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Induction of Phenotypes Resembling CuZn-Superoxide Dismutase Deletion in Wild-Type Yeast Cells: An in Vivo Assay for the Role of Superoxide in the Toxicity of Redox-Cycling Compounds

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Yeast (*Saccharomyces cerevisiae*) lacking the enzyme CuZn-superoxide dismutase (*sod1*Δ) display a large number of dioxygen sensitive phenotypes, such as amino acid auxotrophies, sensitivity to elevated temperatures, and sensitivity to 100% dioxygen, which are attributed to superoxide stress. Such cells are exquisitely sensitive to small amounts of the herbicide paraquat (methyl viologen), which is known to produce high fluxes of superoxide in vivo via a redox-cycling mechanism. We report that dioxygen sensitive phenotypes similar to those seen in *sod1*Δ cells can be induced in wild-type cells by treatment with moderate concentrations of paraquat or diquat, another bipyridyl herbicide, providing strong evidence that the mechanism of toxicity for both of these compounds is attributable to superoxide stress. Certain redox-cycling quinone compounds (e.g., menadione and plumbagin) are also far more toxic toward *sod1*Δ than to wild type. However, treatment of wild-type yeast with menadione or plumbagin did not induce *sod1*Δ-like phenotypes, although toxicity was evident. Thus, their toxicity in wild type cells is predominantly, but not exclusively, due to mechanisms unrelated to superoxide production. Further evidence for a different basis of toxicity toward wild-type yeast in these two classes of redox-cycling compounds includes the observations that (i) growth in low oxygen alleviated the effects of paraquat and diquat but not those of menadione or plumbagin and (ii) activity of the superoxide sensitive enzyme aconitase is affected by very low concentrations of paraquat but only by higher, growth inhibitory concentrations of menadione. These results provide the basis for an easy qualitative assay of the contribution of redox-cycling to the toxicity of a test compound. Using this method, we analyzed the Parkinsonism-inducing compound 1-methyl-4-phenylpyridinium and found that redox cycling and superoxide toxicity are not the predominant factor in its toxic mechanism.

Introduction

Mutant organisms lacking copper−zinc superoxide dismutase (CuZnSOD)[1] have been generated in a number of species and show a surprising range of characteristics. *sod1*−/− mice are phenotypically nearly normal (*1*), while yeast (*Saccharomyces cerevisiae*) lacking the CuZnSOD gene (*sod1*Δ) exhibit a number of debilitating phenotypes. For instance, the mutant yeast strain grows slowly in air and has auxotrophies for lysine, methionine, and leucine. Inactivation of aconitase, a four iron−four sulfur (4Fe-4S) cluster enzyme, is observed (*2*), as well as an increase in electron paramagnetic resonance-detectable "free" iron and (*3*) exquisite sensitivity to redox-cycling compounds, high levels of dioxygen (*4*−*6*), and high extracellular zinc (*7*). The lysine and leucine auxotrophies arise from the selective inactivation by superoxide of 4Fe-4S cluster enzymes involved in the biosynthetic pathways of these amino acids in yeast (*2*). The methionine auxotrophy has been reported to result from a depletion of NADPH, high levels of which are required for methionine synthesis (*8*).

Treatment with drugs such as paraquat (PQ, methyl viologen) and menadione (MD) is widely used to experimentally generate superoxide stress in living organisms. In the cell, these redox-cycling compounds are reduced by one electron forming a radical species (e.g., the monocation radical $PQ^{\bullet+}$ or the semiquinone radical for MD); these radicals react rapidly with dioxygen to generate superoxide anion and regenerate the parent oxidized form (e.g., to PQ^{2+} or the quinone for MD) in a cyclical mode of action (*9*−*11*). Many eukaryotic organisms can limit the redox cycling of quinones through the action of cytosolic NAD(P)H:quinone oxidoreductase (DT

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[1] Abbreviations: CuZnSOD or Sod1p, copper−zinc superoxide dismutase; MnSOD or Sod2p, manganese superoxide dismutase; *sod1*Δ, yeast lacking the CuZnSOD gene; ROS, reactive oxygen species; 4Fe-4S, four iron−four sulfur (cluster); WT, wild-type; PQ, paraquat; DQ, diquat; MD, menadione; PB, plumbagin; BN, 1,4-benzoquinone; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GSH, glutathione.

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diaphorase), which catalyzes the two-electron reduction of quinones to their hydroquinol form, which is unable to redox cycle (*9*, *10*). However, *S. cerevisiae* has not been reported to contain such an enzyme. Yeast do not have a typical complex I in the electron transport chain; instead, they have three NADH dehydrogenases that transfer electrons from NADH to coenzyme Q but do not pump protons. NDI1 is present in the matrix and may carry out a two-electron reduction, although its location on the matrix side of the inner membrane may preclude its involvement in quinone detoxification (*12*). NDE1 and NDE2, present in the IMS of yeast, have been reported to contribute to the production of superoxide under certain conditions (*13*).

