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Short communication

Ascorbic Acid and Thiol Antioxidants Suppress Spontaneous Mutagenesis in a Cu,Zn-superoxide Dismutase-deficient Mutant of *Saccharomyces cerevisiae*

Kohta Nagira, Sayaka Tamura, Shinji Kawano and Shogo Ikeda[1]

Department of Biochemistry, Faculty of Science, Okayama University of Science, Okayama, Japan

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Cu,Zn-superoxide dismutase (SOD1) is a critical enzyme in the cellular antioxidant system. The yeast *Saccharomyces cerevisiae* SOD1 mutant (*SOD1Δ*) exhibits a moderate mutator phenotype under aerobic conditions. The mutation frequency of a *SOD1Δ* strain determined by a *CAN1* forward-mutation assay was about 12-fold higher than that of the parental strain. Base substitutions G·C→T·A, G·C→A·T, and A·T→C·G were most commonly observed in *CAN1* mutants, indicating that the mutations are caused mainly by oxidative DNA damage. The mutation frequency of *SOD1Δ* was reduced in a dose-dependent manner by cultivating it in the presence of ascorbic acid, implying that the *SOD1Δ* mutant can be used as a tester strain for small molecule antioxidants. Exogenous glutathione and *N*-acetylcystein also alleviated the mutator phenotype. The results indicate that ascorbic acid and thiol antioxidants are able to efficiently protect cells against oxidative damage-induced mutagenesis. In this assay, no apparent mutation suppression was seen for other categories of antioxidants including resveratrol, Trolox and melatonin.

Key words: oxidative DNA damage, SOD1, spontaneous mutation, ascorbic acid, antioxidant

Introduction

Reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are produced as normal byproducts of cellular metabolism (1). In the presence of transition metal ions, H_2O_2 is quickly converted to a more powerful oxidant hydroxyl radical (·OH) via the Fenton reaction (2). These ROS are able to oxidize nucleic acids, proteins, and lipids, affecting cellular functions important for viability and genome integrity. In humans, oxidative damage to cellular components is thought to be a cause of cancer, degenerative diseases and ageing. Under physiological conditions, oxidative damage is prevented by intracellular antioxidants including low molecular weight ROS scavengers such as reduced glutathione (GSH) and enzymes that degrade O_2^- and H_2O_2 (3). Natural antioxidants in food, including vitamin C (ascorbic acid, AsA), vitamin E, β-carotene and polyphenols, also serve as radical scavengers.

Cu,Zn-superoxide dismutase (SOD1) is a critical antioxidant enzyme that catalyzes the dismutation of O_2^- into H_2O_2 and oxygen. Although O_2^- is incapable of damaging cellular compounds directly, the accumulation of intracellular O_2^- is thought to accelerate oxidative damage indirectly by participating in the production of ·OH (4). Further, O_2^- rapidly reacts with nitric oxide to yield peroxynitrite, which behaves as a potent oxidant (5). The yeast *Saccharomyces cerevisiae* mutant lacking SOD1 (*SOD1Δ*) is extremely sensitive to many redox-cycling drugs that generate O_2^- (6–8). The *SOD1Δ* strain is hypersensitive to heat and hyperosmotic stress (9,10), lysine and methionine auxotrophy (11), and has a shortened replicative life span (12). These obvious *SOD1Δ* phenotypes could be ameliorated by supplementing AsA at physiological levels, indicating the potent ability of AsA to scavenge endogenous ROS (10,13,14). Therefore, the yeast *SOD1Δ* mutant can be used as a biosensor for low molecular weight antioxidants on the basis of its ability to relieve the oxidative damage-induced phenotype (15–18).

The yeast *SOD1Δ* mutant exhibits a moderate mutator phenotype under aerobic conditions, indicating an important role of SOD1 in preventing oxidative DNA damage (19,20). In this study, we examined the ability of natural antioxidants to prevent spontaneous mutation in *SOD1Δ* cells. The results showed that exogenous AsA and thiol antioxidants quantitatively alleviated the mutator phenotype of *SOD1Δ*. We also applied this as-

[1]Correspondence to: Shogo Ikeda, Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Kita-ku, Okayama 700-0005, Japan. Tel: +81-86-256-9483, Fax: +81-86-256-9559, E-mail: ikeda@dbc.ous.ac.jp
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say to measure the protective effects of several categories of natural antioxidants against endogenous ROS.

