Superoxide radicals have a protective role during H_2O_2 stress

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ABSTRACT **Reactive oxygen species (ROS) consist of potentially toxic, partly reduced oxygen species and free radicals. After H_2O_2 treatment, yeast cells significantly increase superoxide radical production. Respiratory chain complex III and possibly cytochrome *b* function are essential for this increase. Disruption of complex III renders cells sensitive to H_2O_2 but not to the superoxide radical generator menadione. Of interest, the same H_2O_2-sensitive mutant strains have the lowest superoxide radical levels, and strains with the highest resistance to H_2O_2 have the highest levels of superoxide radicals. Consistent with this correlation, overexpression of superoxide dismutase increases sensitivity to H_2O_2, and this phenotype is partially rescued by addition of small concentrations of menadione. Small increases in levels of mitochondrially produced superoxide radicals have a protective effect during H_2O_2-induced stress, and in response to H_2O_2, the wild-type strain increases superoxide radical production to activate this defense mechanism. This provides a direct link between complex III as the main source of ROS and its role in defense against ROS. High levels of the superoxide radical are still toxic. These opposing, concentration-dependent roles of the superoxide radical comprise a form of hormesis and show one ROS having a hormetic effect on the toxicity of another.**

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INTRODUCTION

Most organisms rely on the role of oxygen as a terminal electron acceptor for efficient energy production in the form of ATP. Increased intracellular levels of oxygen, however, are potentially toxic. This toxicity is mainly due to partially reduced forms of O_2 (Gille and Sigler, 1995), since the O_2 molecule per se has low reactivity (Halliwell and Gutteridge, 1990). The molecules and radicals formed by the incomplete reduction of oxygen are termed reactive oxygen species (ROS; Halliwell and Gutteridge, 1989). ROS commonly formed in vivo include the superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$), and their formation can result in damage to proteins, lipids, and nucleic acids (Aung-Htut *et al.*, 2012).

The first intermediate in the sequential reduction of oxygen is often $O_2^{\bullet-}$. This is produced by single-electron reduction of oxygen to a large extent by electron carriers of the respiratory chain in the mitochondria (Lambert and Brand, 2004). Systems that produce $O_2^{\bullet-}$ also produce H_2O_2 as a result of disproportionation reactions (Gille and Sigler, 1995). These reactions are among the main sources of H_2O_2 in vivo and are either nonenzymatic or catalyzed by superoxide dismutases (SODs). In *Saccharomyces cerevisiae* the *SOD1* gene encodes a copper- and zinc-containing enzyme located in the cytoplasm and mitochondrial intermembrane space. *SOD2* encodes a manganese-containing enzyme found in the mitochondrial matrix (Bermingham-McDonogh *et al.*, 1988; Gralla and Kosman, 1992; O'Brien *et al.*, 2004). The main threat to the cell is the subsequent transformation of $O_2^{\bullet-}$ and H_2O_2 to stronger oxidants, in particular the hydroxyl radical, which is the most oxidizing radical known to arise in biological systems (Youngman, 1984; Buettner, 1993).

The incomplete reduction of O_2 primarily occurs at two sites in the respiratory chain: complex I at NADH dehydrogenase (Turrens and Boveris, 1980) and complex III at ubisemiquinone (Boveris *et al.*, 1976; Cadenas *et al.*, 1977; Turrens *et al.*, 1985). It is widely

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Abbreviations used: DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance spectroscopy; ETC, electron transport chain; $O_2^{\bullet-}$, superoxide radical anion; ROS, reactive oxygen species; SOD, superoxide dismutase; WT, wild type.

accepted that radicals derived from the partial reduction of ubiquinone in complex III are the main generators of ROS during respiration. This principally occurs at the semiquinone at the center *o* (Q_o) electron carrier in complex III (Trumpower, 2002; Andreyev *et al.*, 2005). Several studies showed that the complex I and III sites generate mostly $O_2^{\bullet-}$ rather than a mixture of partially reduced species (Turrens, 1997).

S. cerevisiae is a useful tool in the study of mitochondrial function because mutations in mitochondrial DNA or deletion of nuclear genes encoding mitochondrial proteins are not lethal. Although there are differences between the electron transport chain (ETC) of mammals and yeast, mostly at complex I, $O_2^{\bullet-}$ production at complex III results from similar mechanisms in yeast and mammals (Sun and Trumpower, 2003).

Yeast cells have evolved complex defense mechanisms to protect themselves against ROS. These mechanisms use small molecules such as glutathione and D-erythroascorbate and enzymatic defenses such as SOD, catalase, thioredoxins, and glutathione peroxidases, as reviewed in Temple (2005). These defenses are often inducible and are activated by transcription factors such as those encoded by *YAP1* and *SKN7*, forming part of the environmental stress response (Gasch *et al.*, 2000). Other active responses the cell can mount to ROS damage include adaptation, in which exposure to a non-LD of ROS confers resistance to a subsequent and normally LD, and cell cycle delay (Collinson and Dawes, 1992; Flattery-O'Brien *et al.*, 1993; Alic *et al.*, 2001, 2004). These responses have also been linked to upstream transcription factors and signaling pathways such as those encoded by *SWI6*, *YAP1*, *SKN7*, *MPK1*, *ROX1*, and *MGA2* (Alic *et al.*, 2003; Beckhouse *et al.*, 2008; Fong *et al.*, 2008; Ng *et al.*, 2008; Kelley and Ideker, 2009).

The importance of mitochondrial function during oxidative stress has been demonstrated in many studies. ρ^0 petite strains, which contain no mitochondrial DNA, are sensitive to oxidative stress caused by H_2O_2 and $O_2^{\bullet-}$ when compared with their isogenic wild type (WT; Collinson and Dawes, 1992; Jamieson, 1992; Flattery-O'Brien *et al.*, 1993; Lee *et al.*, 2001). Furthermore, strains with deletions in nuclear genes encoding components of the respiratory chain and WT strains treated with respiratory inhibitors are sensitive to H_2O_2 (Grant *et al.*, 1997). This has been highlighted in a global genome-wide study in which nearly half the H_2O_2-sensitive mutants were deficient in electron transport chain function (Thorpe *et al.*, 2004). Of interest, this was specific to H_2O_2-induced stress and not the other oxidative stress conditions examined, suggesting that complex III may have a specific and central role in survival during H_2O_2 stress.

