Cellular factors required for protection from hyperoxia toxicity in *Saccharomyces cerevisiae*

Caryn E. OUTTEN, Robert L. FALK and Valeria C. CULOTTA¹

Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe St. Room 7032, Baltimore, MD 21205, U.S.A.

Prolonged exposure to hyperoxia represents a serious danger to cells, yet little is known about the specific cellular factors that affect hyperoxia stress. By screening the yeast deletion library, we have identified genes that protect against high-O₂ damage. Out of approx. 4800 mutants, 84 were identified as hyperoxia-sensitive, representing genes with diverse cellular functions, including transcription and translation, vacuole function, NADPH production, and superoxide detoxification. Superoxide plays a significant role, since the majority of hyperoxia-sensitive mutants displayed cross-sensitivity to superoxide-generating agents, and mutants with compromised SOD (superoxide dismutase) activity were particularly vulnerable to hyperoxia. By comparison, factors known to guard against H₂O₂ toxicity were poorly represented amongst hyperoxia-sensitive mutants. Although many cellular components

are potential targets, our studies indicate that mitochondrial glutathione is particularly vulnerable to hyperoxia damage. During hyperoxia stress, mitochondrial glutathione is more susceptible to oxidation than cytosolic glutathione. Furthermore, two factors that help maintain mitochondrial GSH in the reduced form, namely the NADH kinase Pos5p and the mitochondrial glutathione reductase (Glr1p), are critical for hyperoxia resistance, whereas their cytosolic counterparts are not. Our findings are consistent with a model in which hyperoxia toxicity is manifested by superoxide-related damage and changes in the mitochondrial redox state.

Key words: glutathione, hyperoxia, mitochondrion, reactive oxygen species, superoxide dismutase, yeast.

INTRODUCTION

Although O₂ is essential for the livelihood of aerobic organisms, exposure to higher than normal O₂ concentrations can be detrimental to cells. In laboratory animals, hyperoxia exposure (> 90 % O₂) causes death within 3–7 days, primarily through progressive damage to the lungs [1]. This damage is thought to be caused by an increase in production of ROS (reactive oxygen species) [2–7]; however, the cellular mechanisms leading to hyperoxia-induced oxidative stress are poorly understood. For example, in mammalian cell models, there are conflicting reports concerning the contribution of mitochondrial ROS to hyperoxic cell damage and death [5,8]. Furthermore, the full range of antioxidant defence systems that respond specifically to this type of stress have not been clearly identified. Despite the potential for oxidative damage, high O₂ is routinely administered during surgery [9,10] and neonatal intensive care [11], and is also used to treat certain medical conditions, such as severe respiratory failure, radiation-induced tissue injury, carbon monoxide poisoning and thermal burns [12]. For these reasons, it is vital to understand how hyperoxia affects cells and which antioxidant systems are required to maintain viability under these conditions.

Aerobic organisms have evolved with numerous antioxidant factors to deal with the production of superoxide (O₂^{•−}), hydrogen peroxide (H₂O₂) and highly reactive hydroxyl radicals (OH[•]), which are by-products of aerobic metabolism. These factors include enzymes such as SODs (superoxide dismutases), catalases and peroxidases, which detoxify these dangerous molecules directly. Cells also have numerous defence systems for maintaining the cellular redox state and repairing oxidatively damaged proteins, DNA and lipids. These systems include the glutathione- and thioredoxin-dependent reduction systems and methionine sulfoxide reductases, all of which ultimately require reductive

equivalents from NADPH for their function [13]. Each of these systems protects against superoxide- and/or peroxide-mediated oxidative stress, as demonstrated in gene-deletion studies in organisms such as yeast [13–15]. However, a role for these factors in protection against hyperoxia has not been investigated extensively.

For many reasons, the yeast *Saccharomyces cerevisiae* provides an ideal organism for studying the effects of hyperoxia exposure. Yeast cells are thought to generate ROS through the same mechanisms as mammalian cells and express many of the same antioxidant factors [13,16]. Additionally, yeast can grow both aerobically and anaerobically, making it easy to identify cell damage that is specific for ROS. The recent development of a complete yeast gene-deletion library, for which each of the approx. 6000 ORFs (open reading frames) was individually and systematically deleted, greatly facilitates the process of phenotypic screening [17].

