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Research Article

Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in *Candida albicans*

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Abstract

Candida albicans **contains copper- and zinc-containing superoxide dismutase but also two manganese-containing superoxide dismutases (MnSODs), one in the cytosol and the other in the mitochondria. Among these, the *SOD2* gene encoding mitochondrial MnSOD was disrupted and overexpressed to investigate its roles in *C. albicans*. The null mutant lacking mitochondrial MnSOD was more sensitive than wild-type cells to various stresses, such as redox-cycling agents, heating, ethanol, high concentration of sodium or potassium and 99.9% O_2. Interestingly, the *sod2/sod2* mutant was rather more resistant to lithium and diamide than the wild-type, whereas overexpression of *SOD2* increased susceptibility of *C. albicans* to these compounds. The inverse effect of mitochondrial MnSOD on lithium toxicity was relieved when the *sod2/sod2* and *SOD2*-overexpressing cells were grown on the synthetic dextrose medium containing sulphur compounds such as methionine, cysteine, glutathione or sulphite, indicating that mitochondrial MnSOD may affect lithium toxicity through sulphur metabolism. Moreover, disruption or overexpression of *SOD2* increased or decreased glutathione reductase activity and cyanide-resistant respiration by alternative oxidase, respectively. Taken together, these findings suggest that mitochondrial MnSOD is important for stress responses, lithium toxicity and cyanide-resistant respiration of *C. albicans*.**

Keywords: ***Candida albicans*****; manganese-containing superoxide dismutase;** ***SOD2*****; lithium; diamide; alternative oxidase**

Introduction

All aerobic organisms inevitably generate a range of reactive oxygen species (ROS), including superoxide radical anion, hydrogen peroxide and hydroxyl radical during oxygen metabolism. If not quickly and effectively eliminated from cells, these ROS initiate a large number of oxidative reactions in cellular systems that possibly lead to cell death (Raha and Robinson, 2000). Mitochondria are a major endogenous source of ROS in eukaryotic cells and extremely susceptible sites toward oxidative damages (Raha and Robinson, 2000). Even under normal physiological conditions, 1–2% of the dioxygen consumed by mitochondria is converted into superoxide radical anions by electron transport chain (Boveris, 1977). Since superoxide radical anion is a charged molecule and does not diffuse easily through the mitochondrial membrane, it has to be scavenged within its matrix. Although superoxide radical anions undergo spontaneous disproportionation into hydrogen peroxide and dioxygen, this reaction is greatly facilitated by superoxide dismutase (SOD). Generally, SODs are categorized into four classes according to their metal co-factors: copper- and zinc-containing superoxide dismutase (CuZnSOD), manganese-containing superoxide dismutase (MnSOD), iron-containing superoxide dismutase (FeSOD) and nickel-containing superoxide dismutase (NiSOD)

(Fridovich, 1995; Youn *et al*., 1996). Among these, MnSOD has generally been found in the mitochondrial matrix of eukaryotes and the cytosolic fractions of prokaryotes (Fridovich, 1995), but unusually in the cytosol of some eukaryotes (Kitayama and Togasaki, 1995; Lamarre *et al*., 2001). The mitochondrial MnSOD has been the subject of particular interest because it represents the first line of defence against superoxide radical anions produced as by-products of oxidative phosphorylation. The lack of mitochondrial MnSOD in *Saccharomyces cerevisiae* resulted in a rapid death in stationary phase (Longo *et al*., 1999) and increased susceptibility to the treatment of elevated oxygen tension or ethanol (Costa *et al*., 1993, 1997; van Loon *et al*., 1986). Moreover, genetic inactivation of mitochondrial MnSOD in mice led to heart and liver complications, metabolic acidosis and early neonatal death (Li *et al*., 1995).

Candida albicans is a major fungal pathogen of humans, and causes not only oral and vaginal thrush but also systemic or life-threatening infections in immunocompromised patients (Coleman *et al*., 1993; Cutler, 1991). Once *C. albicans* infects a host, it inevitably encounters ROS produced by the consequences of its own oxygen metabolism as well as by host phagocytes (Vázquez-Torres and Balish, 1997). Due to its localization, mitochondrial MnSOD is thought to play a protective role by suppressing the production of superoxide radical anion, the first intermediate in the sequential univalent reduction of dioxygen, during the respiration. Interestingly, *C. albicans* possesses a cyanide-resistant respiratory pathway mediated by alternative oxidase (AOX) in addition to the cytochrome respiratory pathway found in all eukaryotes (Huh and Kang, 1999, 2001).

In *C. albicans*, the *SOD1, SOD2* and *SOD3* genes encoding cytosolic CuZnSOD (Hwang *et al*., 1999), mitochondrial MnSOD (Rhie *et al*., 1999) and unusual cytosolic MnSOD (Lamarre *et al*., 2001) were identified, respectively. We previously characterized the cytosolic CuZnSOD (Hwang *et al*., 1999) and mitochondrial MnSOD (Rhie *et al*., 1999), and recently reported that CuZnSOD is required for the protection against oxidative stresses and the establishment of full virulence (Hwang *et al*., 2002). Although superoxide radical anions can induce the expression of AOX (Huh and Kang, 2001; Minagawa *et al*., 1992), it has not been reported until now whether mitochondrial MnSOD is directly involved in its expression. Here, we report that mitochondrial MnSOD is related to various stress responses and changes the expression of AOX of *C. albicans*.

Materials and methods

Organisms and culture conditions

C. albicans strains and plasmids used in this study are listed in Table 1 and 2, respectively. The strains were routinely cultured on YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) at 28 °C. Cells carrying plasmids or disrupted genes were cultured in synthetic dextrose (SD) medium containing 0.67% w/v yeast nitrogen base without amino acids (Difco) and 2% w/v dextrose. Solid media were prepared by adding 1.8% w/v agar to liquid broth. *Escherichia coli* strain DH5α was used for most plasmid construction and maintenance, and the cells were grown at 37 °C in Luria–Bertani medium with appropriate antibiotics.