ROS have been implicated in aging in yeast, with replicatively aged cells showing markers of oxidative stress (Laun *et al.*, 2001). H_2O_2 activates a caspase-like enzyme in yeast that regulates apoptosis, and oxidative stress is intimately involved in the activation and progression of apoptosis in yeast (Madeo *et al.*, 1997, 1999, 2002). This is consistent with the widely held view that ROS and oxidative stress are always harmful to cells. Recent results regarding the role of ROS in chronological aging, however, have offered apparently conflicting views on their contribution. It has been proposed that superoxide radicals mediate chronological aging, whereas it has been suggested that H_2O_2 has a positive effect, increasing chronological lifespan (Mesquita *et al.*, 2010; Weinberger *et al.*, 2010; Lewinska *et al.*, 2011; Pan *et al.*, 2011). To fully understand this, more research into the complex roles of different ROS in the cell is needed to elucidate their exact contribution to cell viability in different conditions.

We therefore sought to determine the role of the mitochondrial electron transport chain and the ROS it produces ($O_2^{\bullet-}$) in the tolerance of H_2O_2-induced oxidative stress in *S. cerevisiae*. The results of these experiments show that mitochondrially produced $O_2^{\bullet-}$ is protective against H_2O_2, providing the first link between complex III as the main source of ROS in the cell and protection against ROS.

RESULTS

H_2O_2 stress increases superoxide radical levels in a mitochondrial-dependent manner

Mitochondrial function is crucial for the survival of yeast treated with H_2O_2 despite mitochondria being a main source of $O_2^{\bullet-}$ production. To investigate the relationship between mitochondrial function and $O_2^{\bullet-}$ levels in cells during H_2O_2-induced oxidative stress, we used electron paramagnetic resonance spectroscopy (EPR) spin trapping using the trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), a method that gives very specific signals with $O_2^{\bullet-}$ (Frejaville *et al.*, 1995).

Cells were grown in yeast extract/peptone/dextrose (YEPD) liquid to late exponential phase (OD_{600} of 4). Half were treated with 4 mM H_2O_2 for 60 min, and cells in both sample and control cultures were washed and incubated with DEPMPO. EPR spectra were obtained on the culture supernatant. Treatment of the WT with H_2O_2 increased superoxide levels to nearly three times that of the untreated control (Figure 1). This was in contrast to the isogenic WT ρ^0 strain, in which H_2O_2 treatment resulted in no increase in $O_2^{\bullet-}$ levels, demonstrating that yeast cells need respiration to increase $O_2^{\bullet-}$ levels in response to H_2O_2 stress. The mitochondrial genome contains only genes that encode proteins that are part of the ETC or transcripts that are required for their synthesis. Consequently, the complexes in the ETC may be the mitochondrial function that is involved in $O_2^{\bullet-}$ production in response to H_2O_2.

The superoxide radical levels observed during H_2O_2-induced oxidative stress depend on cytochrome *b*

The ETC in mitochondria is a major source of endogenous ROS in cells, and since the increase in $O_2^{\bullet-}$ levels in the WT treated with H_2O_2 depended on functional mitochondria, we sought to further investigate their role.

Representative strains covering deficiencies in all complexes in the ETC were chosen, and their $O_2^{\bullet-}$ levels were determined in the absence of added H_2O_2. In general, strains lacking genes encoding for upstream components of the electron transport chain (NADH

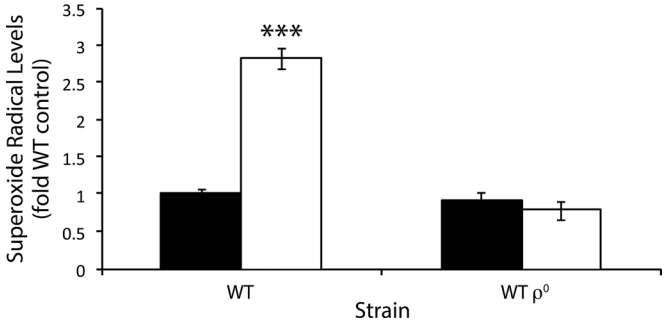

FIGURE 1: Superoxide radical production in the wild type and a petite mutant during oxidative stress. $O_2^{\bullet-}$ was assayed in vivo by EPR using the spin trap DEPMPO and is expressed relative to the unstressed WT cells. Cells were grown in YEPD liquid to late exponential phase (OD_{600} of 4), half were treated with 4 mM H_2O_2 for 60 min, and then cells were washed and incubated with DEPMPO for 20 min. The supernatant was then taken for EPR analysis. Black bars represent the control; white bars represent treatment with H_2O_2. H_2O_2 was compared with the control, ***$p < 0.001$.

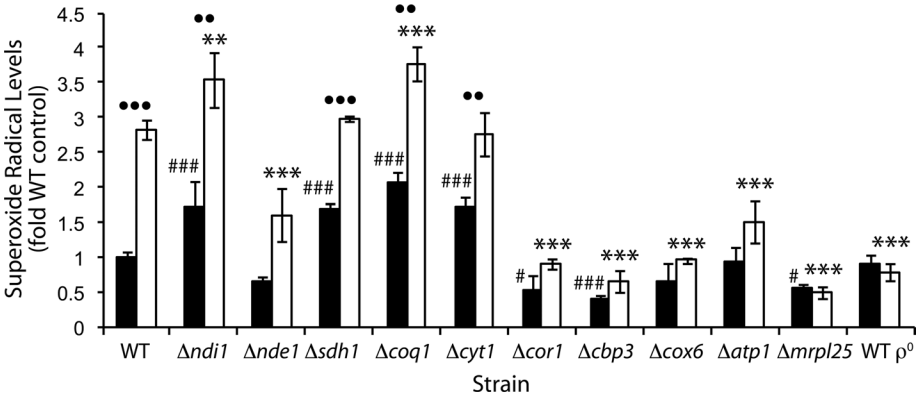

FIGURE 2: Superoxide radical production in electron transport chain mutants during oxidative stress. $O_2^{\bullet-}$ was assayed in vivo by EPR using the spin trap DEPMPO and is expressed relative to the unstressed WT. Cells were grown in YEPD liquid to late exponential phase (OD_{600} of 4), half were treated with 4 mM H_2O_2 for 60 min, and then cells were washed and incubated with DEPMPO for 20 min. The supernatant was then taken for EPR analysis. Black bars represent the untreated control sample; white bars represent treatment with H_2O_2. In the statistical analysis, mutant strains in control conditions were compared with the WT in control conditions (number sign or hash symbol), H_2O_2 treated strains were compared with the H_2O_2-treated WT (asterisks), and for each strain, H_2O_2 treatment was compared with no treatment (dots).

