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Mitochondrial respiratory chain dysfunction variably increases oxidant stress in *Caenorhabditis elegans*

Stephen Dingley [a], Erzsebet Polyak [a], Richard Lightfoot [b], Julian Ostrovsky [a], Meera Rao [a], Todd Greco [b], Harry Ischiropoulos [b], Marni J. Falk [a,*]

[a] Division of Human Genetics, Department of Pediatrics, The Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, PA 19104, USA
[b] Division of Neonatology, Department of Pediatrics, The Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, PA 19104, USA

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ABSTRACT

Mitochondrial dysfunction and associated oxidant stress have been linked with numerous complex diseases and aging largely by *in vitro* determination of mitochondria oxidant production and scavenging. We applied targeted *in vivo* fluorescence analyses of mitochondria-dense pharyngeal tissue in *Caenorhabditis elegans* to better understand relative mitochondrial effects, particularly on matrix oxidant burden, of respiratory chain complex, MnSOD, and insulin receptor mutants displaying variable longevity. The data demonstrate significantly elevated *in vivo* matrix oxidant burden in the short-lived complex I mutant, *gas-1*(*fc21*), which was associated with limited superoxide scavenging capacity despite robust MnSOD induction, as well as decreased mitochondria content and membrane potential. Significantly increased MnSOD activity was associated with *in vivo* matrix oxidant levels similar to wild-type in the long-lived respiratory chain complex III mutant, *isp-1*(*qm150*). Yet, despite greater superoxide scavenging capacity in the complex III mutant than in the significantly longer-lived insulin receptor mutant, *daf-2*(*e1368*), only the former showed modest oxidative stress sensitivity. Furthermore, increased longevity was seen in MnSOD knockout mutants (*sod-2*(*ok1030*) *and sod-2*(*gk257*)) that had decreased MnSOD scavenging capacity and increased *in vivo* matrix oxidant burden. Thus, factors beside oxidant stress must underlie RC mutant longevity in *C. elegans*. This work highlights the utility of the *C. elegans* model as a tractable means to non-invasively monitor multi-dimensional *in vivo* consequences of primary mitochondrial dysfunction.

1. Introduction

Oxidative stress resulting from increased production of reactive species and/or concomitant decline in antioxidant scavenging capacity may damage proteins, lipids, nucleic acids and other cellular structures (Valko et al., 2006). Such multi-faceted cellular damage may contribute to many sporadic (Schapira, 2006) and inherited (DiMauro, 2004) mitochondrial diseases and aging (Rea et al., 2007). Up to 2% of total oxygen consumed by the mitochondrial respiratory chain (RC) in *in vitro* studies, and 0.2% under more physiologic conditions (Balaban et al., 2005), has been shown to be improperly reduced to generate superoxide, which is then released into the matrix primarily from complex I (Murphy, 2009) or intermembrane space exclusively from complex III (Chen et al., 2003; Murphy, 2009). Superoxide dismutase (SOD) offers a primary oxidant defense through rapid conversion of superoxide radicals into hydrogen peroxide and oxygen. Three human SOD proteins localize to different subcellular compartments, where *SOD2* encodes the manganese SOD that functions within the mitochondria matrix.

Assessing the relative balance of oxidant production and scavenging *in vivo* remains a significant challenge (Yang et al., 2007). The development of small, lipophilic molecules that emit fluorescence only in the oxidized form has permitted a method of semi-quantitative evaluation of oxidant burden in cellular model systems. In particular, mitochondria-specific oxidant levels can be assessed using the fluorescent probe, MitoSOX Red, a lipophilic hydroethidine (HE) derivative that accumulates 100- to 1000-fold within mitochondria due to charge attraction of its triphenylphosphonium cation through the mitochondria membrane bilayers into the negatively-charged mitochondria matrix (Robinson et al.,

Abbreviations: *C. elegans*, *Caenorhabditis elegans*; MnSOD, manganese superoxide dismutase; RC, respiratory chain; PB, pharyngeal bulb.