Table 1. Strains used in this study

Strain	Genotype and description	Source or reference
SC5314	Wild-type isolate	Fonzi and Irwin, 1993
CAI4	Δ*ura3::imm434*/Δ*ura3::imm434*	Fonzi and Irwin, 1993
CH201	Δ*ura3::imm434*/Δ*ura3::imm434* Δ*sod2::hisG–URA3–hisG/SOD2*	This work
CH202	Δ*ura3::imm434*/Δ*ura3::imm434* Δ*sod2::hisG/SOD2*	This work
CH203	Δ*ura3::imm434*/Δ*ura3::imm434* Δ*sod2::hisG*/Δ*sod2::hisG–URA3–hisG*	This work
CH204	Δ*ura3::imm434*/Δ*ura3::imm434* Δ*sod2::hisG*/Δ*sod2::hisG*	This work
CAI4(pYPB1–ADHpL)	CAI4 carrying pYPB1–ADHpL	This work
CH204(pYPB1–ADHpL)	CAI4 carrying pYPB1–ADHpL	This work
CAI4(pASOD2)	CAI4 carrying pASOD2	This work

Table 2. Plasmids used in this study

Plasmid	Description	Source or reference
p5921	*hisG–URA3–hisG* in pUC18	Fonzi and Irwin, 1993
p5922	4.1 kb *Hin*dIII/*Sac*I-digested *hisG–URA3–hisG* of p5921 in pGEM-7Z(+)	Hwang *et al*., 2002
pSOD2	3.0 kb *Hin*dIII-digested genomic *SOD2* in pBluescript KS(+)	This work
pCH201	0.77-kb PCR fragment of *SOD1* in pGEM-T Easy	This work
pCH202	*sod2::hisG–URA3–hisG* in pGEM-T Easy	This work
pYPB1–ADHpL	*ADH1* promoter and terminator in *URA3*-marked 2 μm vector	Csank *et al*., 1998
pASOD2	*SOD2* in pYPB1–ADHpL	This work
pRC2312	*URA3*- and *LUE2*-marked *CaARS* vector	Cannon *et al*., 1992
pWK203	*ALO1* and its flanking sequences in pRC2312	Huh *et al*., 2001

DNA manipulation and analysis

Plasmid isolation, polymerase chain reaction (PCR), restriction enzyme digestion, cloning and Southern blot hybridization were carried out by the methods proposed by Sambrook *et al*. (1989). Labelling of a DNA probe was performed with a non-radioactive labelling and detection kit (Roche Molecular Biochemicals). The coding region of *SOD2* in the plasmid, pCH201, was labelled by random-primed incorporation of digoxigenin-tagged dUTP.

Disruption and overexpression of *C. albicans SOD2*

To disrupt both alleles of *SOD2* using the URA blaster technique (Fonzi and Irwin, 1993), the appropriate oligonucleotide primers (5′-CCCTTAG TTGTGGTC-3′ and 5′-AATAAGCATGTTCCCA-3′) were synthesized and used to PCR-amplify its coding region. The resulting 770 bp PCR product was inserted into pGEM-T Easy vector (Promega), yielding pCH201. Then, the disruption construct was created by inserting a *Bgl*II/*Sma*I-digested *hisG–URA3–hisG* cassette from p5922, a derivative of p5921 (Fonzi and Irwin, 1993), into the *Bgl*II/*Hpa*I sites to remove 109 bp from the *SOD2*-coding region of *C. albicans* (Figure 1A). The yielded construct was linearized with *Apa*I/*Sac*I, transformed into CAI4 (a Ura$^-$ derivative of wild-type strain SC5314) (Fonzi and Irwin, 1993), and selected by uracil prototrophy. The integration of these cassettes into the *SOD2* locus was verified by Southern blot analysis (Figure 1B). Spontaneous Ura$^-$ derivatives of the heterozygous disruptants were selected on SD medium containing 5-fluoroorotic acid (625 μg/ml) and uridine (100 μg/ml). This procedure was repeated once more to generate a homozygous *sod2/sod2* mutant strain (CH204).

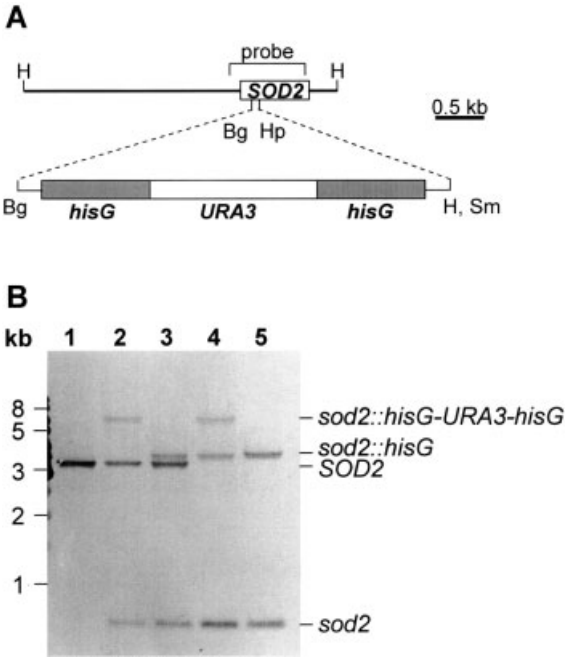

Figure 1. Disruption of the *SOD2* in *C. albicans*. (A) Schematic representation of disruption strategy. Restriction map of the *SOD2* locus and insertion of the *hisG–URA3–hisG* cassette at the indicated *Bgl*II/*Hpa*I sites in *SOD2* coding sequences. The endonuclease restriction sites are as follows: Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; Sm, *Sma*I. (B) Southern blot analysis with the sequences bracketed in (A) used as probe. The DNA digested with *Hin*dIII was from the following strains: CAI4 (lane 1), CH201 (lane 2), CH202 (lane 3), CH203 (lane 4) and CH204 (lane 5)