Materials and Methods

Strains and expression plasmid: The *SOD1* knock-out stain (*SOD1Δ*; clone ID, 16913) of *S. cerevisiae* and the parental strain (BY4742; *Matα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A SOD1 expression plasmid was constructed using pAUR123 vector (Takara Bio, Shiga, Japan) that contains the alcohol dehydrogenase 1 gene promoter for constitutive expression in yeast and the aureobasidin A-resistant gene as a selection marker. The coding sequence of *SOD1* was amplified from *S. cerevisiae* genomic DNA using KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan) and the following primers: 5′-TATGGTACCATGGTTCAAGCAGTCGCAGTG-3′ and 5′-CGCGAGCTCTTAGTTGGTTAGACCAAT GAC-3′. The *SOD1* fragment was inserted into *Kpn*I and *Sac*I sites of pAUR123. Recombinant plasmids were introduced to *SOD1Δ* using the *S. cerevisiae* Direct Transformation Kit (Wako Pure Chemical Industries, Osaka, Japan).

Media: The strains were routinely grown aerobically on YPD medium (1% yeast extract, 2% polypeptone and 2% glucose). The *SOD1Δ* strain harboring the SOD1 expression plasmid (*SOD1Δ* + SOD1) was cultured in YPD containing 0.6 μg/mL of aureobasidin A (Takara Bio). Synthetic complete (SC) medium supplemented with 2.5 μg/mL L-canavanine sulfate salt (Sigma-Aldrich Inc., St. Louis, MO, USA) was used to screen canavaine-resistant (Can^r) colonies. SC medium contains 0.67% Difco yeast nitrogen base w/o amino acids (Becton, Dickinson and Co., Sparks, MD, USA), 2% glucose, 1.5% amino acid/nucleotide base mixture (containing 3.3 mg/mL of each amino acid histidine, leucine, lysine, and methionine, and 1.3 mg/mL uracil).

Assay for spontaneous mutation frequency: Spontaneous mutation frequency of each strain was monitored by selecting forward mutation to canavanine resistance. Cultures were inoculated with single colonies, and grown aerobically in 10 mL of YPD medium on a reciprocal shaker (250 rpm) at 28°C until saturation. Cells were then washed twice with sterile water and the appropriate dilutions were plated on SC medium containing canavanine (SC + Can) to score the Can^r colonies and on complete medium (YPD) plates to count viable cells. The plates were incubated for 3 days at 28°C before counting. The frequency of forward mutation to Can^r at the *CAN1* locus was calculated by dividing the number of Can^r colonies by the viable cell count. At least three independent isolates for each strain were tested for the mutation frequency assay. To evaluate the effect of antioxidants on mutation frequency, cultures were inoculated with 0.1 mL overnight culture of *SOD1Δ* strain, and grown in 10 mL of YPD media containing various concentrations of antioxidants at 28°C for 18 h. After washing and diluting the cells, the mutation frequency in the presence of antioxidants was determined as described above. L-ascorbic acid (AsA), *N*-acetyl-L-cysteine (NAC) and reduced L-glutathione (GSH) were purchased from Sigma-Aldrich. Other antioxidants were from Wako Pure Chemical Industries. Lipophilic antioxidants were dissolved in 95% ethanol.