PQ and diquat (DQ) are bipyridylium compounds, historically utilized as herbicides. Extensive work in *E. coli* has demonstrated that the toxicity of PQ arises through the catalytic production of superoxide (*11*, *14*−*16*) utilizing a cellular reductant such as NAD(P)H as an electron source. Toxicity was enhanced by the metal ions copper and iron (*17*, *18*), while alterations in the media pH (*19*), glucose (*20*), or salt content (*21*) diminished PQ toxicity. Furthermore, treatment with PQ resulted in a 10-fold increase in cyanide resistant respiration (indicating a diversion of intracellular electrons) as well as increased expression of manganese superoxide dismutase (MnSOD) (*14*, *15*). In plant cells, which are extremely sensitive to PQ (hence its use as an herbicide), the target organelle is the chloroplast (*9*); in nonphotosynthetic eukaryotes, the mitochondrion is implicated as the major site of PQ toxicity (*22*−*24*).

In contrast, the toxicity of the quinones MD and plumbagin (PB) is not limited to redox cycling but can arise through a second mechanism involving reactions with cellular nucleophiles. These types of reactions can cause depletion of glutathione (GSH), protein alkylation, and formation of DNA adducts (*10*). Certain quinones (e.g., benzoquinone, BN) act exclusively via the second mechanism at a physiological pH (*25*), while for quinones such as MD experimental evidence for both electrophilic attack and redox-cycling mechanisms has accrued (*10*, *26*, *27*). Superoxide is implicated as the predominant cause of MD cytotoxicity in some studies (*28*, *29*). For example, *S. cerevisiae* treated with MD exhibits fluctuations in SOD levels (*30*) and increased cyanide resistant respiration (*29*), a response reminiscent of that of *E. coli* treated with PQ. Conversely, others have demonstrated a reaction of MD with GSH in *S. cerevisiae* (*31*, *32*) and mammalian endothelial cells (*33*) indicating that a major mode of toxicity for MD does not involve redox cycling. Like PQ, MD likely acts within mitochondria as demonstrated by a requirement for the mitochondrial yeast glutaredoxin, Grx5p, for protection against MD toxicity (*34*).

1-Methyl-4-phenylpyridinium (MPP^+) is a compound structurally similar to PQ that was once used as an herbicide known as cyperquat. It is the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes symptoms resembling Parkinson's disease in humans and other mammals (*35*). There has been some controversy over its mode(s) of toxicity; in vitro data have argued against redox cycling (*36*, *37*) while in vivo data suggest that MPP^+ treatment may result in superoxide stress either directly or by inhibition of complex I (*38*−*41*). Other studies implicate MPP^+ as causing mitochondrial dysfunction through a change in membrane potential, redox status, or energy metabolism (*42*−*45*).

We recently reported that the PQ treatment of wild-type (WT) yeast effectively induced a *sod1*Δ-like phenotype, including amino acid auxotrophies, 4Fe-4S cluster enzyme inactivation (*2*), and increased levels of "free" iron (*3*). Here, we report the expansion of these investigations to other compounds (DQ, MD, PB, BN, H_2O_2, and MPP^+) for the purpose of evaluating the degree to which their toxicity in vivo can be attributed to superoxide stress caused by redox cycling.

Materials and Methods

Yeast Strains, Media, and Growth Conditions. Yeast strains EG103 (WT) and EG118 (*sod1*Δ) were utilized for most experiments (*4*). Strains BY4741 (WT) and BY4741 #6913 (*sod1*Δ) were utilized in a limited fashion for confirmation of results. All experiments in liquid culture were done in synthetic media with added 2% glucose (SD) supplemented with the appropriate amino acids (SDC) as described (*46*), at a pH of 6.0. Cell growth was monitored on a Shimadzu UV-2501PC spectrophotometer by measuring the optical density (OD) of the cells at 600 nm (an OD600 of 1 corresponds to 1×10^7 cells). Yeast were inoculated from 24 h precultures grown in SDC into fresh medium with varying concentrations of test compound or into media lacking methionine (SD-met), lysine (SD-lys), or threonine (SD-thr), as indicated, at an initial OD600 = 0.05. Cultures were grown at 30 °C and 220 rpm for 12 h under normal aeration, using a five to one flask volume to medium volume ratio. For low oxygen conditions, cultures were incubated in a shaking (120 rpm) water bath equipped with a gassing hood with nitrogen continuously streaming through it, attaining a nearly anaerobic condition. (The dioxygen concentration was low enough to completely restore the growth of *sod1*Δ cells, but residual oxygen was sufficient to allow the cells to grow without ergosterol and Tween 80 supplementation.) Similarly, 100% oxygen experiments were done in an enclosed shaking water bath with oxygen streaming.

Chemicals. PQ (methyl viologen), DQ, MD sodium bisulfite, PB, MPP^+, and BN were purchased from Sigma. For PQ, DQ, and MPP^+ studies, a 50 mM stock solution was made in SDC or sterile deionized water. MD sodium bisulfite was dissolved in deionized water to 200 mM, while PB and BN were dissolved in ethanol to 200 mM. Freshly prepared stock solutions were filter sterilized and added to the growth medium at the beginning of the culture period at the concentrations indicated. Note that the water soluble derivative, MD sodium bisulfite, was used instead of the more lipophilic parent compound. The bisulfite adduct is commonly used in place of MD, although it is less toxic on a molar basis (*47*).