dehydrogenase, complex II, and ubiquinone) had increased levels of $O_2^{\bullet-}$ when compared with the WT, whereas strains lacking downstream components (complexes III–V) had reduced levels (Figure 2). Deletion of *NDI1* and *SDH1* resulted in a significant increase in $O_2^{\bullet-}$. This may have occurred due to the electron carriers in the ETC being in a more reduced state, since lack of NADH and succinate dehydrogenase activity may result in reduced flux of electron flow into the ETC. The strain deleted for *COQ1*, which encodes the protein responsible for the first step of ubiquinone biosynthesis, also showed high $O_2^{\bullet-}$ levels. The increased $O_2^{\bullet-}$ levels may be a consequence of the absence of antioxidant activity of ubiquinol, increased superoxide production, or a combination of both. Because the *coq1Δ* strain has no ubiquinol to carry electrons to complex III, complex II and the internal and external NADH dehydrogenases may be the sites of $O_2^{\bullet-}$ production.

The $O_2^{\bullet-}$ levels in the strain deleted for *ATP1* were not significantly different from those in the WT. The *atp1Δ* mutant contains the full complement of genes encoding electron-carrying respiratory complexes but cannot use the membrane potential created to produce ATP. This reinforces the view that the ETC function responsible for $O_2^{\bullet-}$ production comes from the transfer of electrons from NADH and succinate to O_2 rather than the production of ATP.

Complex III was essential for normal $O_2^{\bullet-}$ production in *S. cerevisiae*, since removal of either function resulted in the lowest levels of $O_2^{\bullet-}$ (Figure 2). Two of the three deletion strains that are specifically affected in complex III assembly or function (*cor1Δ* and *cbp3Δ*) had significantly reduced levels of $O_2^{\bullet-}$. In contrast, the strain deleted for *CYT1*, which encodes cytochrome c_1, had significantly increased levels of $O_2^{\bullet-}$. The low $O_2^{\bullet-}$ levels in the *cor1Δ* and *cbp3Δ* strains cannot be explained by the abolition of respiration, since the *cyt1Δ* strain, which is also respiratory deficient, had increased levels of $O_2^{\bullet-}$.

Of interest, the *cor1Δ* and *cbp3Δ* mutants that had low $O_2^{\bullet-}$ levels are deficient in cytochrome *b* function or assembly (Tzagoloff *et al.*, 1986; Gruschke *et al.*, 2011), whereas the *cyt1Δ* strain that did not have reduced superoxide levels is not known to be deficient in cytochrome *b*. Consistent with this, the *mrpl25Δ* strain, which cannot express the mitochondrially encoded cytochrome *b* gene (*COB*), also had reduced levels of $O_2^{\bullet-}$. This strongly indicates that $O_2^{\bullet-}$ production in these strains may depend on cytochrome *b*.

The superoxide levels in the wild type and the foregoing mutants were also determined after exposure to 4 mM H_2O_2 for 60 min. For some mutants this treatment did lead to loss of longer-term viability (in some mutants to as low as 4% survival vs. 57% survival for the wild type) as determined by ability of cells to replicate (by estimation of colony-forming units) but not to loss of viability in the time span used to measure superoxide production, as determined by membrane integrity measurements based on propidium iodide staining (see Supplemental Table S1). During H_2O_2-induced oxidative stress the *cor1Δ*, *cbp3Δ*, *cox6 Δ*, *atp1Δ*, *mrpl25Δ*, and WT ρ^0 strains all showed a significant reduction in $O_2^{\bullet-}$ levels compared with the WT in the same conditions (Figure 2). These strains are affected in the function of complexes III–V. Strains with *NDI1*, *SDH1*, *COQ1*, and *CYT1* deleted had either increased or no significant difference in their $O_2^{\bullet-}$ levels compared with the WT. Therefore H_2O_2 stress resulted in the same general pattern as observed in the absence of stress (Figure 2), that is, higher $O_2^{\bullet-}$ levels in strains deleted for upstream components of the ETC and lower $O_2^{\bullet-}$ levels in strains deleted for downstream components.

The WT and strains deleted for *NDI1*, *SDH1*, *COQ1*, and *CYT1* all had significant increases in $O_2^{\bullet-}$ levels after H_2O_2 treatment when compared with unstressed conditions for each strain (Figure 2). All other strains did not significantly increase their $O_2^{\bullet-}$ levels after H_2O_2 stress from their initial low levels. Because the strains with low superoxide levels could not significantly increase them in response to H_2O_2, it appears that the mechanisms responsible for unstressed $O_2^{\bullet-}$ levels, H_2O_2-treated $O_2^{\bullet-}$ levels, and increased $O_2^{\bullet-}$ levels in response to H_2O_2 stress all rely on complex III and possibly cytochrome *b*.

SOD gene expression does not account for differences in superoxide radical levels

One factor that may influence the $O_2^{\bullet-}$ levels measured in these mutant strains is the detoxification of $O_2^{\bullet-}$ by SOD. To determine whether changes in the amount of SOD due to increased transcription were responsible for the differences in $O_2^{\bullet-}$ levels in some mutants, we determined the transcript levels of the *SOD1* and *SOD2* genes (Figure 3). Differences in the extent of *SOD1* mRNA induction between mutant strains in both test conditions were relatively small. *SOD1* mRNA changes ranged from 0.65-fold for the unstressed *coq1Δ* strain to 1.5-fold for the unstressed *nde1Δ* strain. This strongly suggests that the *SOD1* mRNA levels were not having an effect on $O_2^{\bullet-}$ levels observed in Figure 2. A similar result was found in the case of *SOD2* mRNA, for which we observed small changes (Figure 3B). There was no consistent pattern showing that *SOD1* or *SOD2* mRNA levels were responsible for the differences in $O_2^{\bullet-}$ levels observed in the test strains. Often the opposite was observed, in which strains with lower levels of $O_2^{\bullet-}$ also had lower levels of SOD mRNA. Examples of this included the *cbp3Δ*, *cor1Δ*, and *mrpl25Δ* mutants, which had lower $O_2^{\bullet-}$ levels than the WT in the presence of H_2O_2 while also having slightly lower *SOD1* mRNA levels than the WT.