***CAN1* mutation spectra:** Nucleotide sequences of the *CAN1* locus from independent Can^r colonies of the *SOD1Δ* strain were determined to analyze the spontaneous mutation spectrum. The genomic sequence of the *CAN1* locus was amplified by direct PCR of a single Can^r colony with a high fidelity DNA polymerase, KOD FX (TOYOBO), and the following primers: can(-223)Fw: 5′-GAGTGGTTGCGAACAGAGTAAA CCG-3′ and can(1827)Rv: 5′-GGTGAGAATGCGA AATGGCGTGGAA-3′. The number in parenthesis indicates the nucleotide number of its 5′ end according to Hoffmann (21). Amplification was performed by 25 PCR cycles. After the addition of adenine nucleotide to the 3′ ends of PCR products, the DNA fragments were then cloned into pTA2 vector (TOYOBO). *CAN1* loci were sequenced with the Applied Biosystems 3130 Genetic Analyzer (Life Technology) using the following primers: can(-218)Fw: 5′-GTTGCGAACAGAGTAAA C-3′, can(237)Fw: 5′-ACAGAACGCTGAAGTG-3′, can(1175)Rv: 5′-GAATTTGCGGCAGAAATAATG-3′, can(1430)Rv: 5′-GCTTGCATAAATCTGATGTG-3′, and T3RV: 5′-ATTAACCCTCACTAAAGGGAA-3′.

Results

High frequency mutation in *SOD1Δ* strain: The *SOD1Δ* strain used in this experiment was obtained from a collection of the *Saccharomyces* Genome Deletion Project. Replacement of the *SOD1* gene with the *Kan*MX cassette in the mutant strain was verified by PCR. Defect of SOD1 in the *SOD1Δ* strain was previously demonstrated by a zymographic assay for SOD activity in a crude cell extract (18). Spontaneous mutation frequencies were measured in *SOD1Δ* and parental strains by the *CAN1* forward-mutation assay. The aerobically cultured *SOD1Δ* strain in YPD medium had a mutation frequency about 12-fold greater than that determined for wild-type cells (Fig. 1). The result agrees with previous studies obtained using different *SOD1Δ* mutants, which had mutation rates about 4- to 6-fold higher than that of parental strains (19,20). Exogenous expression of SOD1 sufficiently reduced the occurrence of mutations in the *SOD1Δ* strain, indicating that a high frequency of mutation in the *SOD1Δ* strain is responsible for the loss of SOD1 activity. The 1773-bp *CAN1*

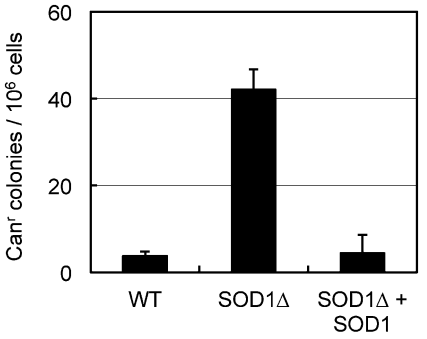

Fig. 1. High spontaneous mutation frequency of *S. cerevisiae SOD1Δ* strain. Spontaneous mutation frequencies of wild type (WT), *SOD1Δ* strain, and *SOD1Δ* harboring SOD1 expression plasmid (*SOD1Δ* + SOD1) were measured as described in the Materials and Methods. Results are the mean ± SD for 3 independent yeast colonies of each strain.

Table 1. Spontaneous mutations in *CAN1* gene of *S. cerevisiae SOD1Δ* strain

Class of mutations	No of occurrences (%)
Plus frameshift	
$A_6 \rightarrow A_7$	1 (2)
Minus frameshift	
$G_2 \rightarrow G_1$	1 (2)
$A_6 \rightarrow A_5$	1 (2)
$T_4 \rightarrow T_3$	1 (2)
Transversion	
G·C → T·A	15 (29)
G·C → C·G	4 (7)
A·T → T·A	2 (4)
A·T → C·G	8 (15)
Transition	
G·C → A·T	14 (27)
A·T → G·C	5 (10)
Total	52 (100)

coding sequence was determined in 45 independent Can^r mutants arising in aerobically cultured *SOD1Δ* cells. Seven clones had two mutation sites, yielding a total of 52 mutations. The changes in sequence were distributed throughout the *CAN1* gene. Single-base substitutions (92%) and single-base frame shifts (8%) were detected (Table 1). The most commonly observed base substitutions were G·C→T·A transversions (29%), G·C→A·T transitions (27%), and A·T→C·G transversions (15%). These mutation spectra are very similar to previous reports that described oxidant-induced mutations in bacterial and eukaryotic cells (20,22–24). These results strongly confirm that SOD1 is a critical enzyme that prevents oxidative damage-induced mutagenesis.