Aconitase Activity. The aconitase activity was determined spectrophotometrically as described by Gardner et al. by monitoring the formation of NADPH at 340 nm (*48*). Briefly, the assay mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$, 2 units of $NADP^+$ isocitrate dehydrogenase, and 200 μL of cell lysate for a final volume of 1 mL. The absorbance change was measured for 5 min, and the activity was calculated from the slope of the linear portion. Cell extracts were prepared from 2.0×10^8 cells. Lysis was performed under nitrogen with 0.5 mm glass beads by vortexing for 30 s followed by incubation on ice for 30 s, repeating the process seven times. It is critical to lyse the cells under nitrogen due to the oxidatively labile nature of aconitase. Cell debris was removed by centrifugation, and the resulting supernatant was assayed.

Results

***sod1*Δ Yeast Are Exquisitely Sensitive to Redox-Cycling Compounds.** Yeast lacking CuZnSOD cannot efficiently decompose superoxide in the cytosol or mito-

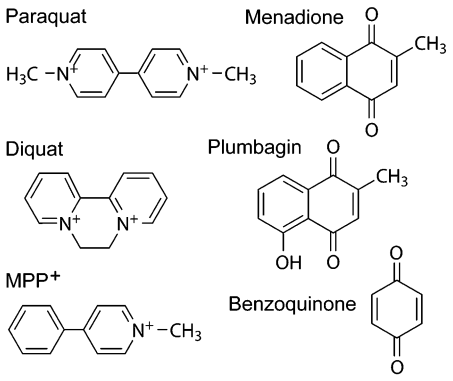

Figure 1. Compounds utilized in this study. Structures of the six chemicals utilized are shown in their reactive forms.

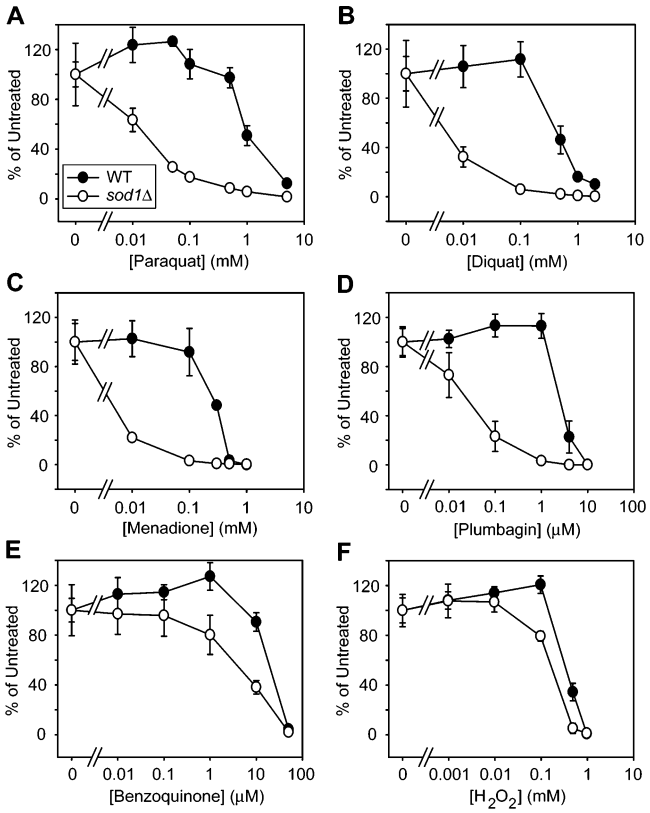

Figure 2. *sod1*Δ is exquisitely sensitive to redox-cycling compounds. EG103 (WT, filled symbols) and EG118 (*sod1*Δ, open symbols) were treated with (A) PQ, (B) DQ, (C) MD, (D) PB, (E) BN, and (F) H_2O_2 and grown for 12 h in SDC, after which the optical density of the culture was measured at 600 nm. Points represent averages of a minimum of six samples (from separate colonies) assayed on at least two different days and are reported as the % of the untreated WT or *sod1*Δ sample.

chondrial intermembrane space and are thus exquisitely sensitive to compounds that redox cycle, including PQ and MD (*4*). As an initial examination of the compounds in this study (Figure 1), we compared the sensitivity of *sod1*Δ and WT yeast to increasing concentrations of the compounds. The assay measures cell density in liquid culture after 12 h of growth. At this point, WT cells are approaching the end of log phase but are still actively growing. Figure 2 shows that *sod1*Δ yeast are 100-fold more sensitive than WT to four compounds that redox cycle, PQ, DQ, MD, and PB (Figure 2A−D), and we conclude that these compounds are all capable of producing superoxide in vivo in yeast. For BN, which is not reported to redox cycle at physiological pH (*25*), a