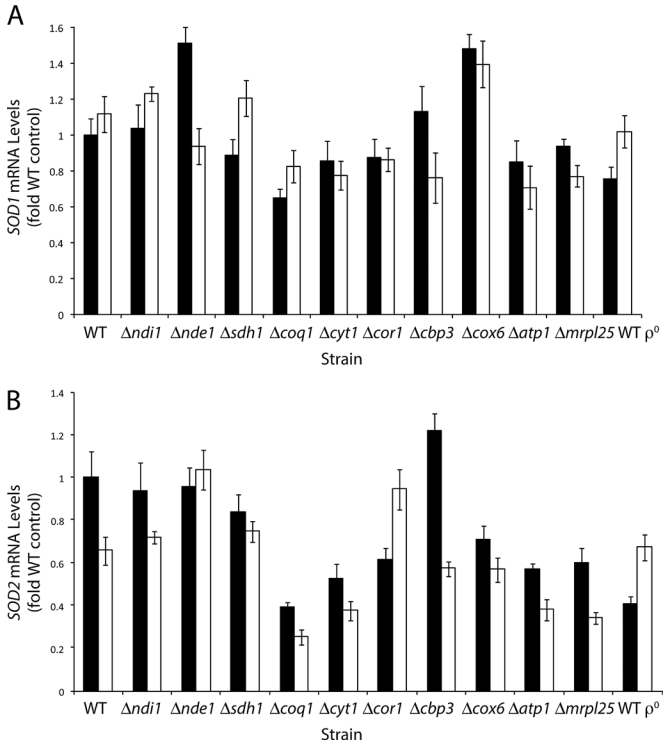

FIGURE 3: CuZnSOD and MnSOD mRNA transcript levels in electron transport chain mutants during oxidative stress. Cells were grown in YEPD to OD_{600} of 4 and then split into one control sample and one sample treated with 4 mM H_2O_2. After 15 min, total cellular RNA was extracted. Purified mRNA was reverse transcribed and quantitative PCR analysis was then performed on the genes (A) *SOD1* (CuZnSOD) and (B) *SOD2* (MnSOD). The relative amounts of the *SOD1* and *SOD2* transcripts were then normalized to the control *ACT1* signal. All mRNA levels are expressed as the multiple relative to the unstressed WT strain. Black bars represent the control; white bars represent treatment with H_2O_2.

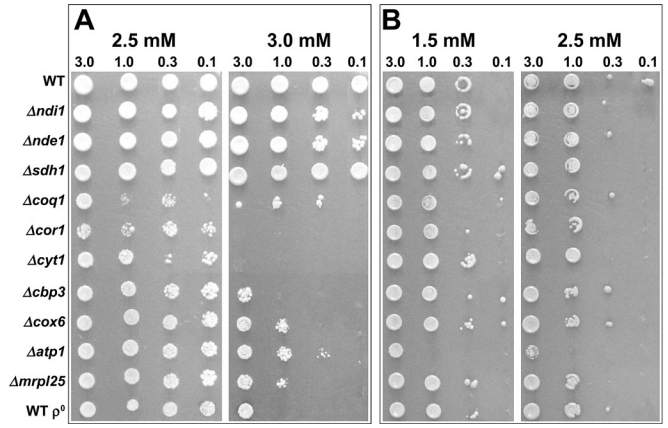

FIGURE 4: Sensitivity of electron transport chain mutants to oxidative stress. Strains were grown in liquid YEPD for 2 d and diluted in YEP to OD_{600} of 3, 1, 0.3, and 0.1. Then 5 µl was spotted onto SC agar containing (A) H_2O_2 and (B) menadione. The plates were incubated at 30°C for 2 d.

Strains deficient in complex III and IV function are sensitive to H_2O_2 but not to superoxide radicals

It would be expected that the increase in $O_2^{\bullet-}$ in the WT and some of the other strains after the addition of H_2O_2 would result in increased oxidative stress and damage to the cell. This is due to the generation of species such as the hydroxyl radical via Fenton chemistry. To investigate this further, we assayed the sensitivities of the ETC mutants to H_2O_2 and the $O_2^{\bullet-}$ generator menadione (Figure 4).

The mutants with different degrees of H_2O_2 sensitivity could be placed into three distinct functional groups: those with extreme sensitivity, high sensitivity, and little or no sensitivity. The strains most sensitive to H_2O_2 (Figure 4A) were those deleted for complex III subunits (*cor1Δ, cyt1Δ*) and those deficient in CoQ biosynthesis (*coq1Δ*). Sensitivity of ubiquinone-deficient mutants to oxidative stress may be due to loss of respiratory activity and the antioxidant function of the CoQ pool (Schultz and Clarke, 1999; James *et al.*, 2004). The next group of strains also showed a high degree of H_2O_2 sensitivity but not to the levels seen in the first group. The functions disrupted in these strains were complex III assembly (*cbp3Δ*), complex IV (*cox6Δ*), complex V (*atp1Δ*), and mitochondrial genome expression (*mrpl25Δ*, and WT ρ^0).

The strains in the group showing little or no H_2O_2 sensitivity were deleted for NADH dehydrogenase (*ndi1Δ* or *nde1Δ*) or succinate dehydrogenase (*sdh1Δ*) activity (Figure 4A). When electron transport is slowed (e.g., decreased supply of electrons into the electron transport chain), electron carriers are mostly reduced. This situation promotes the one-electron reduction of O_2, which increases ROS production (Andreyev *et al.*, 2005), although this does not appear to have any detrimental effect on the cell during H_2O_2-induced stress.

In all strains in which electron flow is completely abolished (*coq1Δ*, strains with mutations in complexes III and IV, and *mrpl25Δ* and WT ρ^0), an extreme or high degree of H_2O_2 sensitivity was observed (Figure 4A). Therefore the ETC is essential for WT H_2O_2 tolerance. The different degrees of sensitivity of these mutants—with complex III mutants the most sensitive—indicate that additional factors may be affecting sensitivity. In the presence of menadione, the *atp1Δ* strain showed the highest degree of sensitivity, whereas all other strains showed either little or no difference in sensitivity compared with the WT (Figure 4B). The differences in $O_2^{\bullet-}$ levels observed in Figure 2 did not seem to affect tolerance of these strains to menadione.

