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Ascorbate Restores Lifespan of Superoxide-dismutase Deficient Yeast

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Yeast (*Saccharomyces cerevisiae*) mutants lacking CuZn-superoxide dismutase (CuZnSOD) are hypersensitive to oxygen and have significantly decreased replicative life span. Both these defects can be ameliorated by exogenous ascorbate. The effect of ascorbate on life span is complicated by auto-oxidation of its compound in the medium. If negative effects of auto-oxidation are prevented by exchange of the medium, ascorbate prolongs not only mean but also maximal replicative life span of the yeast in the atmosphere of air and of pure oxygen. These results demonstrate that life span shortening due to the lack of a vital antioxidant enzyme can be ameliorated by a low-molecular weight antioxidant.

Keywords: Yeast; *Saccharomyces cerevisiae*; Superoxide dismutase; Free radicals; Oxidative stress; Aging

INTRODUCTION

We have isolated the *Saccharomyces cerevisiae* mutants devoid of CuZn-superoxide dismutase (CuZnSOD) for the first time and demonstrated that they are impaired in their ability to withstand exposure to pure oxygen and to superoxide-generating agents and show auxotrophy for lysine and methionine when grown under air.[1] Our subsequent studies have shown that deficiencies in superoxide dismutases (both Cu,ZnSOD and MnSOD) result in substantial shortening of the replicative life span of yeast cells. The negative effects of single mutations in the genes coding for both dismutases are additive.[2] Other studies confirmed that deletion of CuZnSOD decreases the life span of the yeast and pointed out (in contrary to our data) that deletion of catalase may have a similar effect.[3–5] These results suggest that superoxide radical and other reactive oxygen species (ROS) generated secondarily are important factors contributing to lethality of oxygen at high partial pressures and aging, although the role of ROS in yeast aging is not unequivocal.[6] If this premise is true, the effect of SOD deficiency should be ameliorated by antioxidants able to scavenge ROS. The aim of this study was to examine if exogenous ascorbate was able to protect SOD-deficient cells against oxygen toxicity and normalise their replicative life span.

MATERIAL AND METHODS

Yeast Strains

The following yeast strains were used: wild-type SP-4 (MATα leu1 arg4),[7] strain DSCD1-1C devoid of CuZnSOD (strain MATα leu1 arg4 sod1), isogenic to SP-4,[1] a strain devoid of Mn SOD, MnSOD$^-$ (MATα leu2-3,112, his3-11,15 ura3-251,372,328 lac2, sod2# LEU) and its isogenic parent strain DL 1 (MATα leu2-3,112, his3-11,15 ura3-251,372,328 lac2),[8] both obtained from Dr G. Schatz (Biozentrum, Basel).

Media and Growth Conditions

Yeast were grown in a standard liquid YPGlucose medium (1% Difco yeast extract, 1% yeast bacto-peptone, 2% glucose) on a rotary shaker for about 20 h at 28°C until the logarithmic phase was attained ($1–5 \times 10^7$ cells/ml) and were then transferred to

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a solid medium for lifespan determination. In studies of growth kinetics, yeast growth was monitored by determination of turbidity of the cultures at 700 nm. Stock solution of ascorbate sterilised by filtration was added to liquid media immediately before inoculation with yeast cells, and to the agar-containing medium just before pouring into Petri dishes, when its temperature was about 50°C.

Determination of Replicative Life Span

Replicative life span of individual yeast cells were determined by a routine procedure[9] on cells placed on agar plates containing YPGlucose medium with appropriate concentration of ascorbate. Briefly, a fresh bud was isolated by a micromanipulator and formation of successive buds by such a virgin cell was monitored, each appearing bud was being removed using a Narishige MO-202 hydraulic micromanipulator. The number of buds formed by each cell is referred to as its replicative life span, characterised by *mean number of generations* (average number of buds formed by the cells studied) and *maximal number of generations* (number of buds formed by the most viable cell in the population studied). In contrast to the original protocol,[9] the plates were not placed in a refrigerator overnight, to avoid the stress connected with cooling and warming[10] and possible induction of antioxidant enzymes by lowered temperature.[11] In experiments involving exchange of the medium (results shown in Fig. 4), strips of the plate were cut out and replaced by freshly made solid medium containing ascorbate; and budding cells were transferred on the new medium. The time between the appearance of successive buds was monitored and its mean value (mean for all buddings of all cells studied) was referred to as *mean generation time*. The product of the mean number of generations and mean generation time is referred to as *mean chronological life span*.