* Corresponding author. Address: ARC 1002c, 3615 Civic Center Blvd, Philadelphia, PA 19104, USA. Tel.: +1 215 590 4564; fax: +1 267 426 2876.
E-mail addresses: stephen.dingley@gmail.com (S. Dingley), polyake@email.chop.edu (E. Polyak), lightfoot@email.chop.edu (R. Lightfoot), ostrovskyj@email.chop.edu (J. Ostrovsky), meerao@sas.upenn.edu (M. Rao), tgreco@mail.med.upenn.edu (T. Greco), ischirop@mail.med.upenn.edu (H. Ischiropoulos), falkm@email.chop.edu (M.J. Falk).

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2008). Mitochondria-generated oxidants react with MitoSOX to yield two primary fluorescent products, a 2-hydroxyethidium derivative (2-OH-Mito-E$^+$) resulting from superoxide oxidation, and Mito-E$^+$, a non-specific oxidized product (Zielonka et al., 2008). Reliable interpretation of fluorescence analyses that rely upon mitochondria membrane potential (DeltaPsi(m) or $\Delta\Psi_m$) for dye distribution may be subject to diminished or enhanced dye uptake, although several fluorescent dyes are now commonly used for *in vitro* $\Delta\Psi_m$ assessment (O'Reilly et al., 2003). Membrane potential indicator dyes have recently been applied *in vivo* in the model animal, *Caenorhabditis elegans* (Gaskova et al., 2007; Zuryn et al., 2008).

C. elegans offers a facile model in which to study a host of *in vivo* mitochondria functions. Nematodes incorporate ingested fluorescent dyes into their cells and are optically transparent, which permits precise assessment of tissue and cellular dye localization. Furthermore, several well-characterized *C. elegans* strains harboring mutations in nuclear genes encoding mitochondrial proteins are easily accessible (www.wormbase.org). However, assessing mitochondria functions by quantitative analysis of whole animal fluorescence may be limited by non-specific binding of lipophilic, fluorescence-based reagents, particularly within the lipid-rich granules of the gastrointestinal tract (Clokey and Jacobson, 1986). Thus, we sought to identify *C. elegans* tissues having high metabolic activity that might offer a focus for the further study of mitochondria physiology. *C. elegans* actively transports and grinds food (i.e., bacteria) between its mouth and intestines through a muscle-tube-like pharynx, which consists largely of anterior and terminal pharyngeal bulbs (PB) joined by an isthmus (Altun and Hall, 2008). The pharynx shares several similarities with the mammalian heart including near-continuous and intrinsic myogenic electrical activity (up to 250 beats per minute) (Avery and Horvitz, 1989), electrical coupling between muscle cells (Starich et al., 1996), calcium-based action potentials (Shtonda and Avery, 2005), and high mitochondria density (Altun and Hall, 2008). Capitalizing on these anatomical features and the existence of well-characterized *C. elegans* mutants, we evaluated the relative *in vivo* mitochondria oxidant production and mitochondria membrane potential ($\Delta\Psi_m$) in RC and insulin receptor mutants displaying variable longevity, as well as in MnSOD mutants with primary impairment of mitochondria superoxide scavenging capacity. We demonstrate that *C. elegans* offers a powerful model in which to assess the *in vivo*, multi-dimensional consequences of mitochondrial dysfunction, as well as explore the complex relationship between oxidant stress and longevity under varying genetic and environmental conditions.

