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Pyrroloquinoline quinone enhances the resistance to oxidative stress and extends lifespan upon DAF-16 and SKN-1 activities in *C. elegans*

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ABSTRACT

Pyrroloquinoline quinone (PQQ) is linked to fundamental biological processes such as mitochondrial biogenesis and lipid metabolism. PQQ may also function as an essential micronutrient during animal development. Recent studies have shown the therapeutic potential of PQQ for several age-related diseases due to its antioxidant capacity. However, whether PQQ can promote longevity is unknown. Here, we investigate the effects of PQQ on oxidative stress resistance as well as lifespan modulation in *Caenorhabditis elegans*. We find that PQQ enhances resistance to oxidative stress and extends the lifespan of *C. elegans* at optimal doses. The underlying molecular mechanism involves the increased activities of the primary lifespan extension transcriptional factors DAF-16/FOXO, the conserved oxidative stress-responsive transcription factor SKN-1/Nrf2, and upregulation of *daf-16*, *skn-1* downstream targets including *sod-3*, *hsp16.2*, *gst-1* and *gst-10*. Our findings uncover a novel role of PQQ in longevity, supporting PQQ as a possible dietary supplement for overall health improvement.

1. Introduction

Pyrroloquinoline quinone (PQQ) is an aromatic heterocyclic anionic orthoquinone first recognized as an enzyme cofactor in bacteria (Rucker et al., 2009). Trace levels of PQQ have also been found in human and rat tissue, and human milk (He et al., 2003). PQQ functions in a variety of basic physiological processes in mammals as an essential micronutrient (Akagawa et al., 2015). Rodents fed with a PQQ-deficient diet exhibit developmental abnormalities, compromised immune responsiveness and impaired reproductive performance (Rucker et al., 2009; Steinberg et al., 1994). PQQ also interacts with JAK/STAT and Ras-related signalling pathways, leading to upregulation of genes involved in mitochondrial biogenesis and metabolism (Kumazawa et al., 2007; Tchaparian et al., 2010). As well as physiological roles, PQQ is well known for its redox cycling potential, and on a molar basis >100 times more efficient than ascorbic acid, menadione and typical polyphenolic compounds in assays that measure redox cycling potential (Stites et al., 2000). PQQ works in a concentration-dependent manner as an antioxidant. In Jurkat cell experiments up to 10 μM, PQQ works predominantly as an antioxidant, while beyond 50 μM PQQ acts as a pro-oxidant. With regard to pro-oxidant potential, PQQ interacts with the cell culture medium to generate hydrogen peroxide and induce cell death (He et al., 2003; Misra et al., 2012). At optimal concentrations, PQQ is considered to be an antioxidant and shows potential to protect living cells from oxidative damage *in vivo* and protects biomolecules from artificially produced reaction oxygen species *in vitro* (Misra et al., 2012; Nunome et al., 2008). Due to the prominent health enhancement traits of PQQ, many studies have suggested a protective effect of PQQ on cardiovascular diseases and neurodegenerative diseases (Xu et al., 2014; Zhang et al., 2014), including the potential for improving cognitive deficit (Ohwada et al., 2008) as well as decreasing hyperlipidaemia and age-related oxidative stress (Singh et al., 2015).

In mammals, increased resistance to oxidative stress is associated with decreased aging-related diseases and enhanced longevity. Mitochondria are intimately involved in the relationship between aging and oxidative stress, partly due to their critical role in the generation of reactive oxygen species (ROS) from the electron transport chain (Dai et al., 2014). The mitochondrial-free radical theory of aging proposes that aging is caused by damage to macromolecules by mitochondrial ROS. At moderate levels, ROS act as second messengers in different cellular functions and have a prominent effect on cell signalling and homeostasis (Droge, 2002). However, the toxicity of ROS at high levels induces the accelerating senescence and mortality that characterize aging (Jurk et al., 2014). Reducing oxidative damage can extend the lifespan in yeast, *Drosophila* and in mice (Fontana et al., 2010; Perez et al., 2009a). In contrast, the inactivation or overexpression of antioxidant activities in several genetically engineered organisms fails to produce outcomes that support the mitochondrial-free radical theory of aging (Muller et al., 2007; Perez et al., 2009b). Hence, given that mitochondria were one of the targets of PQQ and accumulating evidence showed that PQQ enhances the resistance to oxidative stress *in vitro* and *in vivo*

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(Nunome et al., 2008; Ohwada et al., 2008), we sought to determine whether PQQ could extend the lifespan of *Caenorhabditis elegans*.

