Saccharomyces cerevisiae Exhibits a yAP-1-Mediated Adaptive Response to Malondialdehyde

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Malondialdehyde (MDA) is a highly reactive aldehyde generally formed as a consequence of lipid peroxidation. MDA has been inferred to have mutagenic and cytotoxic roles and possibly to be a participant in the onset of atherosclerosis. The adaptability and oxidative stress response of Saccharomyces cerevisiae is known to generate resistance and adaptation to MDA. A yap1 disruption mutant exhibited the greatest sensitivity and was unable to adapt to MDA, implicating yAP-1 in both the adaptive response and constitutive survival.

The process of respiration requires the uptake of molecular oxygen into the cell. While necessary for aerobic organisms, this can lead to the exposure of cellular components to oxygen. Relatively unreactive and harmless in its ground state, oxygen (O₂) is capable of undergoing excitation or partial reduction to form a number of highly reactive species, including the superoxide anion (O₂⁻), singlet oxygen (O₃), ozone (O₃), and the hydroxyl radical (·OH). These reactive oxygen species (ROS) and the corresponding cellular defense systems activated to protect against them have been extensively studied in the yeast Saccharomyces cerevisiae (19, 26). During oxidative stress, a proportion of the ROS evade the host defenses and can cause oxidative damage to nucleic acids, lipids, and proteins (15, 21, 35). As a result of the oxidative damage to lipids, a variety of lipid hydroperoxides are formed, whose subsequent breakdown products may well be just as toxic as the ROS themselves (5). Malondialdehyde (MDA) is a commonly detected aldehyde (6, 36), which has been used as a measure of lipid peroxidation (13, 20, 27), although such calculations presume MDA cannot be generated from other sources. This presumption is not necessarily accurate (4), since MDA can be generated by free radical attacks on deoxyribose and amino acids and from a number of other sources (1, 6, 14). Thus, levels of MDA may be more indicative of overall cellular oxidative damage, not merely lipid peroxidation (although this may be the predominant source). MDA can exhibit a number of cytotoxic effects and inhibit a variety of enzymes (5, 6). In particular, it has been shown to cause membrane damage, increase fluidity (3, 29), react with DGMP (37), damage DNA (39), and interact with high- and low-density lipoprotein, which contributes to atherosclerosis (16, 33).

Adaptive responses to hydrogen peroxide (H₂O₂) and the superoxide generator menadione, two compounds known to induce oxidative stress, have been demonstrated for S. cerevisiae (2, 7). This ability to acquire resistance to menadione and H₂O₂ raises the possibility that a similar system exists in response to the secondary and tertiary products of oxidative stress, for example, lipid hydroperoxides and breakdown products, such as MDA. Little is known about the consequences of an accumulation of toxic aldehydes, such as MDA, in eukaryotes following oxidative stress or about the systems activated as a response. This study aimed to determine the toxicity of MDA towards yeast cells and assess their ability to detoxify, by way of adaptation or otherwise, this aldehyde.

MATERIALS AND METHODS

Yeast strains and growth conditions. Studies were performed with the wild-type yeast strain CY4 (MATa ura3-52 leu2-3 112 trp1-1 ade2-1 his3-11 can1-100) and its isogenic derivatives CY7 (gsp1::TRP1), CY9 (gsp1::LEU2), CY29 (gsp1::HIS3), and CY4p (petite mutant) (6, 10). Cells were grown in YEPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto Peptone, 2% [wt/vol] glucose) with shaking at 150 rpm at 30°C. Cells were grown to exponential phase (approximately 2 x 10⁸ cells ml⁻¹) and harvested by centrifugation. The effect of pH on MDA toxicity was determined by treating cells in a variety of buffers (0.1 M sodium citrate [pH 3.5 to 5.5], 0.1 M sodium citrate–phosphate [pH 6.0], and 0.1 M sodium phosphate [pH 6.5 to 7.0]) for 1 h at 30°C in a rotary shaker. Samples were removed and diluted in fresh YEPD medium and plated in triplicate to obtain viable counts.

Dose-response curves were generated by treating citrate-buffered suspensions (0.1 M sodium citrate, pH 4.5) with various concentrations of MDA for 1 h at 30°C. Samples were removed, diluted in fresh YEPD medium, and plated in triplicate to obtain viable counts.

For adaptive-response experiments, cells were pretreated with MDA in buffered citrate, pH 4.5, for 1 h at 30°C. Cells were harvested by centrifugation, resuspended in YEPD medium, and incubated at 30°C for 1 h. For protein synthesis inhibition, cycloheximide (50 µg ml⁻¹) was included during pretreatment and YEPD medium incubations.

Determination of MDA concentrations by reversed-phase HPLC. Cells were resuspended in 1% [wt/vol] orthophosphoric acid with 5 mM butylated hydroxytoluene (BHT) and lysed by heating at 90°C for 20 min. Thiodihydroxyacetone (TBA, 0.6%; Sigma) was added, and the reaction mixture was incubated for 30
min 90°C. The chromogen was extracted in n-butanol, 0.50 ml of which was mixed with 0.25 ml of methanol and 0.25 ml of mobile phase (4). Of this mixture, 0.5 ml was mixed with 0.5 ml of mobile phase, and a 50-μl aliquot was loaded onto a Spherisorb octyldecyl silane 25-μm high-pressure liquid chromatography (HPLC) steel column (4.5 by 250 mm). A532 was measured spectrophotometrically, and samples were diluted and plated on YEPD medium to monitor viability. Data are the means of triplicates of a representative experiment.