Survival of H_2O_2 treatment correlates with higher levels of superoxide radicals

To further investigate the relationship between levels of $O_2^{\bullet-}$ and resistance to oxidative stress, we compared $O_2^{\bullet-}$ levels with H_2O_2 sensitivity of each mutant. For H_2O_2 sensitivity, a numerical score was assigned based on growth in the spot test in Figure 4A, with the WT scoring 10 for maximum resistance. In Figure 5, H_2O_2 sensitivity (data from Figure 4A) is compared with the ratio of $O_2^{\bullet-}$ levels during H_2O_2 to control conditions for each strain. There was clearly no linear correlation, but the mutants fell into three distinct functional classes. Group A contained the strains deleted for *MRPL25* and the WT ρ^0, and both mutations result in deficient expression of the entire mitochondrial genome. Because they form a distinct group, the mechanisms by which these strains are sensitive to H_2O_2 may therefore be different from that of the other ETC deletion strains studied. Group B contained all of the other strains that were sensitive to H_2O_2, and these were deleted for genes required for the biosynthesis of ubiquinone and for function and assembly of complexes III–V of the ETC. Group C contained the WT and strains deficient in entry of NADH into the ETC from either side of the inner mitochondrial membrane and succinate dehydrogenase function. This group had

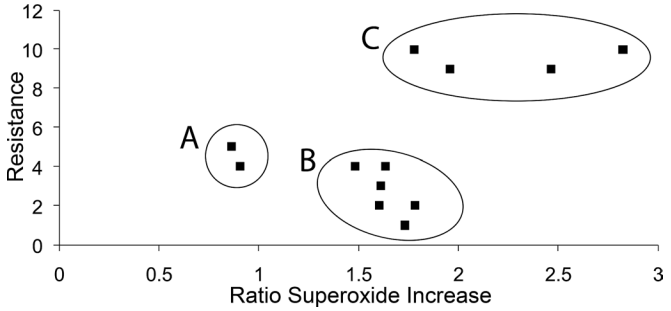

FIGURE 5: The relationship between H_2O_2 resistance and increase in superoxide radical levels. H_2O_2 resistance was plotted against $O_2^{\bullet-}$ level data to determine any correlation. H_2O_2 resistance data from Figure 4 were assigned numerical scores and plotted against the ratio of $O_2^{\bullet-}$ levels during H_2O_2 stress to unstressed conditions, which was calculated for each strain (data from Figure 2). Areas A–C denote the three areas where the plotted data appeared to cluster and are discussed in the text.

the highest resistance to H_2O_2 and included strains among those with the highest $O_2^{\bullet-}$ levels.

The relationship between H_2O_2 sensitivity and $O_2^{\bullet-}$ levels during H_2O_2 stress was examined. The strains were placed into one of four classes based on their relative $O_2^{\bullet-}$ levels and H_2O_2 sensitivity, as shown in Table 1. The WT, *ndi1Δ*, and *sdh1Δ* strains had the highest $O_2^{\bullet-}$ levels and the lowest H_2O_2 sensitivity. The *cor1Δ*, *cbp3Δ*, *cox6Δ*, *mrpl25Δ*, WT ρ^0, and *atp1Δ* strains were the opposite, with all having low or very low $O_2^{\bullet-}$ levels and high or very high H_2O_2 sensitivity (Table 1). These nine strains may provide evidence that there is a correlation between H_2O_2 sensitivity and $O_2^{\bullet-}$ levels during H_2O_2 stress. When $O_2^{\bullet-}$ levels are higher during H_2O_2 stress, resistance to H_2O_2 stress appears to be increased. Therefore it is the overall level of $O_2^{\bullet-}$ that may influence H_2O_2 sensitivity and not the relative increase in $O_2^{\bullet-}$ in response to H_2O_2. The *coq1Δ* strain was very sensitive to H_2O_2 but also had very high levels of $O_2^{\bullet-}$. This may be due to loss of the antioxidant function of the coenzyme Q pool (Schultz and Clarke, 1999; James *et al.*, 2004). Similarly, the *cyt1Δ* strain was very sensitive to H_2O_2 but also had high levels of $O_2^{\bullet-}$. This may be due to the possible presence of cytochrome *b* in this strain, which is not present in most other low-$O_2^{\bullet-}$ strains. Therefore the *coq1Δ and cyt1Δ* strains may deviate from this correlation because of these additional unique factors.

Reducing superoxide levels in the wild type confirms its protective role during H_2O_2 stress

Because low $O_2^{\bullet-}$ levels appeared to correlate with increased H_2O_2 sensitivity, we tested the hypothesis that a decrease in $O_2^{\bullet-}$ levels from overexpressing SOD would increase H_2O_2 sensitivity. Overexpression vectors pRS425 and pRS426 containing the *SOD1* and *SOD2* genes, respectively, were transformed into BY4743, and the resulting strains were then treated with H_2O_2.

Overexpression of SOD increased the sensitivity of the WT to H_2O_2 (Figure 6). This was more pronounced in the case of overexpression of *SOD1* (Figure 6). Overexpression of *SOD2* also made the cell more sensitive to H_2O_2 but to a lesser extent than with SOD1 overexpression. This was not surprising, considering that *SOD1* encodes the CuZnSOD, which in yeast accounts for 90–99% of total SOD activity (Longo *et al.*, 1999; Sturtz and Culotta, 2002). We hypothesized that the increase in H_2O_2 sensitivity observed in the SOD-overexpressing strain could be due to either decreased $O_2^{\bullet-}$ levels (as the substrate of increased SOD) or increased H_2O_2 levels (as the product of increased SOD). To distinguish between these two possibilities, we repeated the experiment in the presence of non-LDs of the $O_2^{\bullet-}$ generator menadione. If increased H_2O_2 was the cause of sensitivity in the SOD-overexpressing strains, the overexpressed SOD would detoxify the menadione-generated $O_2^{\bullet-}$,

	Very low superoxide	Low superoxide	High superoxide	Very high superoxide
Very low sensitivity		*nde1Δ*		WT, *ndi1Δ*, *sdh1Δ*
Low sensitivity				
High sensitivity	*cbp3Δ*, *cox6Δ*, *mrpl25Δ*, WT ρ^0	*atp1Δ*		
Very high sensitivity	*cor1Δ*		*cyt1Δ*	*coq1Δ*

The range of superoxide radical levels during H_2O_2 stress (data from Figure 3) and H_2O_2 sensitivity scores (assigned from data in Figure 5) was split evenly into four categories, and strains were then placed according to their category.