We screened the yeast gene-deletion library for hyperoxia sensitivity and identified 84 genes that function in resistance to this type of oxidative insult. Our studies strongly indicate that superoxide anion is at least in part responsible for hyperoxia toxicity, since SOD enzymes are essential for protection against hyperoxia damage. Furthermore, there exists considerable overlap between mutants that are sensitive to hyperoxia and those that are sensitive to the superoxide-generating agent paraquat. Our studies also indicate that the redox state of the mitochondria is particularly vulnerable to the effects of hyperoxia. As such, factors that help regenerate GSH in the mitochondria are key in preventing damage from high O₂.

EXPERIMENTAL

Hyperoxia-sensitivity screen

The *S. cerevisiae* MATa haploid deletion collection derived from parental strain BY4741 (*MATa his3 Δ1 leu2 Δ0 met15 Δ0 ura3 Δ0*)

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ORF, open reading frame; RNAP II, RNA polymerase II; ROS, reactive oxygen species; SOD, superoxide dismutase; WT, wild-type.

¹ To whom correspondence should be addressed (email vcullotta@jhsph.edu).

Table 1 Classes of genes required for hyperoxia resistance

Functional class	Genes identified in hyperoxia screen
Transcription	<i>TOM1, CYC8, GAL11, PGD1, SIN4, MED2, SNF6, RPB4, SWI6, MET18, SPT4, CTK1, THI3, MAC1</i>
Translation (non-mitochondrial)	<i>CYS4, HOM6, PRO1, PRO2, TRP3, TRP5, TRP2, TOM1, SPT4, CTK1, DEG1, TPD3, ARC1</i>
Vacuole function	<i>TFP1, VMA22, CUP5, VMA9, PPA1, TFP3, VMA10, VMA13, VMA2, VMA21, VMA4, VMA5, VMA7, VMA8, VPH2, PEP12, VPS1, VPS33, PEP7, VPS27, ATG17</i>
Cell structure	<i>SLA1, BEM1, GON7</i>
SOD function	<i>SOD1, SOD2, CCS1, CTR1, MAC1, MTM1</i>
Mitochondrial function	<i>MDM39, OCT1, PIM1, MTG1, MRPS16, FMP21, CAF17</i>
Production of cellular reductants NADPH and GSH	<i>POS5, GND1, GLR1</i>
Manganese and iron homeostasis	<i>PMR1, PER1, ISA1, SSQ1, GRX5</i>
DNA repair and replication	<i>RAD27, RAD9</i>
Fermentation	<i>ADH1, PDC1</i>
Phosphate metabolism	<i>PHO85, PHO86</i>
Heat-shock proteins	<i>HSP12, YDJ1</i>
Stress-related	<i>NRP1, YOR322C, TOM1</i>
Miscellaneous	<i>CAX4, GRR1, NPL6, RMD8, YBL094C</i>

was used in the present study (<http://www-sequence.stanford.edu/group/yeast-deletion-project/deletions3.html>). The 53 96-well plates comprising the collection were thawed, and 5 μ l of cells from each well was added to 100 μ l of YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) in 96-well plates. The freshly inoculated cultures were grown anaerobically in an O₂-depleted culture jar (BBL Gas Pak) [18] at 30 °C for 24 h, then 3 μ l of cells from each well was spotted on to four sets of solid YPD medium (YPD + 2 % agar). Two sets of plates were placed in a chamber flushed with 100 % O₂, while two sets were grown in air (21 % O₂). For growth in 100 % O₂, cultures were placed in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA, U.S.A.) and flushed with 100 % O₂ for 10–20 min before sealing. After incubation at 30 °C for 24 h, growth under atmospheric O₂ and hyperoxic conditions was compared in order to identify deletion strains that are hyperoxia-sensitive. Strains that appeared to show a hyperoxia-sensitivity phenotype were re-checked by spotting 5 μ l of cells, at D_{600} values of 1.0, 0.1, 0.01 or 0.001, on to YPD plates and growing the plates under anaerobic, atmospheric-O₂ and hyperoxic conditions at 30 °C for 48 h. Strains with a slow-growth phenotype in hyperoxia compared with anaerobic conditions were rated on a scale from 1 to 4, with mutants in class 1 exhibiting the strongest sensitivity. Deletion strains with a 1–3 rating were subsequently screened for sensitivity to other oxidants. For these screens, yeast cells were spotted on to YPD plates with added H₂O₂ (2.0 mM) or paraquat (0.5 mM) and grown in air for 2–3 days at 30 °C.

