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Original Contribution

The lipophilic antioxidants α-tocopherol and coenzyme Q_{10} reduce the replicative lifespan of *Saccharomyces cerevisiae*

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ABSTRACT

Reactive oxygen species contribute to cellular ageing and an increased level of oxidative stress is often associated with ageing in many organisms. Supplementation of antioxidants has been advocated to decrease cellular oxidative stress and potentially extend lifespan. A genetically modified K6001 strain of *Saccharomyces cerevisiae* was employed to determine the effect of several antioxidants, including D-erythroascorbic acid, α-tocopherol and coenzyme Q_{10} on yeast cell replicative ageing. The replicative lifespan of the K6001 strain was assessed by absorbance change as cells exhibited a linear growth in glucose medium. In this study, water-soluble D-erythroascorbic acid had little effect on cell replicative lifespan. However, supplementation of the growth medium with the lipophilic antioxidants α-tocopherol increased oxidative stress and decreased cell lifespan. The use of α-tocopherol analogues revealed that the antioxidant activity and the membrane retention ability of α-tocopherol were involved in the lifespan reduction effect. Supplementation with either coenzyme Q_{10} alone, or in combination with α-tocopherol also led to a reduction in yeast replicative lifespan. This study highlights a potential pro-oxidant action of antioxidants.

Introduction

The free radical theory of ageing hypothesizes that oxidative damage induced by reactive oxygen species (ROS) is a major factor contributing to progression of cell ageing [1]. Oxygen acts as electron acceptor in aerobic metabolism to produce ATP via oxidative phosphorylation. However, inevitably, a small proportion of electrons leaks from the electron transport chain and these react with oxygen to generate ROS including the superoxide anion radical, which can lead to generation of hydrogen peroxide, peroxyl and hydroxyl radicals [2]. ROS contribute to the damage of cellular components, including proteins, lipids and DNA that may lead to the impairment of cellular functions. To protect against ROS damage, cells possess numerous antioxidative defenses, which scavenge ROS to prevent cellular damage or remove already damaged components preventing further cellular impairment. Nevertheless, there is a limitation to the capacity of cellular antioxidative defenses and cells experience oxidative stress when ROS generation overwhelms these defenses.

In model organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, a reduction in cellular antioxidative defenses is observed as cells age [3]. For *S. cerevisiae*, genetic and environmental changes that increase the ROS burden by deletion of genes encoding superoxide dismutases [4,5] or catalases [6] lead to a shortening of the life span of mother cells. Old mother cells of *S. cerevisiae* approaching senescence have a markedly increased burden of ROS [7]. Conversely, increasing antioxidant defenses delays ageing by the lowering of oxidative stress [6,8–10]. Some of the "longevity genes" of the nematode *C. elegans* have been shown to play a role in detoxification of oxygen radicals [11,12] indicating the importance of antioxidant defenses during ageing.

A variety of naturally occurring non-enzymatic antioxidants have evolved to increase protection against ROS in conjunction with cellular antioxidative defenses. α-Tocopherol belongs to the group of tocopherols representative of vitamin E and exhibits the highest antioxidant activity of this vitamin [13]. It acts as a chain-breaking antioxidant by scavenging chain-initiating and -propagating radicals. Reaction of α-tocopherol with radicals leads to the formation of the α-tocopheroxyl radical. The effectiveness of α-tocopherol in preventing stress-induced damage has been demonstrated. For example, supplementation of α-tocopherol abrogates ultraviolet radiation-induced ROS levels in fibroblasts [14]. Dietary supplementation of α-tocopherol alleviated oxidative stress and reduces mortality rate of *D. melanogaster* under hyperoxic conditions [15]. Indeed, α-tocopherol has been proposed to increase lifespan in model organisms [16] by means of increasing antioxidative protection against ROS. However, the beneficial effect of α-tocopherol on cellular ageing remains controversial [17,18].

Abbreviations: ROS, reactive oxygen species; ATP, adenosine triphosphate; CoQ, coenzyme Q; SC, synthetic complete; YEPD, yeast extract peptone dextrose; DHE, dihydroethidium; PMC, 2,2,5,7,8-pentamethyl-6-hydroxuchromane.

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Coenzyme Q is composed of a fully substituted benzoquinone 'head' group and a polyisoprenoid side chain. It is known best for its function in transferring electrons from complexes I and II to complex III in the mitochondrial respiratory chain. Its head group can alter between three redox states: (i) the fully oxidized (ubiquinone); (ii) the univalently reduced (ubisemiquinone) and (iii) the fully reduced (ubiquinol). The polyisoprenoid side chain of coenzyme Q facilitates its position in the hydrophobic membrane lipid bilayer. In higher vertebrate and mammalian cells, the side chain consists of 9 or 10 isoprenoid subunits. Coenzyme Q has both antioxidant and pro-oxidant properties. Among its antioxidant activities, ubiquinols regenerate α-tocopherol from its tocopheroxyl radicals [19,20]. In mammalian species, total tissue content of coenzyme Q does not correlate with maximum lifespan, although the relative content of CoQ_9 and CoQ_{10} may be important [21], for presently poorly understood reasons.

Vitamin C (L-ascorbic acid) is a water-soluble antioxidant, which has a broad spectrum of antioxidant activity due to its reactivity with different aqueous free radicals and ROS. L-ascorbic acid is produced in all higher plants and almost all animals, except humans, other primates, guinea pig and some birds [22–24]. Although the biosynthesis of L-ascorbic acid is absent or rare in microorganisms, a five-carbon analogue, D-erythroascrobic acid is present in *S. cerevisiae* [25,26].

