Arch. Pharm. Res. Vol.19, No.3, pp. 178-182, 1996

Effect of Copper Ion on Oxygen Damage in Superoxide Dismutase-Deficient *Saccharomyces cerevisiae*

Jeong Ki Lee, Ji Myon Kim, Su Won Kim, Doo Hyun Nam*, Chul Soon Yong and Keun Huh

College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

(Received January 9, 1996)

Using superoxide dismutase (SOD)-deficient mutants of *Saccharomyces cerevisiae*, the oxidative stresses induced by 0.1 mM of copper ion (Cu^{++}) was studied. In aerobic culture condition, yeasts lacking MnSOD (mitochondrial SOD) showed more significant growth retardation than CuZnSOD (cytoplasmic SOD)-deficient yeasts. However, not so big differences in growth pattern of those mutants compared with wild type were observed under anaerobic condition. It was found that, under aerobic condition, the supplementation of 0.1 mM copper ion (Cu^{++}) into culture medium caused the remarkable increase of CuZnSOD but not so significant change in MnSOD. It was also observed that catalase activities appeared to be relatively high in the presence of copper ion in spite of the remarkable reduction of glutathion peroxidase in CuZnSOD-deficient yeasts, but the slight increments of catalase and glutathion peroxidase were detected in MnSOD-deficient strains. It implies that the lack of cytoplasmic SOD could be compensated mainly by catalase. However, these phenomena resulted in the significant increase of cellular lipid peroxides content in CuZnSOD-deficient yeasts and the slight increment of lipid peroxides in MnSOD-deficient cells. In anaerobic cultivation supplementing copper ion, the cellular enzyme activities of catalase and glutathion peroxidase in SOD-deficient yeasts were slightly increased without any significant changes of lipid peroxides in cell membrane. It suggests that a little amount of free radicals generated by copper ion under anaerobic condition could be sufficiently overcome by catalase as well as glutathion peroxidase.

Key Words : Oxygen damage, Free radical, Superoxide dismutase, *Saccharomyces cerevisiae*, Copper ion, Catalase, Glutathion peroxide, Lipid peroxidation

INTRODUCTION

Superoxide dismutase (SOD), one of free radical scavenging enzymes, is the most important protective enzyme in most aerobic organisms and many anaerobic ones (Deby and Goutier, 1990), by catalyzing the disproportion of the toxic superoxide radicals to dioxygen and hydrogen peroxide ($2O_2^{\cdot-}+2H^+ \rightarrow O_2+2H_2O_2$) (Fridovich, 1974). Lots of researches have been focussed on its protective roles *in vivo*, especially in DNA damage, protein denaturation, lipid peroxidation, aging and carcinogenesis of mammalian animals (Halliwell and Gutteridge. 1989). However, the *in vivo* results did not give an accurate, consistent and quantitative data due to the complexicity of animal bodies.

The yeast strains, as one of the simplest eucaryotic organisms, are known to have 2 different SODs; one is CuZnSOD having Cu^{++} and Zn^{++} at an active site in the cytoplasmic fraction, the other is MnSOD having Mn^{++} in the mitochondrial part, as likely as other eucaryotes (Zitomer and Lowry, 1992). The yeast cells are thought to be a good model system in oxygen toxicity experiments instead of mammalian animals, because of its capability of growing both in aerobic and in anaerobic conditions.

The yeast genes for 2 SODs have been recently cloned and characterized successfully by two research groups (Bermingham-McDonogh *et al.*, 1988; van Loon *et al.*, 1983; Marres *et al.*, 1985), and SOD-deficient yeast strains have been constructed by insertional inactivation of the genes by 2 different groups (Liu *et al.*, 1992; Chang *et al.*, 1991). Using these SOD-deficient yeast cells, we studied already the response against oxidative stress induced by paraquat (Kim *et al.*, 1995), in order to elucidate the role of SOD genes in cellular metabolism and oxygen radical toxicity.