Plasmids and yeast strains

The *GLR1 CEN* plasmids pCO113, pCO114, pCO115, pCO116, pCO117, pCO118, pCO119, pCO121, pCO122 and strain CO205 (*pos5*Δ::*URA2*) were described previously [19,20]. *COQ1* double deletions CO224 (*vma5*Δ *coq1*Δ), CO225 (*vma10*Δ *coq1*Δ), CO229 (*trp5*Δ *coq1*Δ), CO230 (*arc1*Δ *coq1*Δ), CO231 (*met18*Δ *coq1*Δ), CO232 (*nrp1*Δ *coq1*Δ), CO233 (*pgd1*Δ *coq1*Δ) and CO234 (*hom6*Δ *coq1*Δ) were obtained by disruption of the *COQ1* gene (+67 to +850) in the corresponding *kanMX4* single-deletion strain from the BY4741 knockout collection with the *coq1*Δ::*LEU2* plasmid pSG108 [21]. The *sod1*Δ *coq1*Δ double deletion (SG160) was derived from the strain KS105, a *sod1*Δ::*TRP1* derivative of 1783 [22], in which *COQ1* was deleted using pSG108 [21]. Yeast transformations were performed by the lithium acetate procedure [23], and all gene deletions were verified by PCR analysis. Strains were maintained at 30 °C on

either YPD or SD (synthetic defined) medium supplemented with the appropriate amino acids [24].

SOD activity assay

The SOD activity gel assay was conducted as previously described [25]. Briefly, strains were grown in YPD medium, without shaking, to mid-exponential phase, harvested, then homogenized by glass-bead agitation. Protein extracts (60 μ g) were subjected to non-denaturing PAGE on 12 % gels, and subsequently stained for SOD activity using Nitro Blue Tetrazolium (Sigma) as described previously [26].

GSH/GSSG assays

Total glutathione (GSH + GSSG) and GSSG were measured as described previously using the DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] glutathione reductase recycling assay [19]. For comparison of WT (wild-type), *pos5*Δ and *zwf1*Δ strains, cells were grown to mid-exponential phase, with shaking in YPD medium. For comparison of growth under various O₂ tensions, WT BY4741 cells were grown to mid-exponential phase in YPD medium, with constant bubbling of non-humidified N₂, air or 100 % O₂ through the medium. Cells were fractionated into mitochondrial and post-mitochondrial supernatant fractions by converting cells into spheroplasts, followed by gentle lysis by Dounce homogenization and differential centrifugation [27]. Protein concentrations were determined using the Bradford method (Bio-Rad) with BSA as the calibration standard. Extracts were acidified with the addition of 5-sulphosalicylic acid to a final concentration of 1 %. Measurement of total glutathione in acidified extracts was conducted by spectrophotometric analysis of DTNB reduction as described previously [19]. GSSG levels were measured separately by derivatization of GSH with 2-vinylpyridine [19].

RESULTS

Hyperoxia-stress-resistance genes

We screened the *MATa* haploid deletion library (4852 strains) for sensitivity to 100 % O₂ in order to identify genes that provide protection against hyperoxia toxicity. A total of 84 distinct ORFs were identified that, when mutated, resulted in no cell growth or reduced growth in 100 % O₂ compared with anaerobic and atmospheric O₂ conditions (Table 1). These mutants were classified further according to the degree of O₂-sensitivity, with mutants

Figure 1 Hyperoxia-sensitivity screen spot tests

The indicated yeast strains were tested for growth by plating 5 μ l of solution at D_{600} values of 1.0, 0.1, 0.01 and 0.001 on to YPD plates. $-O_2$, growth in anaerobic culture jars; $+air$, atmospheric O_2 growth (21% O_2); $+100\% O_2$, growth in chambers flushed with 100% O_2 . Strains utilized were WT (BY4741) and mutant strains from the BY4741 *MATa* haploid deletion collection, with the exception of *pos5*Δ, which is represented by strain C0205 (BY4741 *pos5*Δ::*URA3*) [20]. Mutant sensitivity is rated on a scale from 1 to 4. Rating 1, no growth detected at any dilution; rating 2, growth only at a dilution of 1.0; rating 3, growth at 1.0 and 0.1 dilutions; rating 4, growth at 1.0, 0.1 and 0.01 dilutions.

ranging from complete absence of growth in 100% O_2 (rating of 1) to impaired, but positive, growth under these conditions (rating of 4). A sampling of such ratings is provided in Figure 1, and the complete list of 84 hyperoxia-sensitive mutants and their sensitivity ratings is given in Supplementary Table 1S (at <http://www.BiochemJ.org/bj/388/bj3880093add.htm>).