AsA suppresses mutation frequency of *SOD1Δ* strain: The effect of AsA on mutations in *SOD1Δ* cells was examined by the *CAN1* forward-mutation assay.

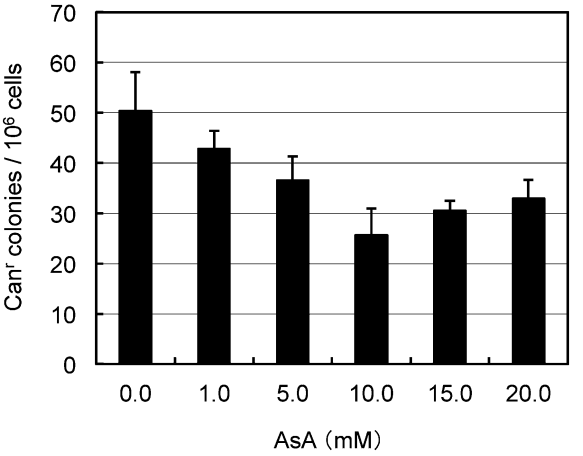

Fig. 2. Effect of AsA on prevention of spontaneous mutation in *SOD1Δ* strain. Spontaneous mutation frequencies of *SOD1Δ* in the presence of various concentrations of AsA were measured as described in the Materials and Methods. Results are the mean ± SD for 3 experiments.

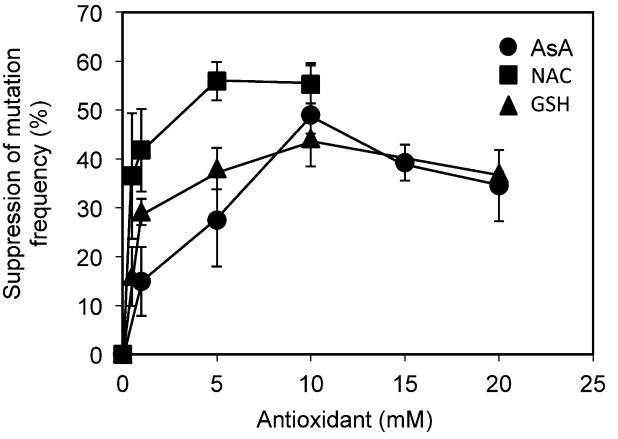

Fig. 3. Suppression of spontaneous mutation in *SOD1Δ* strain by AsA and thiol antioxidants. Spontaneous mutation frequencies of *SOD1Δ* in the presence of various concentrations of antioxidant were measured as described in the Materials and Methods. Suppression of spontaneous mutation was expressed as a percentage reduction of mutation frequency after the addition of an antioxidant. AsA (filled circle); NAC (filled square); GSH (filled triangle). Results are the mean ± SD for 3 experiments.

Spontaneous *SOD1Δ* mutations were substantially reduced after aerobic culture in the presence of various concentrations of AsA (Fig. 2). The protective effect of AsA depended on the concentration, which ranged from 1 to 10 mM. Mutation of *SOD1Δ* cells in the presence of 10 mM AsA was reduced to about 50% compared to cells growing in the absence of AsA (Fig. 3). This result indicates that AsA actually suppresses endogenous oxidant-induced mutagenesis in *SOD1Δ* cells. Higher concentrations of AsA proved less effective in mutation suppression of *SOD1Δ* cells, probably due to adverse effects of an excess of the antioxidant. The quantitative

effects of AsA raise the possibility that this mutation assay can be used for biological evaluation of small molecule antioxidants.

Effects of natural antioxidants on mutation of *SOD1Δ* strain: The same assay was applied to the evaluation of other natural antioxidants. Thiol antioxidants NAC and GSH were also able to protect yeast cells against oxidative damage-induced mutagenesis of *SOD1Δ* cells (Fig. 3). The potency of NAC on mutation suppression was higher than that of AsA and GSH, and NAC at 5 and 10 mM was reduced nearly to 60% compared to cells growing in the absence of antioxidant. GSH suppressed the mutation more efficiently than AsA at 1 mM ($p = 0.030$), and the protective effect was saturated around 40% at a higher concentration (10 mM).