Based on the fact that copper ion generates oxygen damage in a variety of cells (Lee and Hassan, 1987; Galiazzo *et al.*, 1988; Huh *et al.*, 1995), we are here reporting the effects on the cellular enzymes and

Correspondence to: Doo Hyun Nam, College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

metabolites in SOD-deficient *S. cerevisiae*, especially on the activities of catalase and glutathion peroxidase, and the content of lipid peroxides in cell membrane, when exposed to copper ion.

MATERIALS AND METHODS

Microorganisms and culture condition

Four yeast strains were used throughout this work, ; *Saccharomyces cerevisiae* EG103 (*MATα leu2-3,112 his3△1 trp1-289a ura3-52 gal*$^+$) as wild type, *S. cerevisiae* EG110 (same except *sod2△::TRP1*) lacking mitochondrial MnSOD, *S. cerevisiae* EG118 (same except *sod1△A::URA3*) lacking cytoplasmic CuZnSOD, and *S. cerevisiae* EG133 (same except *sod1△A::URA* and *sod2△::TRP1*) deficient of both SOD enzymes, all of which were kindly sent by Dr. Edith B. Gralla at University of California, Los Angeles, CA, U.S.A. The yeast strains were maintained in YPD media, which consists of 1% yeast extract, 2% peptone, and 2% glucose.

In aerobic cultivation, 1% of seed cultures (OD_{600}= 4~5) were inoculated in YPD media and cultivated for 16 hrs at 30°C with vigorous shaking at 150 rpm on a rotary shaker. On the other hand, the tightly closed capped Erlenmeyer flasks with a side arm of spectrophotometric cuvette were employed for the anaerobic cultivation. In order to keep the culture broth anaerobically, 0.03% sodium thioglycollate was supplemented in YPD medium with 1 ppm of resazurin as an indicator. 0.1mM copper sulfate ($CuSO_4$) was added into culture broth, in case of generating the oxygen toxicity.

Analytical procedures

After cultivation, the yeast cells were harvested by centrifugation at 5,000 rpm after 16 hrs-cultivation, washed twice and resuspended in 50mM phosphate buffer (pH 7.5) containing 0.1mM ethylenediamine tetraacetate (EDTA), and then disrupted by sonication for 5 min using Ultrasonic Processor (Model No. XL 2010, Heat Systems, NY, U.S.A.). The supernatant obtained by centrifuging at 15,000 rpm was used as enzyme sources for SOD, catalase and glutathion peroxidase. A part of sonicated suspension (0.4 ml) was extracted with 1-butanol : pyridine (15 : 1) for the analysis of the degree of lipid peroxidation.

The SOD activity was examined by staining the gel with 2.4mM nitroblue tetrazolium (NBT) and 28 M riboflavin after running 10% nondenaturated polyacrylamide gel electrophoresis, according to the procedure of Beauchamp and Fridovich (1971). The catalase activity was measured by the method of Aebi (1969), and expressed as mole of hydrogen peroxide decomposed/min/mg of protein. The activity of glutathion peroxidase was assayed by the procedure of Paglia and Valentine (1967), and described as nmole of NADPH consumed in the reduction of oxidized glutathion/min/mg of protein. The amount of lipid peroxides in cell membrane was determined as malondialdehyde content from 1-butanol/ pyridine (15 : 1) extracts following the procedure of Ohkawa *et al.* (1979), in order to detect the degree of cellular deterioration by oxygen radicals.

The cell mass of yeasts was measured spectrophotometrically at 600 nm, and converted to dry cell weight using standard curve. The protein content in samples was analyzed by Folin-Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

All the results were expressed as the mean values of 3 separate experimental data.