TABLE 1: Relationship between superoxide radical level and H_2O_2 sensitivity.

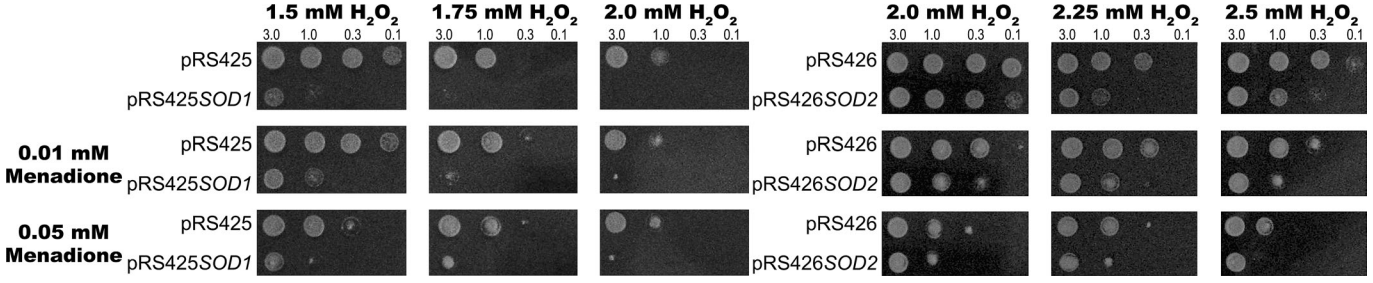

FIGURE 6: The effect of superoxide radical levels on H_2O_2 tolerance. Strains were grown in liquid SD dropout medium to OD_{600} of 1 and suspended in SD minus glucose to OD_{600} of 3, 1, 0.3, and 0.1. Then 5 µl was spotted onto SD dropout agar containing H_2O_2 and menadione (where specified). Top, H_2O_2 only; middle, H_2O_2 of the same concentrations plus 0.01 mM menadione; bottom, H_2O_2 of the same concentrations plus 0.05 mM menadione. The plates were incubated at 30°C for 2 d.

forming even higher levels of H_2O_2, which would further increase sensitivity. Addition of menadione, however, did not increase the sensitivity of the *SOD1*-overexpressing strain, confirming that increased production of H_2O_2 was unlikely to be the cause of sensitivity. Not only did the addition of menadione cause no increase in H_2O_2 sensitivity in the *SOD1*-overexpressing strain, it actually increased its resistance to H_2O_2, as seen in the growth of the test strain compared with that containing the vector-only control (pRS425) under the same conditions (Figure 6). This indicates that overexpression of *SOD1* confers H_2O_2 sensitivity by reducing $O_2^{\bullet-}$ levels. Therefore $O_2^{\bullet-}$ levels appear to have a protective effect during oxidative stress induced by H_2O_2.

The partial rescue of the H_2O_2-sensitive phenotype by adding menadione was accompanied by decreased growth of the control, whereas growth of the *SOD1*-overexpressing strain was mostly unchanged. This loss of yield of the control in increasing menadione concentrations shows that when superoxide levels get too high, a negative effect results. $O_2^{\bullet-}$ levels are therefore protective at low concentrations but toxic at higher concentrations.

DISCUSSION

The increased $O_2^{\bullet-}$ levels observed in the H_2O_2-treated WT cells are unlikely to result directly from metabolism of H_2O_2 since the systems detoxifying H_2O_2 do not directly produce $O_2^{\bullet-}$. Moreover, the differences in $O_2^{\bullet-}$ levels in the various strains did not appear to be due to differences in detoxification of $O_2^{\bullet-}$ since the expression levels of *SOD* genes did not correlate with the different $O_2^{\bullet-}$ levels observed. Therefore differences in superoxide levels observed in the WT appear to be due to a change in $O_2^{\bullet-}$ production, and the dependence on functional mitochondria supports this. Studies using dihydroethidium to detect $O_2^{\bullet-}$ are consistent with these results in the case of the ρ^0 (Reddi and Culotta, 2013) and *mrpl25Δ*, where the *MRPL25* gene was involved in replicative aging, also named *AFO1* (Heeren *et al.*, 2009). The $O_2^{\bullet-}$ observed in our study may not be due to the NADPH oxidase encoded by *YNO1* since $O_2^{\bullet-}$ production by this enzyme is independent of mitochondrial function (Rinnerthaler *et al.*, 2012).

The abolition of complex III and/or complex IV function significantly reduced $O_2^{\bullet-}$ levels below those seen in the WT, presumably due to loss of complex III function, since complex III is recognized as the major in vivo source of $O_2^{\bullet-}$ (Andreyev *et al.*, 2005). Specifically, the $O_2^{\bullet-}$ levels observed depended on cytochrome *b* function. This is consistent with accepted theory that a ubisemiquinone intermediate of the Q-cycle mechanism in complex III is the main source of electrons donated to O_2 to form $O_2^{\bullet-}$. An increased lifetime of the center N ubisemiquinone (Q_o), which is located at cytochrome *b*, is the main cause of higher levels of mitochondrial $O_2^{\bullet-}$ production (Trumpower, 1990; Hunte *et al.*, 2003; Crofts, 2004).

Previously ROS was believed to have only a deleterious role in yeast, apart from activating cellular defenses to combat them and the damage they cause. More recently it was proposed that ROS might have a positive signaling role during chronological aging (Mesquita *et al.*, 2010; Weinberger *et al.*, 2010; Lewinska *et al.*, 2011; Pan *et al.*, 2011). Here we show that $O_2^{\bullet-}$ has a protective signaling role in yeast during H_2O_2-induced oxidative stress. We propose the mechanism for $O_2^{\bullet-}$ production and provide an explanation of hormesis to address the seemingly conflicting positive and negative effects of the $O_2^{\bullet-}$ and ROS. Specifically, in response to H_2O_2, the cell increases $O_2^{\bullet-}$ production in a complex III–dependent manner. Preventing or diminishing this response by gene deletion or increased $O_2^{\bullet-}$ detoxification renders cells more sensitive to H_2O_2.