In this assay, no apparent suppression of spontaneous mutations was observed for other categories of antioxidants, including resveratrol (maximum concentration examined; 1 mM), a vitamin E analogue Trolox (1 mM), and melatonin (0.75 mM). Suppression efficiencies of these antioxidants at higher concentrations could not be examined because of their low solubilities in YPD medium.

Discussion

Genetic mutations caused by oxidative DNA damage are thought to be avoided by ROS-scavenging enzymes and small molecule antioxidants in cooperation with DNA repair systems. The accumulation of O_2^- in *SOD1Δ* cells leads to a substantial increase in the spontaneous mutation rate, even though the DNA repair enzymes are intact in the mutant cells (Fig. 1). In this study, we examined the ability of natural antioxidants to prevent spontaneous mutations in yeast cells lacking SOD1 activity.

Although AsA acts as a strong free radical scavenger *in vitro*, it also has a pro-oxidant effect in the presence of free transition metal ions (25). Therefore, the role of AsA in protecting against oxidative DNA damage in living cells is controversial (26–28). Our result provides evidence that AsA has a protective effect against oxidative damage-induced mutagenesis *in vivo*. The mutation spectra observed in the *SOD1Δ* strain are typical for mutagenesis caused by oxidative DNA damage (Table 1), indicating that AsA effectively suppresses the production of mutagenic adducts in DNA (*e.g.*, 8-hydroxyguanine) and oxidized DNA precursors such as 8-hydroxy-dGTP and 2-hydroxy-dATP (22–24). AsA is also known to alleviate the oxidative stress-induced phenotypes of *SOD1Δ* including growth inhibition by hypertonic stress (10,18), amino acid auxotrophy (14), and shorting of the lifespan (13). Yeast does not synthesize AsA but can synthesize a five-carbon analogue, erythroascorbic acid, which is of limited importance as an antioxidant in cells (29). Taken together, exogenous AsA is able to act as a powerful scavenger for ROS generated in yeast cells.

The addition of thiol antioxidants GSH and NAC also suppressed oxidative damage-induced mutagenesis in the *SOD1Δ* strain in a dose-dependent manner (Fig. 3). GSH is the most important free radical scavenger distributed in nuclei as well as in the cytoplasm and mitochondria at concentrations between 1 and 10 mM (3). Our result implies that intracellular GSH in *SOD1Δ* cells depleted by ROS scavenging is restored to nearly normal levels following the addition of exogenous GSH. NAC has been shown to act as an antioxidant through the ROS scavenging activity via its thiol group (30), as well as through the stimulation of *de novo* GSH synthesis after being converted to cysteine. The mutation frequency was not completely suppressed by AsA and thiol antioxidants even at high concentrations (Fig. 3). A comparison of the *CAN1* mutation spectra in the presence or absence of antioxidants may help to clarify the cause of incomplete suppression and effect on the mutation spectrum of antioxidants.

In this assay, no mutation suppression was seen for resveratrol, Trolox and melatonin, even though these agents are believed to function as antioxidants. The budding yeast can incorporate such hydrophobic antioxidants into cells because these compounds exert physiological effects on yeast cells at concentrations lower than 1 mM, *e.g.*, resveratrol extended the yeast life span by stimulating Sir2 (31), Trolox treatment provided protection against intracellular ROS formation (32), and melatonin attenuated oxidative stress during aging of yeast in a stationary culture (33). At present, the reason for the limited number of antioxidants (AsA and thiol antioxidants) that can suppress spontaneous mutations in the *SOD1Δ* strain is unclear. Tester strains lacking ROS-scavenging enzyme (other than SOD1) in combination with a defect of the DNA repair system may be useful to evaluate the role of different types of antioxidants in mutation suppression.

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Conflicts of interest: None of the authors have any conflicts of interest associated with this study.

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