Here, we take advantage of an invertebrate model *C. elegans* to investigate the potency of PQQ in oxidative stress resistance and in lifespan regulation. *C. elegans* is a small free-living soil nematode widely used as a model organism in the study of aging owing to its unique features such as a fully sequenced genome, short lifespan (2–3 weeks) and life cycle (3 days), and ease of propagation of individuals in a population (Hertweck et al., 2003). Significant progress has been made by employing this simple model organism to delineate the genetic and biochemical pathways involved in aging and identify therapeutic interventions in healthy lifespan extension (Antebi, 2007). One of the first pathways shown to modulate the lifespan in animals was the insulin/IGF-1 (IIS) pathway (IIS) (Kenyon, 2010). Reports demonstrated that inhibition of the IIS pathway enhanced the resistance to oxidative stress, which is the determinant of longevity (Holzenberger et al., 2003). Specifically, restricted IIS signalling extends lifespan and promotes health through the core transcriptional factor forkhead box O transcription factor DAF-16, and its interaction with additional transcriptional factors in the nucleus such as heat shock transcription factor HSF-1 and Nrf-like xenobiotic-response factor SKN-1 (Kenyon, 2010; Henderson and Johnson, 2001). Here, we determine whether PQQ can increase the resistance to oxidative stress, and whether this affects lifespan in *C. elegans*. We demonstrate that PQQ facilitates the nematode's resistance to oxidative damage and increases the lifespan at optimal doses *via* a DAF-16 and SKN-1 dependent pathway.

2. Material and methods

2.1. C. elegans strains and maintenance

The following strains in this study were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota): wild-type N2, TJ356 zIs356 [daf-16:GFP], CF1038 daf-16(mu86), CF1553 muIs84 [pAD76(sod-3::GFP)], EU31 skn-1(zu135) IV/nT1 [unc-?(n754) let-?](IV; V). Worms were maintained at 20 °C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50. PQQ (Shanghai Med Co., Ltd, China; Zhucheng Haotian Pharm Co., Ltd; the purity of the compound >98% by HPLC) was added directly to the NGM agar media as a supplement to a final concentration of 4, 2, 1, 0.5 or 0.25 mM.

2.2. Oxidative stress and heat stress assays

Wild-type N2 *C. elegans* were used to validate the potential antioxidant activity of PQQ. In an H_2O_2-induced stress assay, age-synchronous late L4 worms were pretreated with several doses of PQQ (4, 2, 1, 0.5, 0 mM) in liquid growth medium until day 4 of adulthood, after which the worms were subjected to oxidative stress with H_2O_2 (16 mM) in S basal buffer for 4 h, or paraquat (10 mM) for 5 days in liquid growth medium, or acute heat stress at 35 °C for 12 h. The worms were washed three times with S basal buffer before the stressors were added. Mortality was counted at the end time points for the oxidative stress assay. In the heat stress assay, survivals were counted every 2 h once the experiment commenced. With regard to scoring for oxidative and heat stress resistance capacity, we counted paralysed worms as dead. The final concentration of 50 μM 5-FU was added to the medium to block progeny development.

2.3. Measurement of general reactive oxygen species (ROS)

4-day-old worms with or without 4 days of PQQ treatment were transferred into each well of a black 96-well plate containing 100 μL S basal buffer. 50 μM DCFH-DA (Beyotime, China) was then added to each well. ROS-associated fluorescence levels were measured after 2 h incubation with DCFH-DA, using a fluorescence microplate reader (Tecan Infinite 200 PRO) at 485 nm excitation and 520 nm emission. A final concentration of 50 μM 5-FU was added to the medium to block the progeny development.