For the overexpression of *SOD2*, its coding region was amplified with *Pfu* polymerase (Promega). The primers used were 5′-AA**GGATC C**ATGTTTTCTATCAGATCA-3′ and 5′-**CTCGAG** ACTTAAAATTCAAATCTT-3′ (*Bam*HI/*Xho*I sites

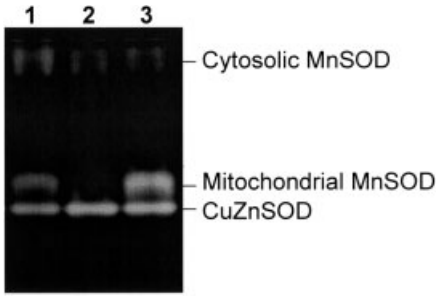

Figure 2. Verification of disruption or overexpression of *SOD2* in *C. albicans* by activity staining. SOD activity was revealed with nitro-blue tetrazolium and riboflavin on 8% native polyacrylamide gel. In each lane, 50 μg total protein from the indicated strains was loaded. Lane 1, CAI4(pYPB1–ADHpL); lane 2, CH204(pYPB1–ADHpL) and lane 3, CAI4(pASOD2)

are emboldened). Each amplification product was digested with *Bam*HI/*Xho*I, and then cloned into the *Bgl*II/*Xho*I sites of plasmid pYPB1-ADHpL (Csank *et al*., 1998). The resultant plasmid pASOD2 was transformed into wild-type (CAI4) or *sod2/sod2* (CH204) strain and then the overexpression of MnSOD was confirmed by activity staining (Figure 2).

Staining to detect SOD activity

The crude extract of *C. albicans* cells was prepared by vigorous shaking of the cell suspension with glass beads for 90 s in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The concentration of protein in the crude extract was determined by the Bio-Rad *DC* Protein Assay kit (Bio-Rad Laboratories), using bovine serum albumin as a reference protein. 50 μg total protein was loaded on to 8% native polyacrylamide gel, in which SOD activity was detected by negative staining (Manchenko, 1994). The gel was incubated in 50 mM phosphate buffer (pH 7.8) for 10 min, in nitro-blue tetrazolium solution (1 mg/ml) for 10 min, and then in 50 mM phosphate buffer (pH 7.8) containing riboflavin (0.01 mg/ml) and N, N, N', N'-tetramethylethylenediamine (3.25 mg /ml) for 10 min at room temperature with gentle shaking. Areas with SOD activity remained clear when the gel was exposed to the light.

Growth assays

To test the effect of various stresses on the growth, *C. albicans* strains were grown overnight in liquid SD medium at 28 °C and then the cultures were adjusted to an absorbance at 600 nm (A_{600}) of 10.0 (2×10^8 cells/ml). After 10-fold serial dilutions, 5 μl of these cell suspensions were spotted onto synthetic complete (SC) medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) dextrose and 0.2% (w/v) drop-out mix (supplemented with all possible amino acids) (Kaiser *et al*., 1994) or SD plates with appropriate chemicals. The plates were photographed after 2 or 3 days incubation at 28 °C. For the culture under normoxic or hyperoxic conditions, exponentially growing cells were streaked on SD plates and then incubated at 28 °C for 3 days in air (20.7% O_2) or in an anaerobic jar filled with 99.9% O_2.

Enzyme assay

For the estimation of glutathione reductase activity, samples were prepared in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. Glutathione reductase activity was spectrophotometrically determined by monitoring the oxidation of NADPH at 340 nm. 1 unit was defined as the amount of enzyme producing 1 μM reduced glutathione/min (Carlberg and Mannervik, 1985).

The activity of D-arabinono-1,4-lactone oxidase (ALO) was measured spectrophotometrically in 0.2 M potassium phosphate (pH 6.1) containing 1 mM EDTA and 50 mM D-arabinono-l,4-lactone and an aliquot of enzyme. The production of D-erythroascorbic acid (EASC) ($\varepsilon_{265} = 13\ 150$/M/cm) was monitored by the increase of the absorbance at 265 nm during the first 1 min of the reaction at 36 °C. One unit of the enzyme was defined as the amount of enzyme which produced 1 μmol EASC/min.

Measurement of respiration

Respiration of the cells in mid-exponential phase was measured polarographically at 25 °C using a YSI 5300 Biological Oxygen Monitor Micro System (Yellow Springs Instruments). Respiration via the cytochrome pathway was inhibited with 1 mM KCN, whereas cyanide-resistant respiration by the alternative pathway was inhibited with 2 mM salicylhydroxamic acid. Oxygen uptake rates remaining after the addition of these two inhibitors were substracted from calculated rates to give

the final values for cyanide-resistant respiratory activity. KCN was dissolved in distilled water adjusted to pH 7.0 and salicylhydroxamic acid in absolute ethanol.

Western blot analysis

50 μg total protein was subjected to 12% SDS–polyacrylamide gel electrophoresis. The resolved protein was transferred onto nitrocellulose filter (Schleicher and Schuell), which was subsequently probed with an antibody raised against the AOX protein from *Sauromatum guttatum* (Elthon *et al*., 1989) at 1 : 1000 dilution. The signals were visualized using a colorimetric detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Results

