OR, USA) while IRDye 680 secondary antibodies were from Li-Cor Biosciences (Bad Homburg, Germany).

2.2. Cell culture

Parental human osteosarcoma cell line 143B, the cybrid cell line derived from NARP skin fibroblasts 143B/206 with 98% penetration of the mtDNA T8993G mutation in ATP6 gene and the Rho0 cell line lacking mitochondrial DNA (King and Attardi, 1996) were gifts from Dr. M. Tanaka from the Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Japan. Cells were cultured in conditions previously described (Wojewoda et al., 2010) and the culture medium was supplemented with 70 nM sodium selenite (Na_2SeO_3) (Sigma Aldrich, St. Louis, MO, USA) for 10–14 days. The basal selenium concentration in DMEM before selenite supplementation varied between 7 and 45 nM, due to FBS addition (List et al., 1996) and, therefore, upon additional supplementation must have been within 77–115 nM. Corresponding cell lines cultured without an additional selenite supplementation were treated as controls. All experiments were performed with cells between the 3rd and 6th passages.

2.3. Mitochondrial mass

Mitochondrial mass was determined with MitoTracker Green FM. Adherent cells were prepared and scanned on a multi-well plate in iCys laser scanning cytometer (CompuCyte Inc., Cambridge, MA, USA). Live cells were rinsed with PBS and incubated with 50 nM MitoTracker Green in the dark at 37 °C for 20 min argon laser (488 nm) was used to excite MitoTracker Green and the emitted green fluorescence was filtered by a 530 nm bandpass filter. In some experiments, cell nuclei were counterstained with 10 μM DRAQ5™. For this purpose, cells were incubated with the dye for 10 min at room temperature and fluorescence at 681 nm (excitation 488 nm) was monitored. A minimal area of 30 μm^2 was defined and 25 pixels were added to cover the entire cell. To collect data without staining of the nuclei, phantom counters were generated.

2.4. Western blotting

Whole cell extracts were obtained as described previously (Wojewoda et al., 2010). Equal amounts of protein were loaded onto a polyacrylamide gel, transferred onto a nitrocellulose membrane and blocked with 5% non-fat dry milk/TBS-0.05%. Subsequently, the blots were incubated with appropriate primary and secondary antibodies and developed with a chemiluminescence detection kit (Bio-Rad Laboratories, Munich, Germany) or Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

2.5. Immunocytochemical imaging and confocal image analysis

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed with 10% FBS/PBS and incubated for 1 h with primary antibodies in 10% FBS/PBS supplemented with 0.2% saponin. After washing three times with 10% FBS/PBS to remove unbound antibody, the cells were incubated for 1 hour with the appropriate fluorescently-conjugated secondary antibodies diluted in 10% FBS/PBS containing 0.2% saponin. Coverslips were washed three times with 10% FBS/PBS, once with PBS, and then mounted on a slide.

To visualise the distribution of mitochondria, cells were incubated with 100 nM MitoTracker CMXRos in the dark at 37 °C for 10 min. The cells were then rinsed with the medium and fixed. CMXRos is a cationic lipophilic dye, preferentially sequestered into the mitochondrial matrix. The dye contains a reactive chloromethyl moiety which forms covalent thioester bonds with thiols of proteins in the mitochondrial matrix.