The mutants fell into several functional groups that are outlined in Table 1. A number of antioxidant factors were found to be important for hyperoxia resistance, including the SODs Sod1p and Sod2p, and enzymes that regenerate the reducing equivalents NADPH (Pos5p and Gnd1p) and GSH (Glr1p). Certain genes involved in DNA repair (Rad9p and Rad27p) were also found to be important, as were genes associated with other types of stress, including the heat-shock protein genes *HSP12* and *YDJ1*, and the *NRP1* and *YOR322C* mutants implicated in stress resistance (Table 1). We additionally identified mutants in metal metabolism, including factors needed for iron-sulphur cluster assembly (Isa1p, Ssq1p and Grx5p) and manganese homeostasis (Pmr1p and Per1p). Currently, there is significant evidence linking oxidative damage to iron and manganese metabolism [28–32]. Consistent with the suggested role of mitochondrial respiration in hyperoxia toxicity [3–7], a number of mutants are implicated in mitochondrial function (Table 1).

In addition to stress-associated genes, a number of hyperoxia-sensitive mutants were found to be important for basic cellular processes, including transcription, translation and vacuole function. We found 14 mutants for genes involved in transcription, including factors required for general RNAP II (RNA polymerase II) transcription and transcriptional activators for RNAP II-transcribed genes. We also identified 15 mutants for translation, including proteins required for amino acid biosynthesis, mRNA

processing and transport, and tRNA biosynthesis and transport. In addition, we identified 15 mutants for genes that either encode subunits of the vacuolar (H^+)-ATPase or are involved in synthesis of this complex, and six mutants for genes implicated in vacuolar morphology and protein trafficking (Table 1). Factors required for protein synthesis (transcription plus translation) and vacuolar function have also been identified in genome-wide screens for mutants sensitive to chemical oxidants and pharmacological agents [14,15]. Therefore these housekeeping processes, which appear to be of widespread importance for general stress resistance, also play a significant role in hyperoxia resistance.

Hyperoxia sensitivity and toxicity from superoxide

Because superoxide and H_2O_2 have been implicated in hyperoxia toxicity [2–6], we examined our mutants for cross-sensitivity to H_2O_2 and paraquat (a superoxide generator). For these studies, we focused on 67 hyperoxia-sensitive mutants that exhibited a particularly strong sensitivity towards high O_2 (ratings 1–3). These results are provided in Supplementary Table 1S (available at <http://www.BiochemJ.org/bj/388/bj3880093add.htm>) and some representative spot tests are shown in Figure 2. Of the mutants screened, 80% showed cross-sensitivity towards the other oxidants, half of which were sensitive to both paraquat and H_2O_2 , and the other half to paraquat alone. H_2O_2 sensitivity was far less prevalent among the hyperoxia-sensitive mutants (Supplementary Table 1S at <http://www.BiochemJ.org/bj/388/bj3880093add.htm>). This result alone might suggest that hyperoxia toxicity is more a problem of superoxide damage, as opposed to H_2O_2 -induced damage.

Figure 2 H_2O_2 and paraquat sensitivities of selected hyperoxia-sensitive mutants

The indicated strains were tested for growth as in Figure 1 under the designated conditions: $-\text{O}_2$, +air and +100% O_2 are oxygen tension conditions as in Figure 1; $+\text{H}_2\text{O}_2$ and +PQ are atmospheric- O_2 growth on YPD plates containing 2 mM H_2O_2 or 0.5 mM paraquat respectively.

Figure 3 H_2O_2 -specific antioxidant proteins are not required for hyperoxia stress

The indicated strains were tested for growth by plating cell dilutions as in Figure 1. $-\text{O}_2$, +air and +100% O_2 are oxygen tension conditions as in Figure 1; $+\text{H}_2\text{O}_2$ is atmospheric- O_2 growth on YPD plates containing 2.5 mM H_2O_2 .