Disruption and overexpression of *SOD2* in *C. albicans*

We have previously reported the characterization of MnSOD and its gene (*SOD2*) from *C. albicans* (Rhie *et al*., 1999). To find out its role in *C. albicans*, the *SOD2* (GenBank Accession No. AF031478) was sequentially disrupted using the URA-blaster technique (Fonzi and Irwin, 1993) and its homozygous disruption was confirmed by Southern blot analysis (Figure 1). Genomic DNA of wild-type CAI4 cell digested with *Hin*dIII revealed one fragment of 3.2 kb with a *SOD2* probe, but the heterozygous *sod2/SOD2* strain (CH201) transformed with the URA-blaster inserted *SOD1*, had three fragments of 6.4, 3.2 and 0.8 kb. The fragment of 3.2 kb is the result of one allele of wild-type *SOD2* and the 6.4 and 0.8 kb fragments are generated due to an added *Hin*dIII site at the right-hand margin of the URA-blaster, which was inserted into the *SOD2* of the other allele. Selection on FOA medium of the CH201 strain led to the isolation of Ura^- cells, in which the *URA3* of the URA-blaster had been deleted by recombination between the *hisG* repeats. In this strain (CH202), the *Hin*dIII-digested genomic DNA revealed three fragments of 3.4, 3.2 and 0.8 kb. Likewise, the Ura^+ *sod2/sod2* strain (CH203) showed three fragments of 6.4, 3.4 and 0.8 kb and the Ura^- *sod2/sod2* strain (CH204) had two DNA bands of 3.4 and 0.8 kb, which represent the correct disruption of two alleles of *SOD2* by one copy of *hisG* (Figure 1B).

When subjected to activity staining in 8% native polyacrylamide gel, the crude extracts of *C. albicans* cells grown up to stationary phase revealed three SOD bands. Among them, two major bands are demonstrated to be CuZnSOD and mitochondrial MnSOD, but the minor smear is likely to be generated from an unusual cytosolic MnSOD, which has recently been characterized (Lamarre *et al*., 2001). We could confirm the localization of cytosolic MnSOD in native gel through disruption of its gene *SOD3* (unpublished data). Since the purified MnSOD had its activity in the upper of the two major SOD bands (Hwang *et al*., 1999; Rhie *et al*., 1999), the correct disruption of *SOD2* could also be confirmed by activity staining (Figure 2). The resultant *sod2/sod2* mutant (CH204) did not show any auxotrophy and grew normally in minimal medium as well as in complex medium. To investigate the effect of the *sod2*-mutation on hyphal growth of *C. albicans*, isogenic Ura^+ prototrophs were grown in liquid or on solid media that induced hyphal growth, e.g. 10% serum, Lee's (Lee *et al*., 1975), Spider (Liu *et al*., 1994), corn meal agar with 0.33% Tween 80 and RPMI 1640 media. The *sod2/sod2* mutant (CH203) exhibited nearly similar cell or colony morphologies to those of the wild-type strain in/on these media. Moreover, we could not observe a significant defect in the virulence of the *sod2/sod2* mutant in a mouse model (data not shown). In contrast, the *sod1/sod1* mutant showed retarded filamentation on Spider medium and attenuated virulence in mice (Hwang *et al*., 2002). Therefore, these results suggest that mitochondrial MnSOD has a different physiological role from CuZnSOD in *C. albicans*.

In order to overexpress mitochondrial MnSOD in *C. albicans*, we constructed the plasmid pASOD2 by inserting the entire coding region of *SOD2* into the plasmid pYPB1–ADHpL (Csank *et al*., 1998) carrying a strong *ADH1* promoter. After these pYPB1–ADHpL and pASOD2 plasmids transformed into wild-type strain CAI4, we could confirm the overexpression of mitochondrial MnSOD through the activity staining on native gel (Figure 2). Like disruption of *SOD2*, its overexpression did not have significant effects on the morphology and virulence of *C. albicans* (data not shown).

Mitochondrial MnSOD contributes to various stress responses in *C. albicans*

Redox-cycling agents such as menadione and paraquat are known to generate superoxide radical anions within cells. While ethanol appears to cause toxic effect through acetaldehyde and ROS produced during its metabolism (Costa *et al*., 1993, 1997), a rise of temperature enhances the uptake of oxygen and a reactivity increase of superoxide radical anion which serve as a stimulant for ROS production and oxidative damages (Piper, 1995). Thus, we examined whether disruption or overexpression of *SOD2* affects the growth of *C. albicans* cells under those stresses. For this purpose, wild-type, *sod2/sod2* or *SOD2*-overexpressing cells were spotted on the indicated solid SD media and treated with various stresses including redox-cycling agents (50 μM menadione and 1.5 mM paraquat), high temperature (43 °C) or 6% ethanol. The *sod2/sod2* mutant of *C. albicans* was more sensitive to those stresses than the wild-type and *SOD2*-overexpressed cells (Figure 3).

Mitochondrial MnSOD inversely affects lithium or diamide toxicity to *C. albicans*

High concentration of salts causes cells to carry not only osmotic stress but also oxidative injury. Recent genetic study of MnSOD in *Schizosaccharomyces pombe* has shown that the *sod2* mutant is hypersensitive to elevated concentration of salt (Jeong *et al*., 2001). To investigate the effect of mitochondrial MnSOD on the growth of *C. albicans* under salt stress, the wild-type, *sod2/sod2* and *SOD2*-overexpressing strains were spotted on the solid SD media including 1.5 M NaCl, 1.5 M KCl or 0.15 M LiCl, respectively. On these media, challenged with high concentrations of either sodium or potassium, the growth of *sod2/sod2* mutant was slightly retarded compared with those of the wild-type or *SOD2*-overexpressing strains (Figure 4). However, the *sod2/sod2* mutant was rather resistant to lithium, unlike sodium or potassium or the wild-type strain. Moreover, the overexpression of *SOD2* resulted in cells that were more sensitive to lithium, indicating that mitochondrial MnSOD was inversely related to the lithium toxicity of *C. albicans* (Figure 4) and osmotic stress appeared not to explain these phenomena. Interestingly, such an effect of mitochondrial MnSOD on lithium toxicity was not observed when *C. albicans* cells were grown on SC medium, which contains various amino acids (data not shown). Hence, we next tested which amino acids could compensate for the cytotoxic effect of lithium affected by the expression of mitochondrial MnSOD. The supplementation with methionine or cysteine could relieve the resistance of *sod2/sod2* mutant and the susceptibility of *SOD2*-overexpressing cells toward lithium on SD medium, while the media with non-sulphur amino acids failed (Figure 5A). Additionally, glutathione or sulphite also could compensate for the inverse effect of mitochondrial MnSOD on lithium toxicity observed (data not shown).