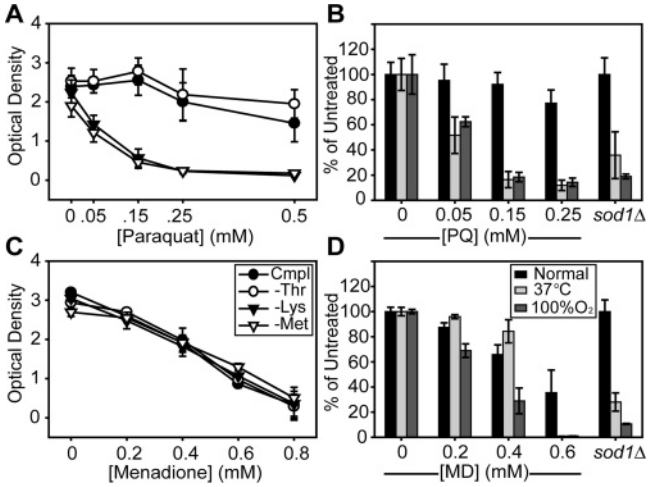

Figure 3. PQ induces superoxide-dependent phenotypes while MD does not. EG103 (WT) was treated with a range of PQ (A,B) and MD (C,D) concentrations, and growth was compared to that of EG118 (*sod1*Δ). Culture turbidity (optical density at 600 nm) was measured after 12 h of growth. (A,C) WT growth was monitored in complete (Cmpl) medium or in medium lacking threonine (−Thr), lysine (−Lys), or methionine (−Met) with the indicated concentration of PQ or MD. (B,D) WT cultures were grown with the indicated concentrations of PQ or MD under standard conditions, at 37 °C, or in 100% dioxygen. Growth of *sod1*Δ yeast under the same conditions is shown for comparison. Points represent averages of a minimum of six samples (from separate colonies) assayed on at least two different days.

dramatic difference in toxicity between WT and *sod1*Δ yeast is not evident, although *sod1*Δ is slightly more sensitive at higher concentrations (Figure 2E). Similarly, H_2O_2 is equally toxic to *sod1*Δ yeast (Figure 2F). It is interesting to note that at low concentrations all of the compounds tested stimulated the growth of the WT strain by as much as 20%. A possible explanation for this finding may be that an adaptive response induced by low concentrations of the agent enabled the cells to better resist the normal oxidative stress of aerobic growth as well as the added stress generated by the agent. This idea is not unprecedented, as many studies have found that the initial treatment of a yeast culture with a sublethal concentration of an oxidant gives that same culture a higher tolerance to subsequent oxidative stress (*49*, *50*).

PQ Treatment of WT Yeast Induces Phenotypes Resembling Those of *sod1*Δ Mutant Yeast. In complete medium, under normal laboratory growth conditions, concentrations of PQ that are quite toxic to the *sod1*Δ mutant have no detrimental effect on the growth of WT yeast, as seen in Figure 2. However, we found that treatment of WT cells with low concentrations of PQ produced phenotypes resembling those of the *sod1*Δ mutant. Figure 3A shows WT cells grown with varying amounts of PQ in complete medium or in medium lacking methionine, lysine, or threonine. Increasing amounts of PQ prevented growth in the absence of methionine or lysine but did not affect growth in complete medium or in the absence of threonine. Medium lacking threonine was included as a control for specificity of the response as its synthesis is not affected in *sod1*Δ mutants. This behavior is analogous to the behavior of the *sod1*Δ mutant grown (without PQ) in the same media (*2*). A similar response was observed in the WT strain BY4741 treated with PQ (data not shown). Figure 3B shows increased sensitivity to elevated temperature (37 °C) and

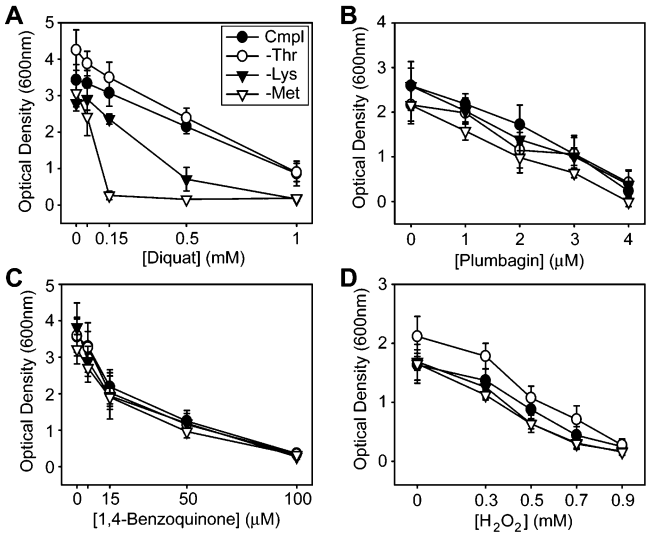

Figure 4. Varied abilities of DQ, PB, BN, and H_2O_2 to induce auxotrophies. EG103 (WT) was treated with a range of concentrations of (A) DQ, (B) PB, (C) BN, or (D) H_2O_2. WT growth was monitored at an optical density of 600 nm in complete (Cmpl) medium or in medium lacking threonine (−Thr), lysine (−Lys), or methionine (−Met) for 12 h with the indicated amounts of compound. All points represent the average of six or more separate cultures grown on at least two different days.

to 100% dioxygen in WT yeast treated with PQ; both phenotypes are characteristic of *sod1*Δ mutants.