2.4. Lifespan analysis

Synchronized late L4 stage worms were placed on PQQ-containing plates with 50 μM 5-FU to block progeny development. The worms were transferred onto fresh plates every other day before the age of 10 days. Thereafter, the worms were counted every other day until death. Those that failed to respond to mechanical touch were scored as dead and were removed from the plates. Worms that died from crawling off the agar were censored from analysis.

2.5. DAF-16 nuclear translocation quantification

TJ356, pretreated with 0, 0.5 or 1 mM PQQ for 4 days from late L4 larva stage, were first exposed to 0 or 1.6 mM H_2O_2 for 3 h then immobilized with azide sodium and mounted on 2% agarose pads on a glass slide. Image acquisition was performed using Motic Pro 205B and Motic Image Advanced 3.2 software. As previously described (Ray et al., 2014), worms were scored as cytosolic, intermediate or nuclear. A final concentration of 50 μM 5-FU was added to the medium to block progeny development.

2.6. Fluorescence quantification of sod-3::GFP

The sod-3::GFP reporter CF1553 was employed. Worms were subjected to PQQ treatments from late larva 4 stage for 48 h and then transferred onto a 96-well plate with 100 worms in 100 μL S buffer per well. Total GFP fluorescence was read by a Tecan Infinite 200 PRO scanner with 485 nm excitation and 530 nm emission. A final concentration of 50 μM 5-FU was added to the medium to block progeny development.

2.7. RNA isolation and quantitative real-time PCR (qPCR) analysis

daf-16, *daf-2*, *sod-3*, *skn-1*, *gst-1*, *gst-10* and *hsp-16.2* mRNA levels were measured in wild-type N2 treated with PQQ for 4 days starting from L4 stage. A final concentration of 50 μM 5-FU was added to the medium to block progeny development. Total RNA was extracted with a ZR RNA Microprep kit (ZYmo RESEARCH, USA), according to the manufacturer's instructions. RNA was quantified with a Nano Drop (Thermo, USA). RT Mastermix (Abm, USA) was used to synthesize cDNA. The cDNA product was stored at −20 °C until use. As previously reported (Hoogewijs et al., 2008), reference gene *ama-1* was set as the internal control. Quantitative real-time PCR (qPCR) was performed using the KAPA SYBR® 2× qPCR Master Mix, according to the manufacturer's instructions, on a Bio—Rad Miniopticon real-time PCR system using cDNA. The primers were as follows: *daf-16*, Forward: 5′-GCGAATCGGTTCCAGCAATTCCAA-3′ and Reverse: 5′-ATCCACGGACACTGTTCAACTCGT-3′; *daf-2*, Forward: GGCCGA TGGACGTTATTTTG and Reverse: TTCCACAGTGAAGAAGCCTGG; *skn-1*, Forward: 5′-AGTGTCGGCGTTCCAGATTTC-3′ and Reverse: 5′-GTCGACGA ATCTTGCGAATCA-3′; *gst-1*, Forward: 5′-CCCTCAAGCTCACGTACTTC-3′ and Reverse: 5'AGCGAGATGACGGATGATAG-3′; *gst-10*, Forward: 5′-AAGAGATTGTGCAGACTGGAG-3′ and Reverse: 5′-AGAACATGTCGAGG AAGGTTG-3′; *hsp-16.2*, Forward: 5′-CTATTTCCGTCCAGCTCAACGT-3′ and Reverse: 5′-GTTAACAATCTCAGAAGACTCAGATGGAG-3′; *sod-3*, Forward: 5′-AGCATCATGCCACCTACGTGA-3′ and Reverse: 5′-CACCACCATTGAAT TTCAGCG-3′; *ama*, Forward: 5′-CCTACGATGTATCGAGGCAAA-3′ and Reverse: 5′-CCTCCCTCCGGTGTAATAATG-3′. Relative-fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

3. Statistical analysis

The results of the lifespan experiments were analysed using Kaplan-Meier survival analysis, and compared among groups scoring