Diamide is a thiol-specific oxidant that can readily oxidize reduced glutathione (GSH) (O'Brien, 1970). Interestingly, the *sod2/sod2* mutant was also more resistant against the treatment of diamide

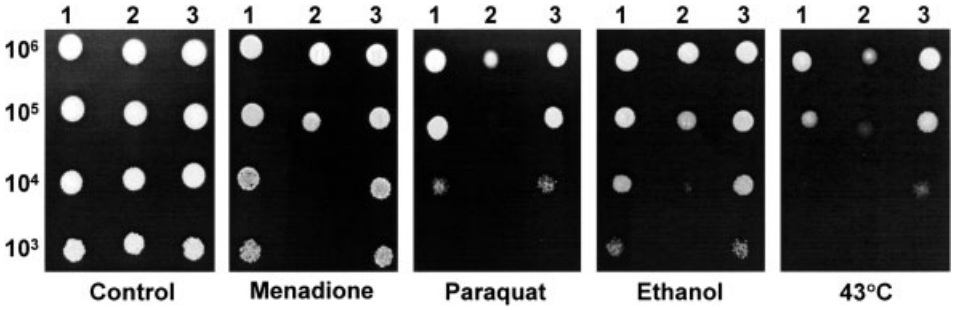

Figure 3. Effect of various stresses on the growth of *sod2/sod2* or *SOD2*-overexpressing *C. albicans* strains. After 10-fold serial dilutions as described in Materials and methods, the wild-type, *sod2/sod2* or *SOD2*-overexpressing cells were spotted onto SD plates with either 50 μM menadione, 0.5 mM paraquat or 6% ethanol, and incubated at 28 °C for 2 days. To test the effect of heat on the growth, each strain was incubated on SD plate at 43 °C for 2 days. The number of spotted cells is represented to the left of the photographs. Lane 1, CAI4(pYPB1–ADHpL); lane 2, CH204(pYPB1–ADHpL) and lane 3, CAI4(pASOD2)

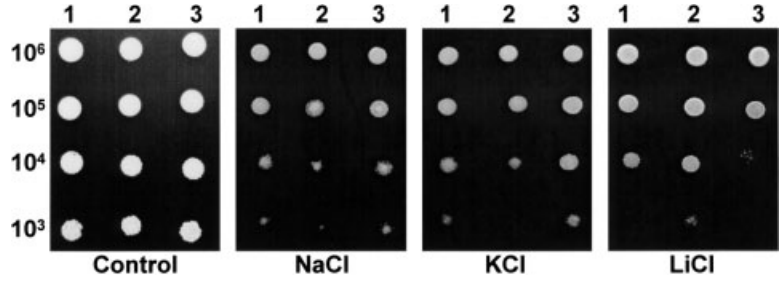

Figure 4. Effect of various salts on the growth of *sod2/sod2* or *SOD2*-overexpressing *C. albicans* strains. After 10-fold serial dilutions as described in Materials and methods, the wild-type, *sod2/sod2, SOD2*-overexpressing cells were spotted onto SD plates containing 1.5 M NaCl, 1.5 M KCl or 0.15 M LiCl and incubated at 28 °C for 3 days. The number of spotted cells is represented to the left of the photographs. Lane 1, CAI4(pYPB1–ADHpL); lane 2, CH204(pYPB1–ADHpL) and lane 3, CAI4(pASOD2)

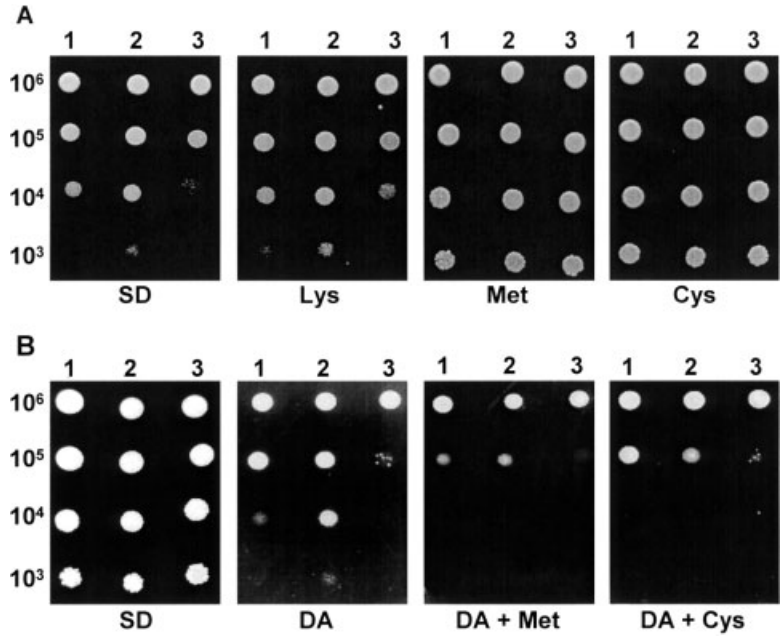

Figure 5. Effect of mitochondrial MnSOD on lithium or diamide toxicity. (A) To test the effect of sulphur compounds on lithium toxicity by mitochondrial MnSOD, the wild-type, *sod2/sod2* or *SOD2*-overexpressing cells, after 10-fold serial dilutions as described in Materials and methods, were spotted onto SD plates with or without either lysine (25 μg/ml), methionine (25 μg/ml) or cysteine (25 μg/ml), and incubated 28 °C for 3 days. Lysine was shown as a representative of non-sulphur amino acids. All these plates contained 0.15 M LiCl. (B) To test the effect of diamide (DA) on the *SOD2*-disrupted or -overexpressing *C. albicans* cells, each strain was spotted onto SD plates with or without either methionine (25 μg/ml) or cysteine (25 μg/ml) in the presence of 1.5 mM diamide at 28 °C for 2 days. The number of spotted cells was represented to the left of the photographs. Lane 1, CAI4(pYPB1–ADHpL); lane 2, CH204(pYPB1–ADHpL); and lane 3, CAI4(pASOD2)

than the wild-type strain, whereas the *SOD2*-overexpressing cells were slightly sensitive to this chemical (Figure 5). Moreover, the *sod2/sod2* mutant increased to 153.4% in glutathione reductase activity, a primary enzyme that reduces oxidized glutathione (GSSG), compared to the wild-type cell, while *SOD2*-overexpressing rather decreased to 85.6% (Figure 6). Furthermore, overexpression of glutathione reductase in *C. albicans* also showed resistance to diamide, but not to lithium, indicating that the resistance of *sod2/sod2* mutant to diamide resulted in part from the