Consistent with the role of superoxide in hyperoxia stress, we noted strong hyperoxia sensitivity in mutants defective in activity for either the largely cytosolic Cu/Zn-SOD1 or the mitochondrial Mn-SOD2 enzyme. Mutations in *SOD1* and *SOD2* have previously been associated with hyperoxia sensitivity [20,33,34], and these were found in the present study to represent some of the most sensitive of the approx. 4800 mutants available. We also show that virtually every factor known to modulate SOD1 activity is also important for hyperoxia resistance. This list includes the Ctr1p copper transporter, the Ccs1p copper chaperone and the Mac1p Cu transcription factor, all needed for SOD1 activity (Table 1 and Figure 2). In addition, Mtm1p needed for SOD2 activity was also found to be important for hyperoxia resistance (Table 1).

While genes for superoxide scavenging were well represented, the major defence systems for peroxide were virtually absent

among hyperoxia-sensitive mutants. For example, the *yap1Δ* and *skn7Δ* mutants (encoding transcriptional regulators) were previously identified in genome-wide screens as being most sensitive towards peroxide [14,15], yet these same mutants show no sensitivity towards hyperoxia (also see [20]). In addition, the H_2O_2 -sensitive *gpx3Δ* (glutathione peroxidase), *trx2Δ* (thioredoxin) and *tsa1Δ* (thioredoxin peroxidase) mutants [14,15] are all resistant towards hyperoxia (Figure 3). If H_2O_2 is contributing to hyperoxia damage, its role would appear to be secondary to that of superoxide.

Since SOD activity is essential for protection from hyperoxia toxicity, a few select mutants were subjected to SOD activity gel assays in order to determine if their SOD activity was altered. As mentioned above, mutants for proteins involved in copper trafficking to SOD1 (*ccs1Δ*, *ctr1Δ* and *mac1Δ*) show very low SOD1 activity, which explains their hyperoxia-sensitivity phenotypes

Figure 4 SOD activity assays of selected hyperoxia-sensitive mutants

Total extracts (60 μ g of protein) prepared from WT BY4741 and the indicated mutant strains grown in YPD were analysed by non-denaturing PAGE and SOD activity staining as described in the Experimental section. Positions of SOD1 and SOD2 are indicated. (A) Mutants with decreased SOD1 activity. (B) Mutants with slightly decreased SOD2 activity. (C) Mutants with normal or slightly increased SOD1 and/or SOD2 activity.

(Figure 4A). A few mutants showed somewhat reduced SOD2 activity, including all the vacuole mutants tested (*vma5* Δ , *vma10* Δ , *vma13* Δ and *vma16* Δ), as well as *pos5* Δ and *pgd1* Δ

(Figure 4B). At this point, it is unclear why these mutants exhibited a loss in SOD2 activity, since SOD2 polypeptide levels were similar to WT (results not shown). However, this reduction in activity was small and cannot completely account for the hyperoxia-sensitivity phenotype, since an *smf2* Δ mutant, encoding a Mn-trafficking factor for SOD2, has a greater loss of SOD2 activity (Figure 4B) [25], but is not hyperoxia-sensitive. Several other mutants showed normal (*pho86* Δ and *sin4* Δ) or even increased SOD activity (*caf17* Δ and *met18* Δ) (Figure 4C). Thus, while the SOD enzymes represent a major means of protecting against hyperoxia toxicity, there are a number of non-SOD pathways that can affect resistance to high O_2 as well.

Mitochondrial respiratory-chain components and hyperoxia toxicity

The role of mitochondrial ROS in hyperoxia toxicity has been a matter of debate. Rho mutations in mitochondrial DNA can suppress hyperoxia damage in some mammalian cells, but not in others [5,7,8]. In studies with *S. cerevisiae*, rho mutations can lead to increased ROS production and oxidative damage [35,36]. We therefore chose a targeted genetic approach.

There are at least two sites in the respiratory chain that can leak superoxide: complex I and the ubisemiquinone anion derivative of coenzyme Q in complex III [37–39]. Although *S. cerevisiae* cells lack complex I, they still express three NADH dehydrogenases (Nde1p, Nde2p and Ndi1p) that are not coupled to ATP production [40,41], yet have been implicated in superoxide production [42]. Our previous studies have shown that deleting the genes encoding these NADH dehydrogenases singly or together had no effect on the hyperoxia toxicity of *pos5* Δ mutants. On the other hand, we did find that disrupting *COQ1* required for synthesis of coenzyme Q helped to alleviate the hyperoxia sensitivity of a *pos5* Δ mutant [20], implicating the semiquinone anion of the respiratory chain in high- O_2 damage. More recently, we tested whether *coq1* Δ mutations globally reverse hyperoxia toxicity. Of the nine mutants tested (*vma5* Δ , *vma10* Δ , *trp5* Δ , *arcl* Δ , *met18* Δ , *pgd1* Δ , *hom6* Δ , *sod1* Δ and *nrp1* Δ), only *nrp1* Δ was partially rescued by a *coq1* Δ mutation (Figure 5). The precise function of Nrp1p is unknown, but it has been implicated in stress responses [43]. Curiously, Nrp1p seems specific to hyperoxia, since it is not required for other forms of oxidative stress (Figure 2) [14,15]. Mutations in *COQ1* also reversed the sensitivity of *sod1* Δ mutants