MD Does Not Induce *sod1*Δ-Like Phenotypes. To probe further the ability of redox-cycling agents to induce *sod1*Δ-like phenotypes, we treated WT yeast with MD and performed the same set of tests. In contrast to PQ, MD repressed growth in a similar, dose-dependent manner regardless of the amino acids present, either in the EG103 background (Figure 3C) or in the BY4741 (data not shown), rather than inducing lysine and methionine auxotrophies. (Note that cysteine is not present in the media and would, therefore, not react with the exogenously added MD.) Figure 3D shows that MD was much less effective at inducing the 37 °C temperature sensitivity. MD treatment had no negative effect on growth at 37 °C until 0.6 mM MD, a concentration that reduced growth to less than 40% of the untreated control at the normal growth temperature of 30 °C. Last, MD induced sensitivity to 100% dioxygen (Figure 3D), although not as dramatically as was seen with PQ. Thus, the mode of toxicity of MD is quite dependent on the precise conditions. It is likely that under high dioxygen conditions the increased availability of dioxygen raises the contribution of redox cycling to MD toxicity, while during growth in ambient dioxygen another mode of MD toxicity dominates, masking any redox cycling effects, at least in WT cells with active SODs.

Induction of *sod1*Δ-Like Phenotypes by Other Related Compounds. The unique nature of the *sod1*Δ amino acid auxotrophies was utilized to test other compounds for their relative ability to cause in vivo superoxide toxicity by looking for the presence or absence of a dose-dependent induction of the lysine and methionine auxotrophies in WT yeast.

Several compounds with varying abilities to induce "oxidative stress" and/or to redox cycle in vitro were chosen. DQ is a bipyridyl herbicide similar to PQ, and like PQ, it induced the lysine and methionine auxotrophies in a dose-dependent manner (Figure 4A). It is interesting to note that DQ more effectively induced the methionine auxotrophy than the lysine auxotrophy, unlike PQ, which induces the auxotrophies equally.

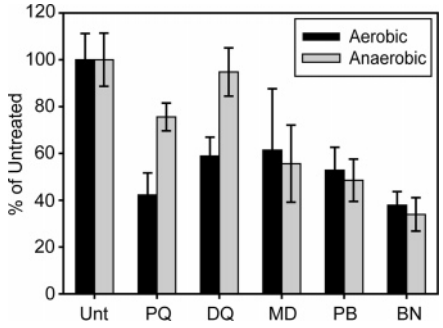

Figure 5. Low oxygen growth diminishes PQ and DQ toxicity. EG103 (WT) was grown for 12 h either untreated (Unt) or treated with 0.5 mM PQ, 0.6 mM DQ, 0.4 mM MD, 4.0 μM PB, or 20 μM BN under a normal aerobic environment or a low oxygen environment created by growth under nitrogen in a shaking water bath. Only the PQ- and DQ-treated samples show a significant difference between the aerobic and the low oxygen conditions. All points represent an average of six or more separate cultures grown on at least two different days.

Two other quinones, PB and BN, were tested. PB is similar in structure to MD, and in our test, PB behaved like MD. It did not induce the lysine and methionine auxotrophies (Figure 4B) and had a similar inhibitory effect on growth regardless of the amino acid content of the medium. BN, which can only redox cycle in vitro at pH values well above those found physiologically and which acts predominantly as an alkylating agent in vivo (*25*), did not induce the methionine or lysine auxotrophies and was equally toxic in all media (Figure 4C).

Attempts to induce the auxotrophies with H_2O_2, a reactive oxygen species (ROS) that does not produce superoxide, were also unsuccessful (Figure 4D). Thus, it appears that the ability to induce the lysine and methionine auxotrophies is a property of molecules that function exclusively or predominantly by catalyzing superoxide production, at least under the normal conditions of this study.

Growth under Low Dioxygen Diminishes the Toxicity of Bipyridyl Compounds but not That of the Quinones. The presence of dioxygen is essential for redox cycling to occur, so the toxicities of PQ, DQ, MD, PB, and BN under aerobic and low dioxygen conditions were compared (Figure 5). As can be seen in Figure 5, low dioxygen growth of cultures treated with either PQ or DQ resulted in a significant increase in growth relative to the aerobic controls, while low dioxygen growth had little effect on the toxicity of the quinones. This dioxygen dependence indicates that a major portion of the toxicity of the bipyridyl herbicides is due to superoxide (or another form of ROS derived from it), while the predominant toxic mechanism for the quinones is unrelated to dioxygen. However, we believe that the relatively large error in the MD samples probably reflects the presence of two modes of toxicity. Depending on the day of the particular experiment, slight rescue of the MD treated cells could be observed, especially at highly toxic concentrations.