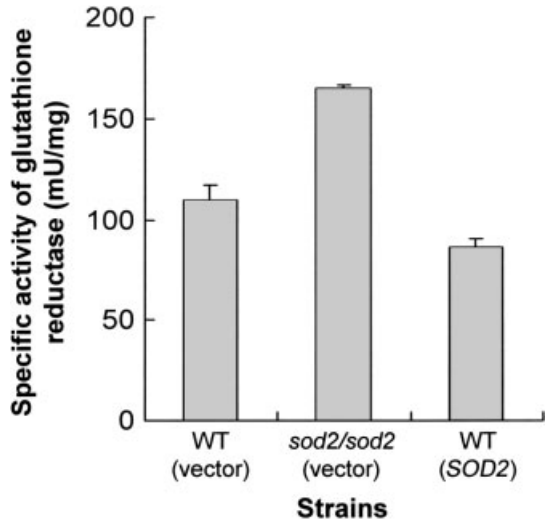

Figure 6. Effect of mitochondrial MnSOD on glutathione reductase activity. After the wild-type [CAI4(pYPB1–ADHpL)], *sod2/sod2* [CH204(pYPB1–ADHpL)] or *SOD2*-overexpressing [CAI4(pASOD2)] *C. albicans* strains were grown exponentially in SD medium, glutathione reductase activity was assayed as described in Materials and methods. Data represent the means ± standard error of three independent experiments

increased glutathione reductase activity (Baek, personal communication). In contrast to the situation with lithium treatment, the resistance of *sod2/sod2* mutant to diamide rather decreased on SD media supplemented with sulphur-amino acids (methionine or cysteine). More interestingly, the *sod1/sod1* mutant also showed the resistance to lithium or diamide (data not shown), indicating that CuZnSOD as well as mitochondrial MnSOD is related to the toxic effect of these compounds. Taken altogether, it is suggested that mitochondrial MnSOD and CuZnSOD of *C. albicans* seem to be linked to lithium or diamide toxicity.

Mitochondrial MnSOD is uniquely essential for the growth of *C. albicans* under hyperoxic condition

In *S. cerevisiae, sod2* mutant is little affected when grown with glucose as the carbon source, but highly sensitive to elevated oxygen tension (van Loon *et al*., 1986). Likewise, the *sod2/sod2* mutant of *C. albicans* showed a growth rate comparable to the parental strain in air (Figure 7). Thus, to determine the effect of *sod2*-mutation on the growth under hyperoxic conditions, wild-type, heterozygote and *sod2/sod2* mutant cells were grown in an anaerobic jar filled with 99.9% O_2. As shown in Figure 7, the *sod2/sod2* mutant failed to grow under that condition. Moreover, the episomal expression of *SOD2* in *sod2/sod2* mutant restored defective growth, indicating that mitochondrial MnSOD is required for the protection of cells from elevated oxygen tension. In contrast, the *sod1/sod1* or *sod3/sod3* strains lacking CuZnSOD or cytosolic MnSOD, respectively, grew as well as wild-type under this hyperoxic condition (data not shown), revealing that mitochondrial MnSOD among SODs of *C. albicans* is exclusively required for growth under hyperoxic conditions.

EASC functions as an important antioxidant molecule in *S. cerevisiae* (Huh *et al*., 1998) and *C. albicans* (Huh and Kang, 2001) and its biosynthesis in *C. albicans* is catalysed by ALO, which is localized within mitochondria (Huh *et al*., 1994). To investigate whether EASC restores the defective growth of *sod2/sod2* mutant under hyperoxic

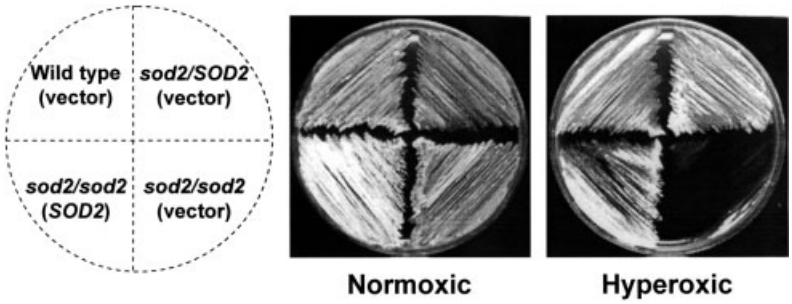

Figure 7. Growth defect of *sod2/sod2* mutant under hyperoxic conditions. The wild-type CAI4, heterozygote CH202 or *sod2/sod2* CH204 strains were transformed with the control pYPB1–ADHpL vector and also the *sod2/sod2* CH204 strain with pASOD2. The indicated strains were streaked on SD plates and then incubated for 3 days at 28 °C in air (20.7% O_2, normoxic) or in an anaerobic jar filled with 99.9% O_2 (hyperoxic)

conditions, the multi-copy plasmid pWK203 (Huh *et al*., 2001) overexpressing ALO was transformed in *sod2/sod2* mutant CH204. Although the ALO activity in *sod2/sod2* mutant increased to 2.1-fold, the overexpression of this enzyme related to oxidative stresses within mitochondria could not rescue the defective growth by *sod2* mutation under 99.9% O_2 (data not shown). Taken together, these results indicate that mitochondrial MnSOD appears to be essential for the survival of *C. albicans* against the elevated oxygen tension.