Estimation of Superoxide Level in Yeast Cells

The level of superoxide production was estimated by measurement of dihydroethidine oxidation.[12] Cells were washed and suspended in 100 mM phosphate buffer, pH 7.0, with 0.1% glucose and 1 mM EDTA, to the density of 1×10^8 cells/ml and added with dihydroethidine (Molecular Probes, OR, USA; 18.9 μM). The kinetics of fluorescence increase was measured using a Hitachi F2500 fluorescence spectrophotometer. Measurement conditions were: $\lambda_{ex} = 518$ nm, $\lambda_{em} = 605$ nm, temperature at 28°C.

All results represent mean ± SD from at least three independent experiments. Evaluation of statistical significance of differences was performed using the Student's *t* test.

RESULTS

CuZnSOD-deficient yeast is hypersensitive to oxygen. Exposure of wild-type SP-4 strain to atmosphere of 100% oxygen for 24 h did not affect yeast survival while cells of the CuZnSOD$^-$ strain (1C) did not survive such exposure. Interestingly, the MnSOD$^-$ mutants were not so sensitive to oxygen (survival of 74% after 24 h exposure to an atmosphere of 100% oxygen, not shown). The presence of ascorbate in the medium had a concentration-dependent protective effect on the survival of the 1C strain, which became close to that of the wild-type strain at an ascorbate concentration of 60 mM (Fig. 1).

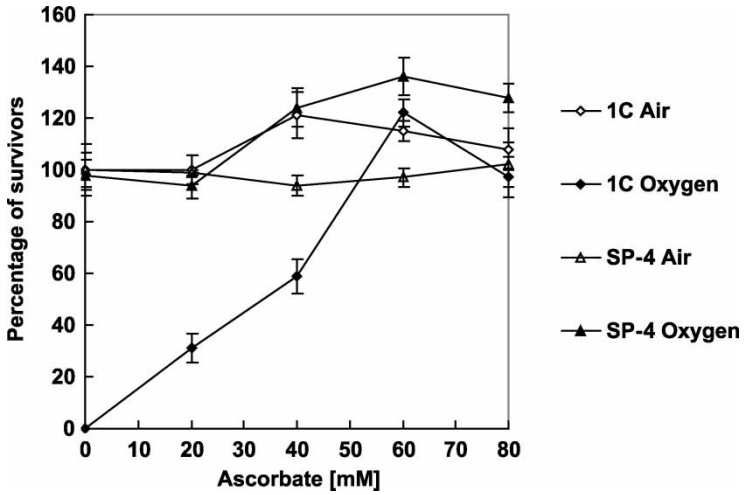

FIGURE 1 Effect of ascorbate on the survival of wild-type (SP-4) and CuZnSOD$^-$ (1C) strains of *S. cerevisiae*. Diluted liquid yeast cultures of known concentration were applied on solid YPGlucose medium and exposed to pure oxygen for 24 h at 28°C. Then the plates were equilibrated with air and number of colonies formed were compared with that on control plates exposed to the atmosphere of air only. Mean values ± SD from four independent experiments. $P < 0.01$ for the effect of ascorbate on the survival of the 1C strain under oxygen, at all concentrations were tested.

The lack of CuZnSOD leads also to diminution of the replicative life span of the yeast.[2] Ascorbate corrected this defect increasing the mean (though not maximal) number of generations of the CuZnSOD$^-$ mutant in the atmosphere of air (Fig. 2A). No discernible effect of ascorbate was seen under anoxic conditions (under the atmosphere of nitrogen). In the atmosphere of pure oxygen, when the replicative life span of the mutant is shortened dramatically and ascorbate increased not only the mean but also the maximal number of generations considerably (Table I). In the case of the MnSOD$^-$ mutant, the effect of ascorbate was very small even in the atmosphere of pure oxygen (Fig. 3), apparently due to the inability of this compound to penetrate mitochondria, expected to be the main site of cellular damage in this mutant.

Another replicative parameter affected by the CuZnSOD$^-$ mutation and restored by addition of ascorbate was the generation time. This time was much longer in the CuZnSOD$^-$ than in the wild-type strain; the increase was attenuated by addition of ascorbate (Table I). Ascorbate had no significant effect on the life span of the wild-type strain (Fig. 2B)

In order to check for the possible adverse effects of ascorbate auto-oxidation in the course of the experiment, the influence of ascorbate on the replicative life span of the yeast was studied under conditions of exchange of the solid medium for freshly prepared one of every two buddings. Change of medium resulted in a significant prolongation of not only the mean but also of maximal number of generations of yeast cells by 80 mM ascorbate under the atmosphere of air (Fig. 4).