Aconitase Inactivation, an Indicator of the Presence of Superoxide. The activity of aconitase, a 4Fe-4S cluster enzyme with a labile, solvent-exposed iron atom, is often used as a sensor of in vivo superoxide stress (*51*). Enzymes of this type are specifically inactivated by

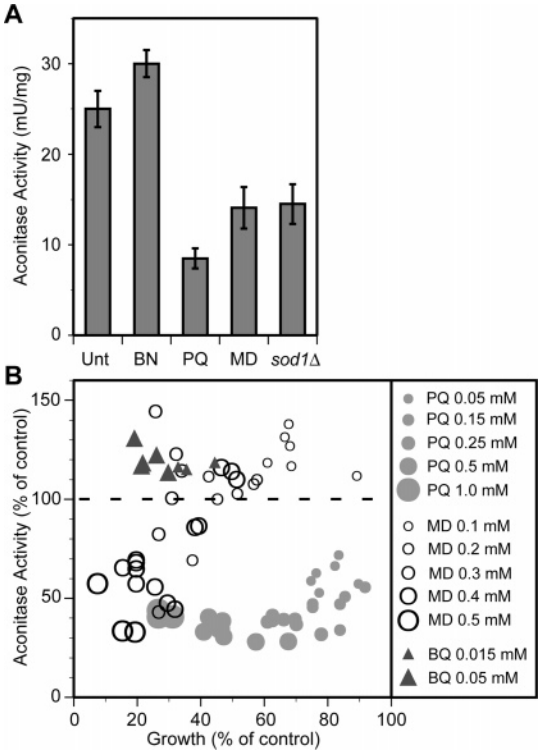

Figure 6. Aconitase activity indicates that PQ and MD redox cycle but with varied effectiveness. Aconitase activity was analyzed in EG103 (WT) samples treated with various concentrations of either PQ, MD, or BN after the cells were allowed to grow to an optical density of approximately 1.0 and compared to the activity observed in EG118 (*sod1*Δ) grown under normal aerobic conditions. (A) The effect of 0.5 mM PQ, 0.4 mM MD, or 10 μM BN on aconitase activity. (B) Depiction of the relationship of cell growth after 12 h to aconitase activity as a function of concentration; 0.05−1 mM PQ (closed circles), 0.1−0.5 mM MD (open circles), and 0.015−0.05 mM BN (triangles). Different concentrations are represented by different symbol sizes, with larger sizes representing higher concentrations. Each point represents one colony.

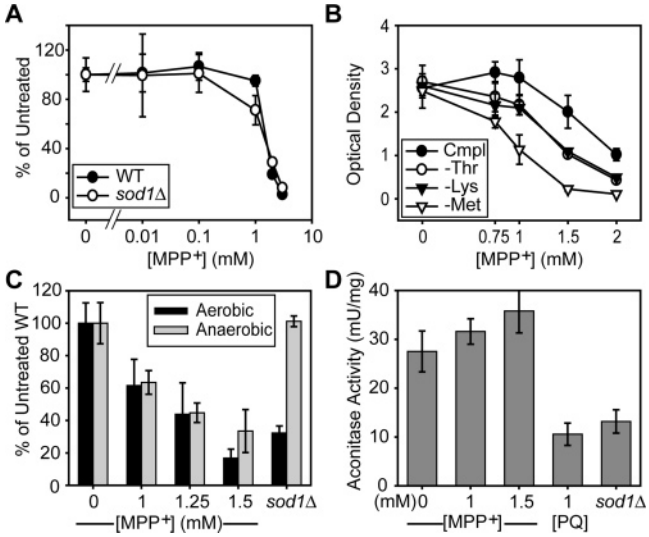

Figure 7. MPP^+ is unable to redox cycle in vivo. (A) Differential sensitivity of *sod1*Δ and WT yeast to MPP^+ was not observed. Yeast strains EG103 (WT, closed circles) and EG118 (*sod1*Δ, open circles) were grown for 12 h with varying concentrations of MPP^+, and the optical density (600 nm) was measured. Data are shown as a percent of the growth of the untreated control. (B) MPP^+ does not induce amino acid auxotrophies in WT yeast. Growth was examined by measuring the optical density (600 nm) after 12 h of growth in SDC (Cmpl), SD-Thr (−Thr), SD-Lys (−Lys), and SD-Met (−Met). (C) Normal aerobic growth conditions were compared to growth under low oxygen for an untreated WT sample, WT treated with 1.0, 1.25, or 1.5 mM MPP^+, and *sod1*Δ. (D) MPP^+ does not inhibit aconitase. Cultures were grown as in Figure 6 in the presence of 1.0 mM PQ, 1.0 mM MPP^+, or 1.5 mM MPP^+. A parallel sample of untreated *sod1*Δ was also prepared and assayed. All data are representative of at least six different samples (separate cultures from different colonies) assayed on at least two different days.

the superoxide-catalyzed removal of the labile iron atom (*52*). Aconitase is particularly useful as a marker of superoxide stress because it is not rate-limiting in its pathway and thus its activity can be substantially reduced without major effects on cellular metabolism. As a further test of the ability of these compounds to produce superoxide in vivo, we analyzed the aconitase activity in WT yeast grown in SDC medium with added PQ, MD, or BN and compared it to that found in *sod1*Δ yeast. Figure 6A shows that treatment with 0.5 mM PQ resulted in a 65% reduction in aconitase activity, while 0.4 mM MD caused a 42% reduction. These observed decreases in activity compare well to the 41% reduction seen in the *sod1*Δ strain. BN treatment did not inhibit aconitase activity and instead caused a small but reproducible increase over the untreated control. These results support the conclusion that PQ and MD redox cycle while BN acts solely as an alkylating agent.