RESULTS AND DISCUSSION

Growth pattern of SOD-deficient yeasts

Firstly, the growth pattern of yeast strains lacking SOD genes was observed under aerobic and anaerobic conditions, and compared with that of wild yeast

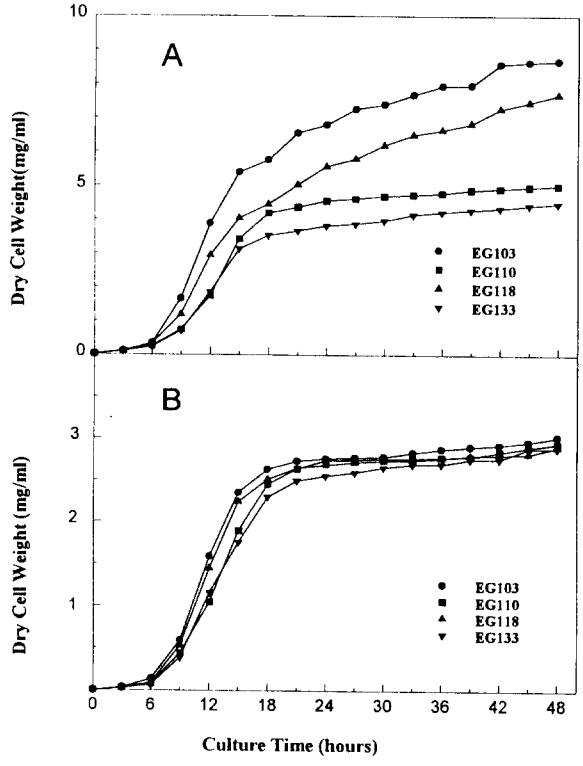

Fig. 1. Growth profiles of SOD-deficient yeasts under aerobic or anaerobic culture condition. A; aerobic cultivation, B; anaerobic cultivation. EG103, *S. cerevisiae* EG103 (wild type); EG110, *S. cerevisiae* EG110 (lacking mitochondrial MnSOD); EG118, *S. cerevisiae* EG118 (lacking cytoplasmic CuZnSOD); EG133, *S. cerevisiae* EG133 (deficient of both SOD enzymes)

strain. Fig. 1 shows the growth retardation of SOD-deficient yeast strains compared to wild strain (*S. cerevisiae* EG103) during aerobic cultivation. The growth retardation as well as low achievement of cell masses of SOD-deficient strains implies that these strains could not overcome the oxygen toxicity generated in aerobic condition due to their loss of SOD activities, the most important enzyme in oxygen radical scavenging system. From the fact that yeasts lacking MnSOD gene (*S. cerevisiae* EG110 and EG133) were more severely affected than CuZnSOD-deficient strain (*S. cerevisiae* EG118) in aerobic condition, it could be assumed that MnSOD (mitochondrial SOD) could play more important roles than cytoplasmic CuZnSOD in protecting cells from oxygen radical. In contrast, not so big differences in growth kinetics were observed in anaerobic culture condition. It is probably due to the generation of a little oxygen radical in anaerobic condition, which was likely not to give any adverse effects on cell growth of SOD-deficient yeasts.

Adaptative responses of SOD-deficient yeasts when exposed to copper ion

In order to examine the effects of SOD deficiency in yeast cells on intracellular adaptation to free radicals generated by copper ion, the changes of cellular enzyme activities and lipid peroxidation during cultivation were carefully determined.

When the change in SOD activities of yeast mutants was examined by activity staining on 10% nondenaturated polyacrylamide gels, it was observed that CuZnSOD activities in wild yeast (*S. cerevisiae* EG 103) and MnSOD-deficient yeast (*S. cerevisiae* EG 110) were not only significantly increased by copper sulfate under aerobic condition, but also slightly increased under anaerobic condition, as shown in Fig. 2. However, CuZnSOD-deficient yeasts (S. cerevisiae EG118 and EG133) did not show any marked changes in MnSOD activities. This result implies that copper ion might generate free radicals mainly in cytoplasmic fraction, but not in mitochondrial fraction.