The rate of dihydroethidine oxidation, a measure of the level of production of ascorbate able to react with an exogenous substrate, was higher in the CuZnSOD$^-$ mutant than in the wild-type strain. Ascorbate decreased the rate of oxidation of dihydroethidine close to the baseline level measured under anoxic conditions, the magnitude of the effect being bigger in the CuZnSOD$^-$ mutant (Fig. 4).

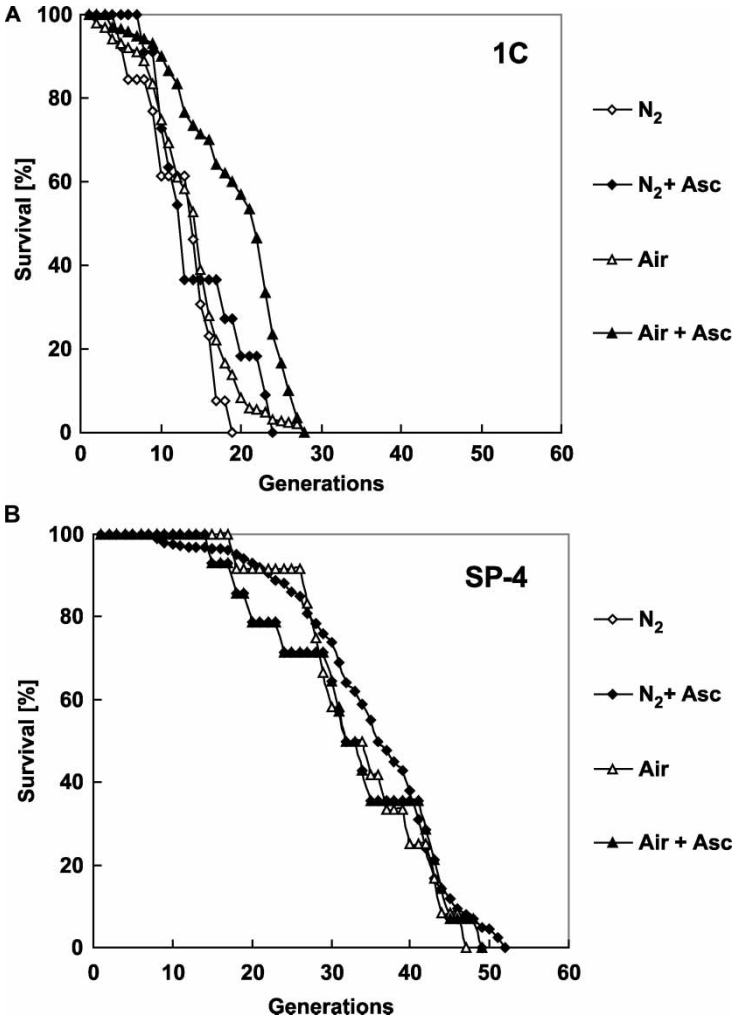

FIGURE 2 Effect of ascorbate (80 mM) on the replicative life span of: (A) ZnSOD- (1C) and (B) wild-type (SP-4) strains of *S. cerevisiae* under the atmosphere of air and in anoxia. Results shown are mean values of 40 observations.

TABLE I Effect of ascorbate on the replicative life span, generation time and mean chronological life span of the CuZnSOD$^-$ strain of *S. cerevisiae*. The data are mean ± SD from observations on 40–60 individual cells

Strain	1C				SP-4	
Atmosphere/Ascorbate [mM]	Air/0	Air/80	Oxygen/0	Oxygen/80	Air/0	Oxygen/0
Mean number of generations	13 ± 2	19 ± 2*	1.0 ± 0.2	12 ± 1*	34 ± 3	31 ± 5†
Maximal number of generations	27	27	2	15	51	43
Mean generation time [min]	121 ± 5	99 ± 3*	201 ± 19	103 ± 4*	89 ± 2	97 ± 5†
Mean chronological life span [h]	26 ± 5	31 ± 4*	4 ± 1	21 ± 3*	51 ± 6	51 ± 8

* $P < 0.001$ (effect of ascorbate). $^\dagger P < 0.001$ (oxygen vs. air).