While both PQ and MD are able to redox cycle, the propensity for each compound to do so and the contribution of redox cycling to the overall toxicity in normal cells differs significantly. Figure 6B shows a plot of aconitase activity vs cell growth for a series of samples treated with various concentrations of PQ, MD, or BN. The increasing concentration of each agent is indicated by the increasing size of the symbol. Low amounts of PQ diminish the aconitase activity to the lowest observed level—about 30% of untreated control—at concentrations that have minimal effect on cell growth (about 80% of untreated control). BN, on the other hand, shows excess aconitase activity (120% of untreated) at concentrations that are extremely inhibitory of cell growth (20% of untreated control). MD falls between these two extremes. Low levels that decrease growth by less than approximately 50% cause a slight increase in aconitase activity, but at more toxic levels (growth below 40% of untreated), aconitase activity is inhibited. Thus, it appears that PQ is a more efficient redox cycler than MD, that superoxide on its own is not extremely toxic (since levels that inhibit aconitase activity very effectively do not greatly effect cell growth), that MD is toxic to WT cells due to its alkylating ability even though at high concentrations redox cycling may make a contribution, and that BN does not redox cycle.

Test of MPP^+ as a Redox-Cycling Compound. We next applied our assays to an "unknown" sample, MPP^+, the active metabolite of MPTP that causes Parkinsonian-like symptoms in mammals. There has been some dispute in the literature about whether this compound acts by redox cycling, making it an ideal test case (*35*−*45*). Figure 7 shows the results of this group of tests, which support the conclusion that redox cycling is not the toxic mode of action of this compound, for the following reasons. First, *sod1*Δ yeast are no more sensitive than WT to this compound (Figure 7A). Second, aconitase activity is not decreased by MPP^+ treatment and is in fact slightly increased, even at high (toxic) concentrations (1.5 mM) (Figure 7D). Third, MPP^+ does not induce the lysine auxotrophy in WT cells (Figure 7B). Fourth, the

toxicity of MPP^+ is still evident under low oxygen growth conditions (Figure 7C). We did notice a small improvement in growth under low oxygen at high concentrations of MPP^+, and the methionine auxotrophy is observed to be somewhat induced by MPP^+. Taken together, these results strongly suggest that MPP^+ does not exert its toxicity by redox cycling in vivo but indicate that there may be a small portion of the toxicity that is oxygen-dependent. (It should be noted that our observation of toxicity implies that the MPP^+ is getting into the cells.)

Discussion

A number of drugs are commonly used to induce superoxide production in vivo, including PQ, MD, and PB. Superoxide production by these compounds occurs via a catalytic redox cycling process: Biological reducing agents chemically reduce the compound by one electron, the reduced form is reoxidized by dioxygen, forming superoxide and regenerating the original form of the compound, and then the compound can be rereduced for another cycle (*9*−*11*).

The phenotype of *sod1*Δ yeast can be attributed to excess superoxide anion and includes a range of specific characteristics—lysine and methionine auxotrophies, slow growth in air, temperature sensitivity, sensitivity to high dioxygen, and decreased aconitase activity. In addition, these cells are extremely sensitive to redox-cycling drugs, including PQ, DQ, MD, and PB (Figure 2 and refs *4* and *5*). The presence of dioxygen is required for the manifestation of these phenotypic characteristics (refs *4* and *5* and data not shown). We reasoned that, if superoxide production is the primary means by which these compounds are toxic under normal conditions, treatment of WT yeast with these compounds should induce a *sod1*Δ-like phenotype, and we tested a series of compounds for their ability to do this.

We chose two bipyridyl herbicides, PQ and DQ, and three quinones, MD, PB, and BN. MD and PB are often used to generate superoxide in vivo; BN is not known to redox cycle in vivo (*25*). Only the bipyridyl compounds fully induced the *sod1*Δ phenotype, and only PQ treatment exactly mimicked the *sod1*Δ phenotype—inducing all of the phenotypic characteristics of *sod1*Δ yeast listed above (Figures 3, 4, and 6) as well as increasing free iron levels (*3*). Treatment with the other bipyridyl compound, DQ, caused good but not perfect mimicry, the most notable difference being its relatively poor induction of the lysine auxotrophy. This difference could be due to variations in cellular localization of these two compounds, since the methionine auxotrophy is thought to result from events in the cytosol (*8*) while the lysine auxotrophy is due to inactivation of homoaconitase, which is located in the mitochondrial matrix (*2*). In addition, the growth inhibitory action of both of these compounds was much more pronounced under aerobic conditions, indicating that dioxygen is required for the toxic effects (Figure 5).