Figure 5 Respiratory-chain deletions and hyperoxia sensitivity

The indicated strains were tested for growth by plating cell dilutions at D_{600} values of 2.0, 0.2, 0.02 and 0.002 on to YPD plates. (A) Strains utilized were WT (BY4741), *nrp1* Δ (*nrp1* Δ ::*kanMX4*) and CO232 (*nrp1* Δ *coq1* Δ). (B) Strains utilized were WT (1783), KS105 (*sod1* Δ) and SG160 (*sod1* Δ *coq1* Δ).

Figure 6 Effects of NADPH sources on mitochondrial compared with cytosolic GSH

WT (BY4741), *pos5Δ* (CQ205) and *zwf1Δ* (*zwf1Δ::kanMX4*) strains were grown overnight to mid-exponential phase in YPD medium with shaking. Cells were lysed and fractionated, then assayed for GSH and GSSG as described in the Experimental section. The percentage of GSSG (% GSSG/total glutathione) in crude mitochondrial (A) and post-mitochondrial supernatant (PMS) (B) fractions was calculated from total glutathione and GSS levels in each extract ($GSS = 2 \times GSSG$). Results are the means $\pm$ S.D. for three independent experiments.

towards atmospheric (21 %) O_2 , but not towards hyperoxic O_2 (Figure 5); and there was no further rescue by additional mutations in the NADH dehydrogenases (results not shown). Therefore, although the semiquinone anion may contribute in part to hyperoxia damage, other cellular sources must be involved as well (see the Discussion).

Mitochondrial redox state, GSH and hyperoxia stress

One of most O_2 -sensitive mutants isolated in this screen is *Pos5p*, the mitochondrial source of NADPH [20]. Compared with *pos5Δ* cells, mutants that affect cytosolic production of NADPH showed only mild (*gnd1Δ*) (Figure 2) or no sensitivity (e.g. *tkl1Δ* and *zwf1Δ*) towards hyperoxia [20]. This result would suggest that mitochondrial reductants are more critical in hyperoxia protection.

One role of NADPH is to provide the reducing equivalents needed to regenerate GSH via glutathione reductase. To test whether glutathione is affected in *pos5Δ* mutants, we measured GSSG and GSH in the mitochondria and cytosol of these cells. The results were compared with that of *zwf1Δ* mutants encoding the main cytosolic source of NADPH [22,44]. As seen in Figure 6(A), there is a striking elevation in the mitochondrial GSSG/GSH ratio, but not that of the cytosol (Figure 6B). Hence, *Pos5p* and NADPH are needed to reduce mitochondrial glutathione. In contrast, the GSSG/GSH ratio in *zwf1Δ* cells shows the opposite trend, with a dramatic increase in the cytosolic GSSG/GSH ratio, but no change in the mitochondria, reflecting the influence of this cytosolic NADPH source only on glutathione redox status in the cytosol. Apparently, this increase in cytosolic

redox potential does not affect resistance to hyperoxia, since *zwf1Δ* mutants are not hyperoxia-sensitive [20]. In comparison, the increase in mitochondrial GSSG/GSH of *pos5Δ* cells is clearly associated with hyperoxia sensitivity.

Regeneration of GSH also requires the *Glr1p* glutathione reductase, and we noted that *glr1Δ* mutants are hyperoxia-sensitive (Table 1 and Figure 7). Since *GLR1* encodes both cytosolic and mitochondrial GSH reductase [19], we sought to determine which of these isoforms is required for hyperoxia resistance. Our previous studies have shown that the mRNA encoding *Glr1p* is translated from two alternative start sites, producing mitochondrial and cytosolic isoforms of the protein [19]. We could therefore control *Glr1p* localization using mutant forms of the translational start sites that favour cytosolic over mitochondrial localization. Strains expressing such *Glr1p* variants were tested for hyperoxia sensitivity. The cytosolic versus mitochondrial localization of each *Glr1p* variant tested has been published previously [19], with the locations indicated in Figure 7. From this Figure, it is clear that only the mitochondrial form of *Glr1p* is critical in protection from high- O_2 toxicity, even though it represents only 5–10 % of the total cellular pool of *Glr1p*. Together with our studies of *Pos5p*, it would appear that maintenance of the mitochondrial redox state by GSH is critical for hyperoxia resistance.