DISCUSSION

The primary consequences of the lack of SOD in the yeast cell are: an increase in the steady-state level of the superoxide radical anion and increased rates of superoxide-dependent reactions. These phenomena lead to a considerable decrease of the replicative life span of the yeast which seems to be a direct confirmation of the validity of the free radical theory of aging.[13] If so, one should expect attenuation of the life span shortening by introduction of another antioxidant able to scavenge superoxide and/or ROS derived from it, or chemically repair oxidative damage. The data presented here demonstrate that such an effect can indeed be observed for ascorbate. The protective effects of ascorbate include the mean and maximal replicative life span of yeast cells under hyperoxic and normoxic conditions.

A perplexing facet of the shortening of the life span of *S. cerevisiae* differs by deletion of CuZnSOD is the persistence of this effect under anoxia.[6] Ascorbate compensated for the effect of lack of CuZnSOD in the presence of oxygen but was ineffective under the atmosphere of nitrogen. These results indicate that the effect of ascorbate is due to amelioration of some aspects of redox consequences of oxygen atmosphere, but the influence of SOD on the life span of the yeast includes also other effects, e.g. copper homeostasis[14] which may become critical under anoxic conditions.

Although any antioxidant can show prooxidative action under certain conditions, this property has often been addressed for ascorbate. Many studies demonstrated both prooxidative action of ascorbate in purely chemical or cellular systems, especially in the presence of transition metal ions known to catalyse auto-oxidation of ascorbate leading to formation of free radicals[15] and of cytotoxic dehydroascorbate.[16] I.a., ascorbate has been demonstrated to promote protein oxidation in the presence of metal ions,[17] induce apoptosis[18,19] and enhance iron toxicity in hemochromatosis.[20] It seems probable that prolonged presence of ascorbate in cell culture medium or in animal feed, in the presence of transition metal ions may create conditions for manifestation of prooxidative rather than antioxidative action of ascorbate. Ascorbate has been shown to be unstable in mammalian cell culture media.[21] It could be suspected that this effect did take place also in yeast life-span experiments when the cells were incubated in ascorbate-containing media for tens of hours. In order to check for this possibility, measurements of the effect of ascorbate on the life span of the 1C mutant were repeated

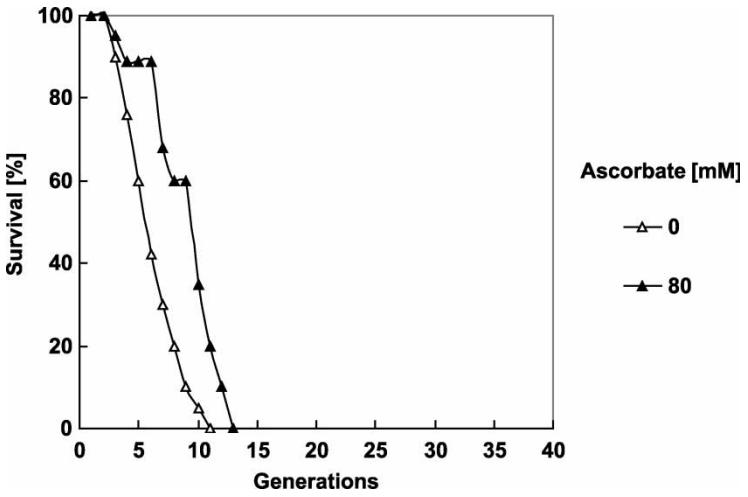

FIGURE 3 Effect of ascorbate (80 mM) on the replicative life span of MnSOD$^-$ strain of *S. cerevisiae* under the atmosphere of pure oxygen. Results shown are mean values of 40 observations.

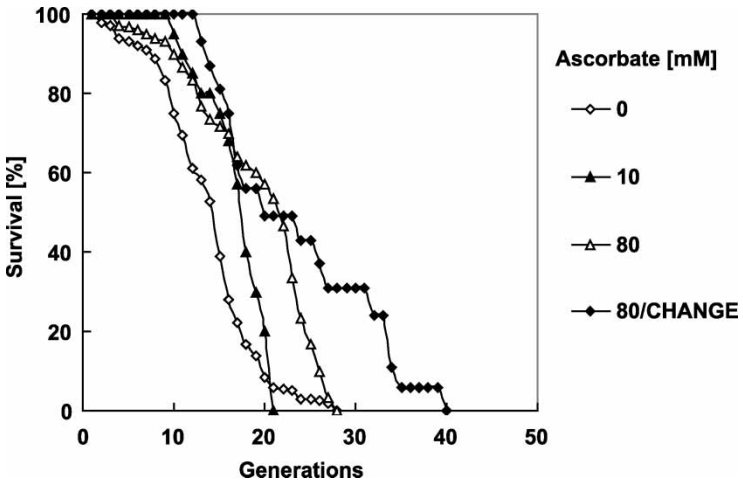

FIGURE 4 Effect of ascorbate on the replicative life span of the CuZnSOD$^-$ (1C) strain of *S. cerevisiae* in the atmosphere of air. CHANGE: medium changes for every two generations. Results shown are mean values of 40 observations.