The mitochondrial electron transport chain is crucial for cell viability during H_2O_2 stress, with complex III implicated as having critical importance (Grant *et al.*, 1997; Thorpe *et al.*, 2004). One possible explanation for the importance of the ETC during H_2O_2 stress could be a requirement for energy (Grant *et al.*, 1997). Because there may be little or no proton-motive force in the H_2O_2-sensitive strains, they may therefore be unable to make ATP at complex V. Furthermore, the *atp1Δ* strain, which has an intact electron transport chain but is unable to synthesize ATP, was sensitive to H_2O_2. A previous large-scale screening study, however, highlighted only strains lacking respiratory chain functions to be H_2O_2 sensitive and not ones lacking other mitochondrial energy-generating reactions such as the trichloroacetic acid cycle (Thorpe *et al.*, 2004). Moreover, these studies were carried out in cells primarily generating energy by fermentation rather than respiration. The metabolic adaptation to loss of mitochondrial function induced by the retrograde response also offers no explanation for H_2O_2 sensitivity since the ρ^0 strain showed little difference in sensitivity or $O_2^{\bullet-}$ levels compared with other mitochondrial mutants.

The specific involvement of the electron transport chain in H_2O_2 tolerance may therefore be due to other factors, such as ROS production. Here we found that increased $O_2^{\bullet-}$ levels resulted in cells becoming more resistant to H_2O_2. This correlation with $O_2^{\bullet-}$ levels is interesting because elevated levels of the radical in the presence of H_2O_2 would be expected, on the basis of Fenton chemistry, to increase oxidative stress by generating the hydroxyl radical and therefore render the cells more sensitive to H_2O_2. Other studies support our finding; for example, grande strains have been proposed to produce more ROS than petite strains (Guidot *et al.*, 1993; Longo *et al.*, 1999), but they are more resistant to oxidative stress elicited by H_2O_2 and menadione than petites (Collinson and Dawes, 1992; Jamieson, 1992; Flattery-O'Brien *et al.*, 1993). Moreover, a ρ^0 mutant that showed significant viability loss during chronological aging produced low amounts of ROS (Trancikova *et al.*, 2004).

How can lower levels of $O_2^{\bullet-}$ production result in increased sensitivity? The answer may lie in $O_2^{\bullet-}$ having a protective effect. The normal response to H_2O_2-induced stress in WT cells was to increase $O_2^{\bullet-}$ production, and when this was inhibited by either increased SOD levels or inhibition of complex III function, sensitivity to H_2O_2 increased. The protective effect of increased $O_2^{\bullet-}$ is distinct from the adaptive response, since pretreatment with menadione does not protect against LD of H_2O_2, and adaptation occurs in strains lacking mitochondrial function (Collinson and Dawes, 1992; Jamieson, 1992). Therefore adaptation and $O_2^{\bullet-}$ signaling are two distinct examples of the many overlapping defenses against H_2O_2, and full activation of H_2O_2 defenses may only happen in response to both H_2O_2 and $O_2^{\bullet-}$. Consequently, one function of the electron transport chain during H_2O_2 stress may be to produce increased levels of $O_2^{\bullet-}$, which then has a signaling role capable of activating antioxidant defenses.

Although our results show that $O_2^{\bullet-}$ produced in the mitochondria has a protective effect at low concentrations, high concentrations were still detrimental. The importance of $O_2^{\bullet-}$ concentration means that SODs may have an important role during H_2O_2 stress beyond simply detoxifying $O_2^{\bullet-}$. The levels of $O_2^{\bullet-}$ must also be tightly regulated to promote their protective signaling effect. Supporting this, it was proposed that in vivo SOD activity can either increase or decrease ROS damage, depending on the conditions (Lushchak *et al.*, 2005). Furthermore, SOD overexpression has been shown to extend chronological lifespan but reduce replicative lifespan (Harris *et al.*, 2003; Fabrizio *et al.*, 2004). This concentration-dependent dual role of $O_2^{\bullet-}$, in which at low concentration it can

have a positive effect and at higher concentration a negative effect, is a form of mitochondrial hormesis. The concept of mitochondrial hormesis has been proposed as an explanation for the role of ROS in aging in higher organisms and humans (Linnane *et al.*, 2007; Yang and Hekimi, 2010; Ristow and Schmeisser, 2011). In yeast, $O_2^{\bullet-}$ has been proposed to provide an adaptive signal extending chronological lifespan, and the levels of H_2O_2 have been proposed to have a hormetic effect on chronological aging (Mesquita *et al.*, 2010; Pan *et al.*, 2011). Here we show the hormetic effect of $O_2^{\bullet-}$ on tolerance of another ROS, H_2O_2.

$O_2^{\bullet-}$ production in yeast and mammalian complex III appears to occur via a similar mechanism (Sun and Trumpower, 2003), and yeast is an important model for research into mitochondrial function and oxidative stress. This hormetic signaling role of $O_2^{\bullet-}$ has implications for the role of ROS and mitochondria in oxidative stress, aging, and apoptosis in yeast and higher organisms and consequently a possible role in human diseases such as cancer (Singh, 2004).

MATERIALS AND METHODS

Strains and plasmids

S. cerevisiae strains used were in the genetic background of BY4741 (*MAT**a**; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and BY4742 (*MATα; his3Δ1; leu2Δ0; lysΔ0; ura3Δ0*), with BY4743 (*MAT**a**/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; MET15/met15Δ0; lysΔ0/LYS2; ura3Δ0/ura3Δ0*) the diploid product of these two strains (Brachmann *et al.*, 1998). The term wild type is used to refer to BY4743. Diploid deletion strains, homozygous at the deleted locus, constructed as part of International *Saccharomyces* Gene Deletion Project (Winzeler *et al.*, 1999), were obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis, Frankfurt, Germany. To confirm that strains deleted for nuclear genes were grande, haploid strains were mated with a haploid strain lacking mitochondrial DNA, and the resultant diploid was able to grow on a respiratory carbon source. The BY4743 ρ^0 petite (WT ρ^0) was generated by growth of the parent strain on YEPD agar containing 20 mg/l ethidium bromide. The plasmids used in the study were the pRS42X series (Christianson *et al.*, 1992). pRS425*SOD1* and pRS426*SOD2* were obtained from John Park (Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI; Park *et al.*, 1998).