The cellular activity of catalase was significantly increased in MnSOD-deficient mutants (*S. cerevisiae* EG110), whereas slightly higher activity was observed in CuZnSOD-deficient strains (*S. cerevisiae* EG118 and EG133), when exposed to copper ion during aerobic cultivation (Fig. 3). On the other hand, the cellular activity of glutathion peroxidase, one of the alternative radical scavenging systems, was remarkably decreased in yeast strains lacking CuZnSOD in the presence of copper ion, but slight increment was found in MnSOD-deficient mutant, when cultivated aerobically (Fig. 4). It strongly suggests that the free radical scavenging system might shift to catalase rather than glutathion peroxidase when cytoplasmic CuZnSOD or mitochondrial MnSOD is inactivated. However, these results are slightly different from those obtained by inducing oxidative stress with paraquat (Kim *et al.*, 1995), in the case of which glutathion peroxidase was much more activated than catalase in yeast cells lacking of CuZnSOD. It is still unclear whether this difference may be attributable to the control at genetic level or enzyme level. But, in wild yeasts, the similar

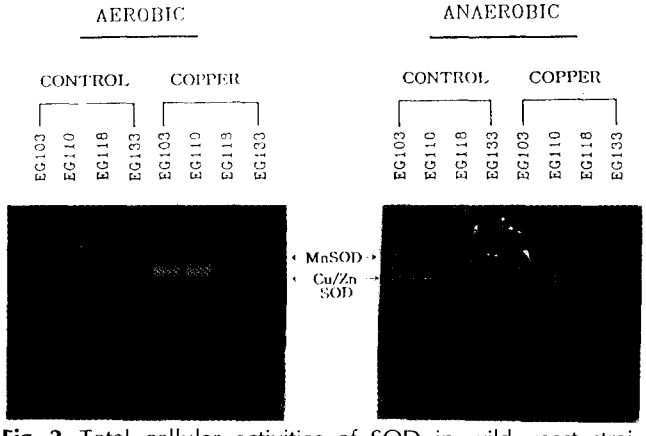

Fig. 2. Total cellular activities of SOD in wild yeast strain and SOD-deficient mutants during aerobic or anaerobic cultivation in the medium containing copper ion. After sonication of yeast cells, the supernatants were subjected to 10% nondenaturated polyacrylamide gel electrophoresis, and the activities of SOD were stained as described in Materials and Methods. EG103, *S. cerevisiae* EG103 (wild type); EG110, *S. cerevisiae* EG133 (lacking mitochondrial MnSOD); EG118, *S. cerevisiae* EG118 (lacking cytoplasmic CuZnSOD); EG133, *S. cerevisiae* EG110 (deficient of both SOD enzymes)

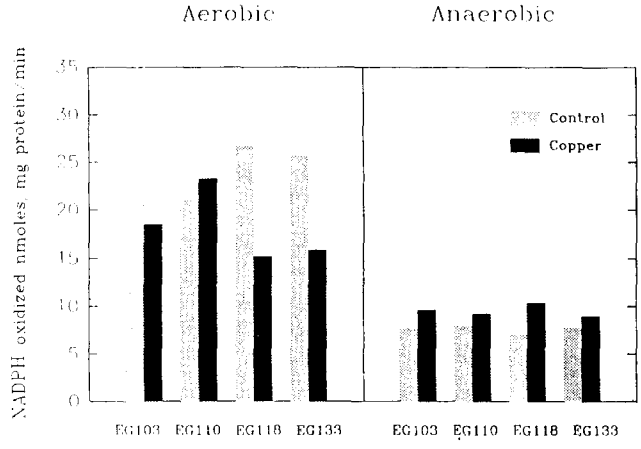

Fig. 3. Total cellular activities of catalase in wild yeast strain and SOD-deficient mutants when cultivated aerobically or anaerobically in the presence of copper ion. EG 103, *S. cerevisiae* EG103 (wild type); EG110, *S. cerevisiae* EG110 (lacking mitochondrial MnSOD); EG118, *S. cerevisiae* EG118 (lacking cytoplasmic CuZnSOD); EG133, *S. cerevisiae* EG133 (deficient of both SOD enzymes)