To investigate further the role of mitochondrial GSH in hyperoxia protection, we measured glutathione levels in cells grown under three O_2 conditions: anaerobic (cells bubbled with nitrogen), atmospheric (with 21 % O_2) and hyperoxic (with 100 % O_2). Cytosolic and crude mitochondrial fractions were assayed independently. The assay used specifically measures free glutathione levels, although there is also a possibility of changes in levels of mixed glutathione–protein disulphides in the different O_2 tension conditions. In the case of the cytosol, levels of total glutathione remain relatively constant under different O_2 tensions (Figure 8A), while there was a small increase in the percentage of oxidized glutathione from atmospheric O_2 to hyperoxia (Figure 8B). By comparison, effects with mitochondrial glutathione were more pronounced. Total mitochondrial glutathione increased by 80 % from anaerobic to atmospheric O_2 conditions (Figure 8C). In going from atmospheric to hyperoxia conditions, there was a 55 % increase in the percentage of GSSG, with no change in total glutathione (Figures 8C and 8D). This increase in mitochondrial GSSG indicates that the redox state of the mitochondria is particularly vulnerable to high O_2 , with the regeneration of GSH by *Pos5p* and mitochondrial *Glr1p* representing an important defence against hyperoxia damage.

DISCUSSION

Toxicity from high O_2 tension has long been known to cause cellular damage, and yet very little is understood regarding the determinants of hyperoxia toxicity. Using a high-throughput genetic screen in *S. cerevisiae*, we found that the factors that modulate hyperoxia sensitivity are diverse and cover a wide variety of functions. Hyperoxia resistance requires a number of stress-response factors, including those involved in antioxidant defence, heat shock, DNA damage repair and metal homeostasis. In addition, a large class of hyperoxia-resistance genes participate in more housekeeping activities, including transcription, translation and vacuole function.

We compared these findings with results obtained recently from genome-wide screens for mutants sensitive to chemical oxidants [14,15]. Some overlap with hyperoxia is apparent, particularly in genes for transcription, translation and vacuole

Figure 7 Mitochondrial Glr1p is required for hyperoxia protection

The indicated strains were tested for growth by plating cell dilutions as in Figure 1. Strains utilized were WT (BY4741) and *glr1Δ* strains transformed with pAA1 (vector), pC0113 (WT Glr1p), pC0114 (M17L), pC0115 (Δ1-16), pC0116 (M1L), pC0117 (FS1), pC0118 (FS2), pC0119 [U₃A(M1)], pC0121 [U₃A(M1)/M17L] or pC0122 (M1L/M17L). A short description of the different mutant constructs as well as the subcellular location of Glr1p for each (as determined in [19]) is shown on the right. M, mitochondria; C, cytosol.

Figure 8 O₂ tension effects on glutathione levels

WT (BY4741) strains were grown overnight to mid-exponential phase in YPD medium with N₂, air or 100% O₂ bubbling through the culture during growth. Extracts were prepared as described in Figure 6, and were assayed for total glutathione (GSH + GSSG) in the post-mitochondrial supernatant (PMS) (A) and mitochondrial (C) extracts. The percentage of GSSG (% GSS/total glutathione) was calculated from total GSH and GSS levels in the PMS (B) and mitochondrial (D) extracts (GSS = 2 × GSSG). Results are means ± S.D. for three independent experiments.

function/protein trafficking. Such classes of genes have been defined as MCS ('multi-chemical sensitive') [15] and are thought to help replenish proteins that become damaged during stress

[14]. Although there was considerable overlap between hyperoxia and other oxidative-stress inducers, there are a number of genes unique to hyperoxia toxicity, including the *CAF17*, *FMP21*, *MTG1* and *MTM1* genes, which are needed for mitochondrial respiration, *NRPI*, implicated in stress signalling to the nucleus, and the *HSP12* and *TOM1* genes, involved in numerous stress responses. Likewise, a number of antioxidant factors identified in the chemical oxidant studies were absent in our hyperoxia screen, including the thioredoxin Trx2p, glutathione biosynthesis proteins Gsh1p and Gsh2p, glutathione peroxidase Gpx3p, thioredoxin peroxidase Tsa1p, cytochrome *c* peroxidase Ccp1p, and the Yap1p and Skn7p transcription factors [14,15]. It has been suggested that different types of oxidative stress require distinct sets of genes for resistance [14]. Hyperoxia, likewise, involves defence pathways that are both unique and overlapping with other oxidative insults.