Media and chemicals

Cells were grown in YEPD (2% [wt/vol] D-glucose, 2% [wt/vol] bactopeptone, and 1% yeast extract), SC (2% [wt/vol] D-glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.074% complete supplement mixture from Difco [Franklin Lakes, NJ]), and SD minimal medium (2% [wt/vol] D-glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate). SD medium was supplemented with appropriate auxotrophic requirements to the concentrations prescribed for SC medium (Adams *et al.*, 1998). Leucine auxotrophs were also supplemented with isoleucine and valine. Agar plates were solidified with 2% (wt/vol) agar (type 1 agar for SD and SC; type 3 agar for YEPD). Dropout plates lacking only the selected nutrient were used to select transformants with acquired prototrophic genes. *S. cerevisiae* was grown at 30°C with shaking and aeration when in liquid media. DEPMPO was purchased from Sapphire Bioscience (Waterloo, Australia). Diethylenetriaminepentaacetic acid (DTPA), ethidium bromide, and menadione sodium bisulfate were obtained from Sigma-Aldrich (Castle Hill, Australia).

Sensitivity to oxidative stress

Sensitivity to a chronic exposure of oxidative stress was performed as spot tests on agar plates. Liquid cultures were grown to the growth phase specified, centrifuged, and resuspended in 0.17% yeast nitrogen base and 0.5% ammonium sulfate to an OD_{600} of 3.0, 1.0, 0.3, and 0.1. A 5-μl amount of each dilution was spotted onto SC or SC-dropout agar plates if strains contained a plasmid, with oxidants of differing concentrations, and incubated at 30°C for 2 d. All agar was cooled to 50°C before addition of the oxidant and then immediately allowed to set.

Viability estimation

Cells of the wild type and mutants were grown in YEPD to OD_{600} of 4, and each culture was split into one control sample and one sample treated with 4 mM H_2O_2 for 60 min. Samples of treated and untreated cultures were diluted appropriately for plate counts on YEPD plates and also taken for propidium iodide staining to determine cell viability as measured by retention of membrane permeability. For the propidium iodide (PI) staining, cells were harvested by centrifugation, resuspended in 1 ml phosphate-buffered saline (PBS), and stained with propidium iodide (10 μg/ml) for 20 min in the dark and washed twice with PBS, and the level of PI staining was analyzed by flow cytometry. Stained cells were analyzed with a FACS Canto II (BD Biosciences, San Diego, CA) instrument equipped with a laser that excites at 488 nm, and emission was detected at 617 nm using a 556-nm long-pass filter and a 585/42-nm broad-pass filter.

Measurement of superoxide radicals

Intracellular superoxide radicals were estimated based on the method of Heeren *et al.* (2004). Before the experiment, 100 μl of methanol was added to each 50-mg tube of DEPMPO, and all tubes to be used in the experiment were pooled together. DEPMPO aliquots were transferred to individual Eppendorf tubes so that the final concentration in 350 μl of buffer was 100 mM. Cells were grown in YEPD to the specified OD_{600}/growth phase, and a sample was taken to determine viable cells per milliliter. If pretreatment was involved (H_2O_2-induced oxidative stress), this was applied at the specified concentration for 1 h. Cultures were centrifuged (4 min, 2000 rpm) and washed twice in 10 mM potassium phosphate buffer, pH 7.0. Triplicate samples of 2.25×10^8 cells were resuspended in 350 μl of 10 mM potassium phosphate buffer, pH 7.0, and 0.1 mM DTPA. Cell concentration calculations were based on OD_{600} derived from a standard curve obtained from the WT and checked 2 d later from viable colony counts. The 350 μl of cells in PI/DTPA buffer was transferred to an Eppendorf tube containing DEPMPO and incubated for 20 min at room temperature with shaking, with lid open to allow aeration, and protected from light. After the 20-min incubation, samples were centrifuged and the supernatant taken for subsequent EPR analysis. The supernatant was immediately frozen in liquid N_2 and stored protected from light until immediately before EPR analysis.

EPR spectra were recorded at room temperature using a Bruker EMX spectrometer with 100-kHz modulation equipped with a cylindrical (ER4103TM) cavity. Samples were contained in a flattened aqueous sample cell (Wilmad WG-813-SQ), and recording of the spectra was initiated within 2 min of the start of the reaction. Hyperfine coupling constants were measured directly from the field scan and confirmed by spectral simulation with the program WINSIM. Typical EPR spectrometer settings were as follows: gain, 2×10^6; modulation amplitude, 0.1 mT; time constant, 163 s; scan time, 83 s; resolution, 1024 points; center field, 348.5 mT; field scan, 12.0 mT; power, 20.068 mW; and frequency, 9.658 GHz; with eight scans averaged. The relative concentrations of the O_2^- adducts to DEPMPO were determined from measurement of peak-to-peak line heights of the EPR absorption lines specific to this adduct, relative to the WT control spectra.

RNA extraction and quantitative real time-PCR

Total cell RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA), with optional on-column DNase digestion. Extracted RNA was quantified using the Bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The absence of genomic DNA in samples was confirmed using non–reverse-transcribed RNA as a template in real-time PCR assays. RNA was then reverse transcribed into cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

Oligonucleotides were designed using Primer3 (Rozen and Skaletsky, 2000). The primers used to amplify a 144–base pair section of *ACT1* were CTGCCGGTATTGACCAAACT (forward) and CGGTGATTTCCTTTTGCATT (reverse). A 129–base pair section of *SOD1* was amplified using CACATGGTGCTCCAACTGAC (forward) and CAACGGAGGTAGGACCGATA (reverse). A 131–base pair sequence of *SOD2* was amplified using AACCAGGATACCGTCACAGG (forward) and TTCCAGTTGACCACATTCCA (reverse). PCRs were performed in quadruplicate samples using the iTaq SYBR Green Supermix With ROX and analyzed on a Chromo4 Real-Time PCR Detection System (Bio-Rad), with a no-template control included in each assay. The thermocycling program consisted of 95°C for 150 s, followed by 40 cycles of 20 s at 95, 58, 72°C. Melting-curve data were collected to verify PCR specificity and absence of contamination and primer dimers. PCR efficiency was determined using the dilution series method, and data were analyzed using Opticon Monitor Software (Bio-Rad). Expression relative to *ACT1* was determined with efficiency correction (Pfaffl, 2001).

Statistics

Values in graphs are means ± SD. Student's *t* test was used for the statistical analysis of each strain in control conditions compared with H_2O_2 treatment. A $p < 0.01$ was deemed indicative of a statistically significant difference for these tests. Analysis of mutants strains compared with the WT was performed using a one-way analysis of variance followed by Dunnett's test for multiple comparisons. A $p < 0.05$ was deemed indicative of a statistically significant difference for these tests. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

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