under different conditions: instead of using the same medium throughout the experiments, the cells were transferred onto a fresh medium every two generations. The stronger effects of such a procedure, including a significant prolongation of not only the mean but also of maximal replicative life span of yeast cells in the atmosphere of air (Fig. 4) demonstrate that the beneficial effects of ascorbate can indeed be complicated by its auto-oxidation.

Available data concerning the effect of ascorbate on the cellular and organismal life span-related phenomena are rather contradictory, e.g. telomere shortening of human vascular endothelial cells was slowed down by treatment with an auto-oxidation-resistant derivative of ascorbate, ascorbate-2-*O*-phosphate.[22] However, administration of 2% ascorbate decreased instead of increasing the life span of *Drosophila melanogaster*.[23] In our opinion, this discrepancy of the effects of ascorbate can be related to the prooxidative effects of ascorbic acid under some experimental conditions. Under conditions of our experiments, significant rescue effects required high concentrations of ascorbate when the prooxidative effects of ascorbate and its oxidation products were overcome by large excess of the reduced form. Such conditions were perhaps not met in some experimental systems.

Ascorbate is not produced in yeast cells. A related compound, erythroascorbate, has been detected in *S. cerevisiae*; however, this antioxidant is present in micromolar amounts in the cells.[24] Under some conditions (e.g. when supplemented with L-galactose, L-galactono-1,4-lactone and L-gulono-1,4-lactone), *S. cerevisiae* can synthesise low amounts of L-ascorbate.[25] However, the cells can take up external ascorbate, probably via a hexose uptake system.[26–28]

The mechanism of the protective action of ascorbate on the CuZnSOD$^-$ yeast is intriguing. A trivial explanation would be that ascorbate, due to auto-oxidation, depletes medium of oxygen. However, this argument is not relevant to the conditions of lifespan determination where the cells are grown on solid medium and about 80% of their surface are in contact with the gaseous atmosphere. A different parameter might be the redox potential of the medium which is affected by ascorbate. Redox potential of the medium has been reported to affect the growth of bacterial cultures independent of oxygen concentration[29,30] and a similar phenomenon can take place in yeast cultures. However, the simplest explanation would be that ascorbate acts intracellularly as a competitive substrate for superoxide. The rate constant for the reaction of ascorbate with superoxide was reported to be $2.7 \times 10^5\,M^{-1}\,s^{-1}$ at pH 7.4.[31] This value is much lower than that of CuZnSOD ($2.6 \times 10^9\,M^{-1}\,s^{-1}$).[31] If CuZnSOD is present in the yeast cytosol at concentrations of an order of 1 μM, intracellular

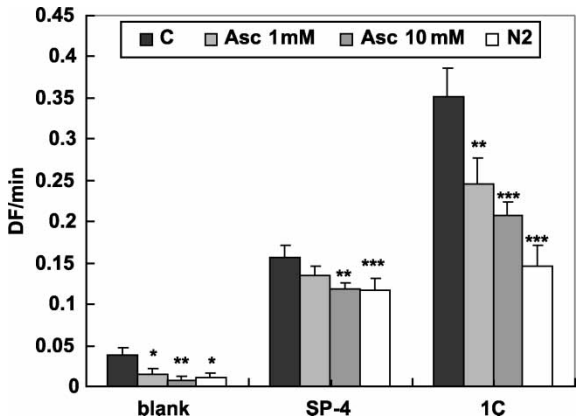

FIGURE 5 Effect of ascorbate on the rate of dihydroethidine oxidation (a measure of the level of superoxide production) in the wild-type (SP-4) and CuZnSOD$^-$ (1C) strains of *S. cerevisiae*. Blank: buffer containing no cells.

ascorbate concentrations of an order of 10 mM would be needed to substitute for the lack of CuZnSOD, as it was observed in this study. We studied the effect of ascorbate on the level of superoxide production in yeast cells by measurements of dihydroethidine oxidation and found a significant decrease of the rate of oxidation of the probe (Fig. 5). These results indicate that, although other explanations should be also considered, the protective action of ascorbate is mainly due to the scavenging of superoxide.

Acknowledgements

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