Under hyperoxia toxicity, O₂ itself may not be the main culprit of damage, but rather products of O₂ metabolism, specifically superoxide, lead to toxicity. Mutants that result in loss of SOD activity are among the most sensitive towards hyperoxia, and the majority of hyperoxia-sensitive mutants show cross-sensitivity to superoxide. In comparison, factors that are known to protect against H₂O₂ (e.g. Yap1p, Skn7p, Gpx3p and Tsa1p) [13,45] were poorly represented in our collection of hyperoxia-sensitive strains. It has long been thought, largely based on studies with isolated mitochondria, that both superoxide and H₂O₂ are the culprits in hyperoxia-related damage [3,4,6,38]. Yet, based on our genetic studies, it would appear that the superoxide anion is the key damaging agent.

Where is the superoxide coming from? The mitochondrial respiratory chain would seem a likely candidate. However, inconsistent findings have been obtained with rho mutations in cells that lack mitochondrial DNA. Rho mutations suppress hyperoxia damage of HeLa cells [5], but not of human HT1080 cells [8]. In *S. cerevisiae*, rho mutations can suppress O₂ toxicity in *sod2Δ* mutants, but not in *sod1Δ* mutants [46,47]. Because rho mutations

can increase oxidative damage in yeast [35,36], we specifically deleted the NADH dehydrogenases or the semiquinone anion sources of mitochondrial superoxide [37–39,42]. Yet again, there was no suppression of the hyperoxia toxicity of *sod1* Δ mutants in the present study. Targeted deletion of the respiratory-chain components did help alleviate the hyperoxia toxicity of a few mutants (e.g. *nrf1* Δ and *pos5* Δ), yet the effects are limiting. Hence, under hyperoxia, it appears that non-mitochondrial sources of ROS may pose an additional danger to the cell. These potential sources include numerous redox-active enzymes that have the capacity to reduce molecular O₂ in other cellular compartments, such as the cytosol, peroxisomes, endoplasmic reticulum and the cell surface [48]. With high O₂ tension, perhaps the possibility to produce ROS inadvertently at multiple intracellular sites increases. In future studies, it would be interesting to screen for hyperoxia sensitivity that is specific to mitochondrial ROS, e.g. by growth under conditions that specifically require mitochondrial respiration, such as growth on a non-fermentable carbon source.

An important outcome of our findings was the observed role of mitochondrial redox state in hyperoxia resistance. The redox environment of the cell is largely controlled by the oxidation state of the abundant thiol glutathione [49], and we find that two factors required for the reduction of mitochondrial glutathione, namely Pos5p and mitochondrial Glr1p, are important in hyperoxia defence. In comparison, the equivalent system for reducing glutathione in the cytosol (namely cytosolic Glr1p and factors in the pentose phosphate pathway) is of less significance. Moreover, we find that, during hyperoxia, mitochondrial GSH is more susceptible to oxidation than cytosolic GSH. In the absence of the Pos5p/Glr1p systems for regenerating GSH, mitochondrial GSSG/GSH must be exceedingly high during hyperoxia, leading to cell death (GSSG/GSH could not be measured in *pos5* Δ strains grown in hyperoxic conditions, since this mutant cannot survive in these conditions). Why is mitochondrial GSH so vulnerable? During oxidative stress, GSH is consumed to reduce cysteine residues in proteins and to drive antioxidant reactions that involve glutaredoxins and peroxidases [13,16]. GSH can also act as a radical scavenger itself by reacting with ROS to produce oxidized glutathione [13]. Such a demand on GSH may be particularly high in the mitochondria when aerobic respiration is increased during hyperoxia. These findings on GSH in yeast may be extended to mammalian cells, since targeting glutathione reductase to the mitochondria helped to alleviate hyperoxia toxicity of pulmonary epithelial cells [50]. Mitochondrial GSH may be a key determinant in hyperoxia resistance of diverse organisms and cell types.

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