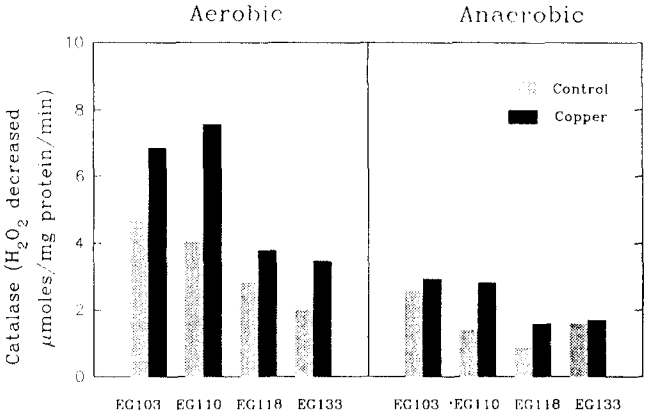

Fig. 4. Total cellular activities of glutathion peroxidase in wild yeast strain and SOD-deficient mutants when exposed to copper ion under aerobic or anaerobic culture condition. EG103, *S. cerevisiae* EG103 (wild type); EG110, *S. cerevisiae* EG110 (lacking mitochondrial MnSOD); EG118, *S. cerevisiae* EG118 (lacking cytoplasmic CuZnSOD); EG133, *S. cerevisiae* EG133 (deficient of both SOD enzymes)

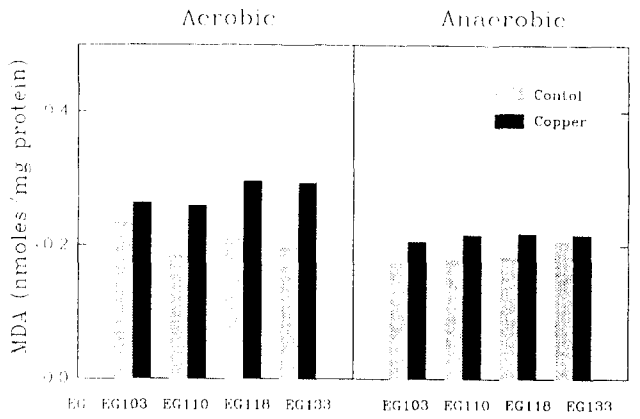

Fig. 5. The content of lipid peroxides, determined as malondialdehyde, in wild yeast strain and SOD-deficient mutants cultured aerobically or anaerobically in the presence of copper ion. EG103, *S. cerevisiae* EG103 (wild type); EG110, *S. cerevisiae* EG110 (lacking mitochondrial MnSOD); EG 118, *S. cerevisiae* EG118 (lacking cytoplasmic CuZnSOD); EG133, *S. cerevisiae* EG133 (deficient of both SOD enzymes)

phenomena have been reported that aerobic growth in the presence of copper ion causes an induction of housekeeping enzyme activities of oxygen metabolism including catalase and glutathion peroxidase (Galiazzo *et al.*, 1988).

On the other hand, the significant increment in the content of lipid peroxides in CuZnSOD-deficient mutants (*S. cerevisiae* EG118 and EG133), which was expressed as malondialdehyde, and its slight increment in wild type (*S. cerevisiae* EG103) and MnSOD-deficient mutant (*S. cerevisiae* EG110) was observed when exposed to copper ion under aerobic condition (Fig. 5). This result shows that the catalase activation following to the significant inactivation of glutathion peroxidase in the absence of CuZnSOD could not remove perfectly the free radicals generated in cytoplasm to cause membrane damage, whereas MnSOD could be somewhat compensated by increased amount of catalase and glutathion peroxidase. The similar phenomena were observed when oxygen damage was generated by paraquat, as reported previously (Kim *et al.*, 1995). However, not so significant effects was recognized in anaerobic culture. It means that small amounts of free radicals generated under anaerobic condition could not give the severe damage on membrane lipids by detoxication through catalase and glutathion peroxidase, in spite of the absence of SOD enzymes.