Mitochondrial MnSOD regulates cyanide-resistant respiration of *C. albicans*

Due to its localization, mitochondrial MnSOD seems to be related to the respiratory chain. Particularly, *C. albicans* carries a cyanide-resistant respiratory pathway mediated by AOX as well as cytochrome-involved pathway (Huh and Kang, 1999, 2001). We previously showed that AOX activity and its gene expression of *C. albicans* increased after the treatment of menadione and paraquat (Huh and Kang, 2001), which are known to generate superoxide radical anions. Based on this fact, it is suggested that AOX may play a role as an antioxidant defence mechanism by keeping the ubiquinone pool sufficiently oxidized to prevent the auto-oxidation of reduced quinone and the subsequent formation of ROS, when electron transport flow through the cytochrome pathway becomes limited (Wagner and Moore, 1997). Therefore, to examine the effect of disruption or overexpression of *SOD2* on the respiration of *C. albicans*, mid-exponentially growing wild-type, *sod2/sod2* or *SOD2*-overexpressing cells were treated with or without 1 mM cyanide, and then the oxygen consumption rates were assayed. The total respiration rates of these strains in the absence of cyanide were little influenced by the disruption or overexpression of MnSOD (Figure 8A). However, the cyanide-resistant respiration of the *sod2/sod2* or *SOD2*-overexpressing strains increased or decreased to 168.7% or 61.5% compared to that of the wild-type, respectively (Figure 8A). In accordance with the measurement of respiration, Western blot analysis using the antibody against AOX from *S. guttatum* (Elthon *et al*., 1989) revealed that the AOX with a molecular mass of 39.2 kDa from *C. albicans* (Huh and Kang, 1999) inversely expressed by mitochondrial MnSOD (Figure 8B).

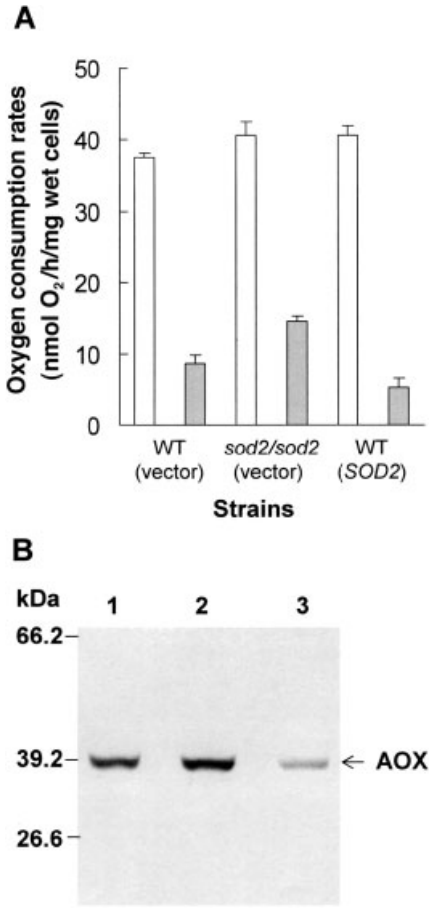

Figure 8. Respiratory characteristics of the *sod2/sod2* or *SOD2*-overexpressing *C. albicans* strains. (A) Oxygen consumption rates of wild-type, *sod2/sod2* or *SOD2*-overexpressing strains were measured in the absence (open bar) or in the presence (closed bar) of 1 mM KCN. Data shown represent means ± standard error of three independent experiments. (B) Western blot analysis of AOX from wild-type, *sod2/sod2* or *SOD2*-overexpressing cells. Lane 1, CAI4(pYPB1–ADHpL); lane 2, CH204(pYPB1–ADHpL) and lane 3, CAI4(pASOD2)

These results indicate that mitochondrial MnSOD not only removes superoxide radical anion but also modulates AOX expression through the control of its level within mitochondria and that AOX functions as an antioxidant enzyme against ROS produced in mitochondria.

Discussion

In the present study, we investigated the roles of mitochondrial MnSOD in *C. albicans* through

disruption or overexpression of its gene, *SOD2*. As expected, deletion of *SOD2* rendered *C. albicans* cells more susceptible to redox-cycling agents, high concentration of salts (potassium or sodium), 6% ethanol, elevated temperature (43 °C) or 99.9% oxygen. In addition, inactivation of MnSOD in *S. cerevisiae* (Costa *et al*., 1993, 1997; Davidson *et al*., 1996; Pereira *et al*., 2001) and *Sz. pombe* (Jeong *et al*., 2001) also results in increased sensitivity toward those various stresses. Mitochondrial MnSOD of *C. albicans* is accordingly required for protecting cells from oxidative damages generated by various external stresses like other yeasts. However, the *SOD2*-overexpressing cells, which retained increased mitochondrial MnSOD activity clearly (Figure 2), showed few different phenotypes from wild-type in response to these stresses (Figures 3 and 4). Indeed, the lack of apparent phenotypes to those oxidative stresses by mitochondrial MnSOD overexpression, in comparison with its disruption, seems to result in part from the nature of SOD, which disproportionates superoxide radical anions into dioxygen and hydrogen peroxide. One product of this reaction, hydrogen peroxide, is freely diffusible and fairly reactive and may generate a different spectrum of cellular damage relative to the superoxide radical anion. Thus, the disadvantages inherent from hydrogen peroxide production by SOD appear to counteract the advantages obtained from the removal of superoxide radical anions. Even in the case of cultured rat glioma cells, it is reported that MnSOD overexpression made cells more sensitive to injuries by radiation and carcinogens, suggesting that overexpression of MnSOD is as problematic as its deficiency (Li *et al*, 1998; Zhong *et al*., 1996). Alternatively, the anti-parallel decrease of other enzyme activity by MnSOD overexpression may contribute to indistinguishable phenotypes between the wild-type and *SOD2*-overexpressing cells against those stresses described above; particularly, glutathione reductase and alternative oxidase activities were significantly reduced when the mitochondrial MnSOD was overexpressed in *C. albicans* (Figures 6 and 8).