The unicellular eukaryote *S. cerevisiae* displays characteristic markers associated with cellular ageing, and many of the pathways that influence yeast cell ageing are conserved with those involved in mammalian cell ageing [27]. The lifespan of *S. cerevisiae* can be defined by the numbers of generations that an individual mother cell undergoes before reaching senescence, known as the replicative lifespan. Yeast replicative lifespan has a limited potential with loss of cell reproductive capacity occurring when mother cells reach a threshold age. Although on average yeast cells divide for approximately 25 generations, the average lifespan can vary between strains. Replicative ageing is asymmetrical, with newly born daughter cells regaining their full potential lifespan regardless of the age of their mother [28,29], although daughter cells produced by very old mother cells have a shortened replicative lifespan due to the loss of asymmetrical division [30]. In yeast, ROS accumulate in aged mother cells [7] and oxidatively damaged proteins are distributed asymmetrically with daughter cells having reduced damaged proteins relative to mother cells [31].

In this study, we aimed to determine the effect of exogenous antioxidants, D-erythroascorbate and α-tocopherol on yeast replicative lifespan in the absence of additional stress. We demonstrate that α-tocopherol decreases yeast replicative lifespan, possibly via a pro-oxidant activity.

Materials and methods

Yeast strains and media

Saccharomyces cerevisiae strain K6001 in the strain W303 background has genotype: *MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, GAL, psi+, ho::HO::CDC6 (at HO), cdc6::hisG, ura3::URA3 GAL-ubiR-CDC6* (at *URA3*) [32,33]. The haploid strain BY4741 has genotype *MATa, his3Δ1, leu2Δ0, met15Δ0, lys2Δ0, ura3Δ0.*

Cultures were grown in synthetic complete (SC) medium (2% w/v glucose or 2% w/v galactose, 0.5% ammonium sulfate, 0.017% yeast nitrogen base without amino acids) supplied with appropriate amino acid supplementation. YEPD contained 2% (w/v) glucose, 2% (w/v) peptone and 1% yeast extract. Media were solidified by adding 2% (w/v) agar. α-Tocopherol (Sigma Aldrich) or α-tocopherol acetate (Sigma Aldrich) was dissolved in ethanol and added as less than 1% of total cell culture volume. Trolox (Sigma Aldrich) and PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane) (Sigma Aldrich) were prepared in water. For solid medium, α-tocopherol was added to molten agar cooled to 49 °C prior to pouring plates. The K6001 strain was grown exponentially in SC medium supplemented with galactose (SC galactose) and grown linearly in SC supplemented with glucose (SC glucose). The BY4741 strain was grown in SC glucose. Cells were grown at 30 °C with aeration.

Replicative lifespan determination

The replicative lifespan of the modified strain K6001 was estimated by measuring the change in its growth following transfer from galactose- to glucose-containing medium. K6001 cells were grown to exponential phase ($OD_{600\ nm}$ 0.5) in SC galactose medium for propagation. Cells were washed with sterile water to remove residual galactose and transferred into SC glucose medium. K6001 grows linearly in SC glucose medium until mother cells undergo senescence [33]. To determine the replicative lifespan of K6001, optical density was measured at 600 nm at 3 h intervals from an initial $OD_{600\ nm}$ 0.01. Different concentrations of α-tocopherol or α-tocopherol analogues were added to SC glucose medium. Cells were aerated and cultured at 30 °C.

Replicative lifespan of the wild type BY4741 strain was assayed by conventional micromanipulation [34]. 90-110 Virgin mother cells were positioned on the matrix of an SC glucose agar plates containing α-tocopherol. Newly produced daughter cells were removed from the mother cells at each cell division cycle by micromanipulation. The number of daughter cells removed was recorded, representing the number of generations that mother cells had undergone. Agar plates for the manipulation assay were stored at 4 °C overnight preventing the over-growth of mother cells.

Cell viability

Cells were treated with different concentrations of α-tocopherol or α-tocopherol analogues for 16 h in liquid SC glucose medium. To determine cell viability, total numbers of cells were counted using a hemocytometer, and cells were then plated on YEPD agar plates for recovery after each treatment.

Measurement of reactive oxygen species and lipid peroxidation

Cells were grown in SC glucose and treated with different concentrations of α-tocopherol or ubiquinone for 4 h and 20 h to determine the short- and long-term effects of the antioxidant. Cellular levels of ROS were assessed by dihydroethidium (DHE), which is oxidized to fluorescent ethidium upon exposure to ROS, especially superoxide anion [35]. Cells were washed with PBS and treated with 0.5 μL DHE (1 mg/mL) for 10 min at room temperature in the dark, before fluorescence was measured at 633 nm. Cellular lipid oxidation was assessed by C11-Bodipy (Molecular Probe), a lipophilic probe, localizes to cellular membranes and changes its emitted fluorescence from red to green upon oxidation [36]. Fluorescence was measured at 520 nm using a flow cytometer FACSorter.

Results

α-Tocopherol reduces the replicative lifespan of the K6001 strain

α-Tocopherol is a potent antioxidant *in vitro*, however, its effect on cell ageing remains controversial. The effect of increasing concentrations of added α-tocopherol on yeast cell ageing was determined using the modified strain K6001. This strain can grow exponentially on galactose-containing medium, but only mother cells can grow in glucose-containing medium. The linear increase of the mother cell population following the shift to glucose-containing medium provides an estimate of the replicative lifespan of the population of cells and its use has been validated under a range of conditions [33]. This assay has