By contrast, treatment with the quinones—MD, PB, and BN—did not induce methionine and lysine auxotrophies in WT cells (Figures 3 and 4), and their growth inhibitory action was independent of the presence of dioxygen (Figure 5), indicating a mechanism of toxicity in WT cells under normal conditions that does not involve superoxide anion and which most likely involves their ability to react with cellular nucleophiles. Nevertheless, for MD and PB, redox cycling is part of their repertoire because (i) the *sod1*Δ mutant was extremely sensitive to them, (ii) their growth inhibitory action increased in 100% dioxygen (Figure 3D), and (iii) they caused a decrease in aconitase activity at higher concentrations (Figure 6). Some accounts attribute the toxicity of MD-like quinones to redox cycling while others cite their ability to react with cellular nucleophiles as alkylating agents (*10*, *26*, *27*). Our work provides evidence for a complex biological activity that involves both toxic mechanisms of these quinones. Which one predominates is highly dependent on the precise conditions, including the level of SOD protection and the partial pressure of dioxygen. WT yeast are apparently quite able to cope with the increased superoxide flux due to redox cycling of MD but less able to cope with its other reactivity. On the other hand, there was no indication that BN could ever act by redox cycling: It did not induce any of the *sod1*Δ phenotypes in WT yeast, *sod1*Δ yeast showed no hypersensitivity to it, and it did not decrease aconitase activity.

The experiments described herein can be used to obtain a qualitative assessment of the degree to which superoxide production by redox cycling contributes to the total toxicity of a chemical. The ability to induce the lysine and methionine auxotrophies in WT yeast indicates that redox cycling is the major mechanism of toxicity for these compounds in normal cells. Differential toxicity for *sod1*Δ as compared to WT and inhibition of aconitase activity are strong indicators that a compound can redox cycle in vivo, even if that is not its primary toxic mechanism. The toxicity under various dioxygen levels can also help determine whether redox cycling contributes to toxicity under some conditions. If high dioxygen increases the toxicity, then redox cycling is implicated, at least under some conditions. Conversely, if a compound has similar toxicity under aerobic and low oxygen conditions, it indicates the presence of another mode of toxicity and little involvement of redox cycling in the primary toxicity. In some organisms, the presence of a diaphorase enzyme (cytoplasmic NAD(P)H:quinone oxidoreductase) could further limit redox cycling by quinones and decrease its contribution to toxicity, but this kind of enzyme is not known to be present in yeast.

Some medically relevant drugs are thought to act, at least in part, by redox cycling. These include the compound MPP^+, which causes symptoms that resemble Parkinson's disease. There has been some disagreement in the literature as to whether MPP^+ works by redox cycling (*35*−*45*) so we decided to use it as a test case for our system. Although MPP^+ is toxic to yeast, our results indicate that redox cycling is not a route to its toxicity under any conditions (Figure 7). Cells lacking CuZnSOD are not dramatically more sensitive than WT cells to MPP^+, and aconitase activity is not inhibited. Both of these results strongly indicate that excess superoxide is not generated by treatment with MPP^+. Correspondingly, MPP^+ does not induce the lysine auxotrophy in WT cells. However, growth of MPP^+-treated cells was slightly rescued under low oxygen and a weak methionine auxotrophy was observed, both of which were very surprising in light of the nonsensitivity of *sod1*Δ cells. We believe that the methionine auxotrophy must result from something other than excess superoxide. For example, MPP^+ is thought to deplete GSH in some systems (*39*, *53*, *54*), and methionine is a good source of reduced sulfur, so it is possible that in the absence of methionine growth is slowed because the cells must devote significantly more

energy to processing sulfur for GSH synthesis. Alternatively, MPP^+ may specifically inhibit some step in methionine synthesis or deplete NADPH in another way.

There are a few lessons here for researchers utilizing redox-cycling compounds to generate superoxide in vivo. It is important to be aware of the differences among the variety of compounds available before selecting a compound. Because their main toxicity derives from superoxide production, the bipyridyl herbicides, particularly PQ, should be considered the preferred method for inducing production of superoxide in vivo, at least in nonphotosynthetic organisms. WT yeast treated with a moderate amount of PQ (0.25−1.0 mM) can be appropriately used as a substitute for a strain deficient in CuZnSOD activity but MD-treated cells cannot. Additionally, the conditions of the experiment are critical. For instance, we have observed that PQ is at least five times more toxic to yeast at pH 6.0 than at pH 4.0 (data not shown), which is important not only for the initial medium preparation but also for the length of time the cells are allowed to grow, due to the tendency of yeast to lower the culture pH. Similar pH effects were also observed in *E. coli* (*19*).

Many assays for superoxide production in vivo have been presented in recent years, usually employing added reagents such as spin traps, tetrazolium blue, or lucigenin and luminol to detect superoxide. While common, many of these assays are inherently flawed to some degree by variables associated with the reagent itself or the conditions of an in vivo experiment (*51*). The present work presents an alternative assay for the qualitative assessment of a compound's ability to produce superoxide toxicity in vivo that relies upon phenotypic responses attributable to increased superoxide and thus minimizes the flaws observed in other assays. While not an assay in the classical sense, these experiments can be utilized to effectively discern the likelihood that a compound will redox cycle in vivo and whether this is its primary toxic mechanism. The techniques used are fairly simple and can be utilized for any number of compounds that are capable of entering a yeast cell in a bioactive form.

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