It is very interesting that, despite sensitivity to most of oxidative stresses, the *sod2/sod2* mutants are resistant to lithium and diamide, whereas the *SOD2*-overexpressing cells show slight sensitivity to them in comparison to wild-type strain (Figures 4 and 5). Reportedly, lithium toxicity appears related to sulphur metabolism in other yeasts. For example, the supplementation of methionine or sulphite could suppress the defective growth of *S. cerevisiae* (Dichtl *et al*., 1997) or *Sz. pombe* (Miyamoto *et al*., 2000) caused by lithium toxicity, respectively, and genetic studies also have revealed that lithium is involved in the accumulation of adenosine-3′,5′-bisphosphate (pAp), a side product of sulphur assimilation, which is highly toxic to cells (Dichtl *et al*., 1997; Miyamoto *et al*., 2000). Similarly, the resistance of *sod2/sod2* cells to lithium could be relieved by the supplementation with sulphur compounds (methionine, cysteine or sulphite), which repress sulphate assimilatory pathway and thereby block the accumulation of pAp (Dichtl *et al*., 1997). Thus, it is conceivable that excess or deficient mitochondrial MnSOD may directly or indirectly control the accumulation or degradation of pAp. However, it can not exclude other possibilities that the expression of *SOD2* inversely affects some lithium-inhibited targets, such as inositol monophosphatases (Berridge *et al*., 1989) and phosphoglucomutase (Masuda *et al*., 2001).

Several lines of evidence indicate that the resistance of *sod2/sod2* cells to diamide may be linked to glutathione metabolism. First, glutathione reductase activity was enhanced in the *sod2/sod2* mutant, whereas it was slightly reduced in *SOD2*-overexpressing cells. In addition, overexpression of glutathione reductase in *C. albicans* made cells resistant to diamide (data not shown). Second, the resistance of *sod2/sod2* mutant to diamide was significantly diminished by the supplementation of sulphur amino acids (Figure 5). In *S. cerevisiae*, a direct link between the metabolism of sulphur amino acids metabolism and glutathione biosynthesis is demonstrated by the result that transcription factors regulating the methionine biosynthetic pathway are also responsible for the repression of glutathione synthesis (Dormer *et al*. 2000). Consequently, it is possible that sulphur amino acids render the *sod2/sod2* cells sensitive to diamide through the repression of glutathione synthesis. Third, it seems likely that *SOD2* expression is inversely related to total glutathione levels, considering the report that *S. cerevisiae* mutants lacking glutaredoxins or thioredoxins show resistance to diamide due to elevated

glutathione levels (Eleanor *et al*., 2002). Nonetheless, in addition to glutathione metabolism, some unknown factors of the oxidative stress response controlled by *SOD2* expression can be important for resistance to diamide, since even in *S. cerevisiae* the *gsh1* mutant lacking glutathione is more resistant to diamide than wild-type (Grant *et al*., 1997). Furthermore, genome-wide study in *S. cerevisiae* shows that diamide induced various genes involved in protein folding, respiration, cell wall synthesis and secretion and processing in the endoplasmic reticulum as well as oxidative stresses (Gasch *et al*., 2000). The level of *SOD2* expression in *C. albicans*, therefore, seems to be associated with other pleiotropic effects of diamide in addition to oxidative stresses. Although lithium and diamide had similar effects on *SOD2*-disrupted or -overexpressing cells, the underlying mechanisms seem to be different; sulphur amino acids reduced the resistance of *sod2/sod2* mutant to diamide in contrast to lithium, and the overexpression of glutathione reductase did not confer a resistance to lithium to *C. albicans* cells, unlike diamide. Further detailed investigation is therefore required to elucidate the relationship between mitochondrial MnSOD and lithium or/and diamide toxicity.

Many studies have shown that *AOX* gene expression is induced by respiratory inhibitors and ROS, such as H_2O_2 and superoxide radical anions (Minagawa, *et al*., 1992; Wagner and Moore, 1997). Interestingly, *Candida albicans* bears the *AOX* gene family consisting of *AOX1a* (Huh and Kang, 1999) and *AOX1b* (Huh and Kang, 2001). β-galactosidase reporter assays showed that, whereas *AOX1a* is expressed constitutively, the expression of *AOX1b* is induced in response to growth phase or by oxidants and respiratory inhibitors (Huh and Kang, 2001).Therefore, the change of AOX expression in *SOD2*-disrupted or -overexpressing cells seems to be relevant to *AOX1b* induction. Unlike the situation with plant AOX (Maxwell *et al*., 1999), the disruption or overexpression of *AOX* did not cause any difference in intracellular levels of ROS within *C. albicans* (Huh and Kang, 2001). Moreover, disruption and overexpression of *AOX* did not have any effect on the resistance of *C. albicans* to oxidants, suggesting that some antioxidant enzymes scavenging ROS may be coordinately regulated by AOX expression (Huh and Kang, 2001). Nonetheless, the anti-parallel expression of AOX by mitochondrial MnSOD raises the likelihood that AOX can play an important role as an antioxidant defence system to cope with an abrupt increase of ROS, particularly when the activities of antioxidant enzymes such as mitochondrial MnSOD are impaired.

In conclusion, our present study shows that mitochondrial MnSOD is required for *C. albicans* to resist various external stresses, such as redox-cycling agents, high concentration of salts, ethanol or hyperoxic conditions. We have also demonstrated that mitochondrial MnSOD inversely affects lithium or diamide toxicity and the cyanide-resistant respiration of *C. albicans*. Therefore, our results would provide an important clue for understanding the roles of mitochondrial MnSOD in stress responses, lithium toxicity and cyanide-resistant respiration of *C. albicans*.

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