It could be concluded that copper ion could induce oxygen toxicity in cytoplasmic fraction of SOD-deficient yeasts. It was also an interesting finding that catalase could be activated for scavenging the oxygen radicals instead of SODs but could not remove the radicals completely in the cytoplasm of yeast cells.

ACKNOWLEDGEMENT

This work was kindly supported by Yeungnam University for the academic research promotion (1995), and partly by Ministry of Education for Biochemical Engineering Program (1995). The authors would like to express their deep thanks to Dr. Edith Butler Gralla (University of California, Los Angeles) for his kindness to provide yeast strains.

REFERENCES CITED

Aebi, H., Catalase *in vivo*. *Methods Enzymol.*, 105, 121-126 (1984).

Beauchamp, C.O. and Fridovich, I., Superoxide dismutase : improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.*, 44, 276-287 (1971).

Bermingham-Mcdonogh, O., Gralla, E.B. and Valentine, J.S., The copper, zinc- superoxide dismutase gene of *Saccharomyces cerevisiae* : cloning, sequencing, and biological activity. *Proc. Natl. Acad. Sci. USA*, 85, 4789-4793 (1988).

Chang, E.C., Crawford, B.F., Hong, Z., Bilinski, T. and Kosman, D.J., Genetic and biochemical characterization of Cu,Zn superoxide dismutase mutants in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 266, 4417-4424 (1991).

Deby, C. and Goutier, R., New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. *Biochem. Pharmacol.*, 39, 399-405 (1990).

Fridovich, I., Superoxide dismutase. *Ann. Rev. Biochem.*, 44, 147-159 (1975).

Galiazzo, F., Schiesser, A. and Rotilo, G., Oxygen-independent induction of enzyme activities related to oxygen metabolism in yeast by copper. *Biochim. Biophys. Acta*, 965, 46-51 (1988).

Halliwell, B. and Gutteridge, J.M.C., *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford (1989).

Huh, K., Shin, U.S. and Lee, S.I., Effect of heat-treated ceruloplasmin on the hepatic xanthine oxidase activity and type conversion. *Arch. Pharm. Res.*, 18, 56-59 (1995).

Kim, J.M., Nam, D.H., Yong, C.S. and Huh, K., Oxygen toxicity of superoxide dismutase-deficient Saccharomyces cerevisiae by paraquat. *Korean J. Biotechnol. Bioeng.*, 38, 561-567 (1995).

Lee, F.-J.S. and Hassan, H.M., Biosynthesis of superoxide dismutase and catalase in chemostat culture of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, 26, 531-536 (1987).

Liu, X.F., Elashvili, I., Gralla, E.B., Valentine, J.S., Lapinskas, P. and Culotta, V.C., Yeast lacking superoxide dismutase. *J. Biol. Chem.*, 267, 18298-18302 (1992).

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Marres, C.A.M., van Loon, A.P.G.M., Oudshoorn, P., van Steeg, H., Grivell, L.A. and Slater, E.C., Nucleotide sequence analysis of the nuclear gene coding for manganese superoxide dismutase of yeast mitochondria, a gene previously assumed to code for the Rieske iron-sulfur protein. *Eur. J. Biochem.*, 147, 153- 161 (1985).

Ohkawa, H., Ohishi, N. and Yaki, K., Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, 95, 351-358 (1979).

Paglia, E.D. and Valentine, W.N., Studies on the quantitative and qualitative characterization of erythrocyte glutathion peroxidase. *J. Lab. Clin. Med.*, 70, 158-169 (1967).

van Loon, A.P.G.M., Marres, C.A.M., Riezman, H. and Grivell, L.A., Isolation, characterization and regulation of expression of the nuclear genes for the core II and Rieske iron-sulfur proteins of the yeast ubiquinol-cytochrome c reductase. *Gene*, 26, 261-272 (1983).

Zitomer, R.S. and Lowry, C.V., Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. *Microbiol. Rev.*, 56, 1-11 (1992).