2. Methods

2.1. Strains growth and maintenance

C. elegans worm strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN) (Table 1). Strains studied included wild-type (N2 Bristol), three mitochondrial RC mutants in complexes I, II, and III [*gas-1*(*fc21*), *mev-1*(*kn1*), *isp-1*(*qm150*), respectively], and the insulin receptor mutant (*daf-2*(*e1368*)). All superoxide scavenging (MnSOD) mutant strains studied harbored loss-of-function mutations, including two *sod-2* alleles [*sod-2*(*gk257*) is homozygous for a complex mutation characterized by an 18 basepair insertion and 159 basepair deletion; *sod-2*(*ok1030*) has a 900 basepair homozygous deletion] and a single allele of *sod-3* [*sod-3*(*gk235*) is homozygous for a complex rearrangement characterized by a six basepair insertion and 390 basepair deletion] (www.wormbase.org). Nematodes were grown at 20 °C on nematode growth media (NGM) plates spread with OP50 *Escherichia coli*. For all fluorescent studies, synchronous young adult populations were obtained by bleaching gravid adults on NGM plates (Hope, 1999), plating the recovered eggs onto unspread NGM plates overnight, and then transferring L1-arrested animals the next day to NGM plates spread with OP50 *E. coli*. Upon reaching adulthood (defined by the presence of eggs laid on the plate), nematodes were washed off in S. basal (3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 5.85 g NaCl, 1 l water, pH 7.0) and adults were separated from eggs by gravity. For mass spectrometry study of whole worm aliquots (see below) as well as for mitochondria isolation, nematodes were grown at 20 °C on 20 large NGM plates spread with OP50 *E. coli* for two generations, and then grown in 1 l liquid culture flasks with K12 *E. coli* at 200 rpm in 20 °C incubators (I26/R Series, New Brunswick Scientific, Edison, NJ). Worms were harvested from liquid culture, as previously described (Falk et al., 2006; Hope, 1999).

2.2. Lifespan assessment

Animals were maintained at 20 °C throughout the experiment. Synchronized nematode cultures were initiated by bleaching young adults to obtain eggs. Collected eggs were allowed to hatch overnight on 10 cm unspread NGM plates, after which L1-arrested larvae were transferred to 10 cm NGM plates spread with OP50 *E. coli*. Upon reaching the first day of egg laying, synchronous young adults were moved to fresh 3.5 cm NGM plates seeded with OP50 *E. coli* (lifespan experiment "Day 0"). For purposes of higher-throughput lifespan screening by preventing offspring from nema-

Table 1
C. elegans strain description, homology, and lifespan. The role of *in vivo* oxidant stress on longevity was studied in three RC subunit missense mutants, three MnSOD knockout mutants, and an insulin receptor missense mutant. FUDR-based median lifespan assessment was largely consistent with previously reported lifespan findings in these strains.

C. elegans strain name	Gene function (human homolog)	Mutation type	Human-worm protein similarity (%)	Lifespan (20 °C)			
				Interpretation	Median	Mean	Maximum
N2 Bristol	–	Wild-type	–	vs. RC Mutants	14	16.9	21
				vs. MnSOD mutants*	17	19.9	23
gas-1 (*fc21*)	Complex I subunit (*NDUFS2*)	Missense	83.4	Short-lived	12	15.6	20
mev-1 (*kn1*)	Complex II subunit (*SDH-C*)	Missense	75.3	Unchanged	14	16.4	22
isp-1 (*qm150*)	Complex III subunit (*UQCRFS1*)	Missense	97.5	Long-lived	20	22.4	31
daf-2 (*e1368*)	Insulin receptor (*IR*)	Missense	62.0	Long-lived	31	31.1	38
sod-2 (*gk257*)	Mitochondrial MnSOD (*SOD2*) – *C. elegans* chr. X	Knockout	97.3	Long-lived*	19	20.7	24
sod-2 (*ok1030*)	Mitochondrial MnSOD (*SOD2*) – *C. elegans* chr. X	Knockout	97.3	Long-lived*	20	21.6	25
sod-3 (*gk235*)	Mitochondrial MnSOD (*SOD2*) – *C. elegans* chr. I	Knockout	97.2	Long-lived*	18.5	20.0	23

All other strains were concurrently studied with the unmarked wild-type (N2 Bristol) control. RC, respiratory chain. Lifespan plots and animal number studied for each mutant are provided in Supplementary Fig. S4.
* Indicates concurrent wild-type (N2 Bristol) control for MnSOD mutant lifespan comparison.