**RESULTS AND DISCUSSION**

**MDA toxicity increases at lower pH.** In aqueous solutions, MDA exists predominantly in the cyclic enol form rather than as a diadehyde and readily ionizes at a physiological pH (Fig. 1a). To determine the toxicities of both the ionized and the undissociated forms of MDA, exponential-phase yeast cells were treated with 5 mM MDA in buffers with a range of pHs. Greater sensitivity was observed at low pHs, whereas low pH alone had little effect on cell viability (Fig. 1b). We therefore chose a pH at which approximately 50% of the compound was ionized and a significant loss of viability was observed for further studies of the effect of MDA on yeast. The increased toxicity of MDA at the lower pHs can be attributed to the greater proportion of MDA in the undissociated state and the increased rate of diffusion of this molecule across the cell membrane under these conditions. As a consequence, higher levels of intracellular MDA accumulated at lower external pHs. Since intracellular yeast pH is maintained at between 5.7 and 7.2, even when external pH falls to as low as 3.6 (17), exogenous MDA ionizes and exists in the same form as MDA produced within the cells as a result of oxidative or other forms of damage.

**Yeast cells are sensitive to MDA.** Various yeast strains were tested for their sensitivities to MDA. These included a wild-type yeast strain; an isogenic respiratory incompetent petite mutant; and strains lacking GLRI, which encodes glutathione reductase, GSHI, which encodes γ-glutamylcysteine synthetase, and YAPI, which encodes a yeast transcription activator with homology to the mammalian AP-1 protein. Strains were grown to exponential phase (A600 = 1) and exposed to various concentrations of MDA for 1 h to generate a dose-response curve (Fig. 2). All strains exhibited a concentration-dependent loss of viability following exposure to MDA, with a dose of 5 mM resulting in greater than 95% cell death. The

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** Toxicity of MDA varies according to treatment pH. (a) MDA undergoes keto-enol tautomerism and ionizes at a pKₐ of 4.46. (b) Wild-type yeast cells were grown to exponential phase and treated with MDA (5 mM) in buffers of various pHs for 1 h. Control cells received treatment in buffer alone. The proportions of undissociated MDA were determined by the Henderson-Hasselbach equation and confirmed spectrophotometrically. Samples were diluted and plated on YEPD medium to monitor viability. Data are the means of triplicates of a representative experiment.

![Diagram](https://via.placeholder.com/150)

**FIG. 2.** Sensitivities of yeast cells to MDA. Yeast strains CY4 (wild type), CY7 (glr1), CY9 (gsh1), CY4p (petite mutant), and CY29 (yap1) were grown to exponential phase and treated with various concentrations of MDA for 1 h. Samples were diluted and plated on YEPD medium to monitor viability. Data are the means of triplicates of a representative experiment.

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toxicity of MDA was comparable to those of H₂O₂ and the superoxide anion, for which concentrations of 2 and 6 mM, respectively, resulted in about a 95% loss of viability (2, 7). Wild-type cells demonstrated greater resistance than any of the mutants, indicating that none of the mutations conferred resistance to MDA. At low concentrations (up to 3 mM) all strains, except the yap1 mutant, retained a relatively high level of viability, but higher concentrations resulted in rapid, exponential death. This threshold is indicative of either a detoxification system that is overwhelmed at concentrations of greater than 3 mM or a requirement for multiple-site damage for loss of viability by a cell. The lower threshold exhibited by the yap1 mutant tends to rule out the latter possibility and implicates a yAP-1-mediated detoxification system in protection against MDA.

In comparison to the wild type, the glutathione (GSH) metabolism ghr1 and gsh1 mutants exhibited greater sensitivity to 4 or 5 mM MDA. Sensitivity was most significant at 5 mM, with the gsh1 mutant exhibiting the greatest sensitivity of these three strains. This mutant lacks the enzyme required to catalyze the first step in GSH biosynthesis and completely lacks this metabolite (28), whereas the ghr1 mutant (CY7) lacks glutathione reductase activity and is unable to recycle oxidized GSH to reduced GSH. The ghr1 mutant contains elevated levels of GSSG but maintains wild-type levels of reduced GSH, presumably by de novo synthesis (8). The absence of GSH in the gsh1 mutant renders the yeast highly MDA sensitive, whereas in the ghr1 mutant, a buildup of oxidized GSH, with the concomitant disruption of redox equilibrium, appears to result in a mild sensitivity. These results indicate a correlation between GSH metabolism and MDA resistance.

A deficiency of GSH in yeast has previously been described to lead to a disruption of respiratory ability, caused by loss of mitochondrial DNA (9, 22). It was speculated that mitochondrial damage occurs as a result of an accumulation of oxidants produced in the respiratory chain, with similar results observed in higher eukaryotes (18). As a consequence of such DNA damage, gsh1 strains are effectively petite mutants and may therefore be affected in all mitochondrial activities. Respiratory-deficient strains have been shown to exhibit increased sensitivities to oxidants (2); hence, it is not clear whether the phenotype of the gsh1 mutant is due to the petite genotype or the gsh1 defect. To resolve this, an isogenic petite strain generated by ethidium bromide treatment was included for comparison (Fig. 2). Interestingly, the petite strain (CY4p) was significantly more sensitive than the gsh1 strain and extremely sensitive relative to the wild type. This indicates that mitochondrial function is required for maximum resistance to MDA but that an absence of GSH fails to render petite mutants more sensitive.

Wild-type yeast cells exhibit an adaptive response to MDA. Having established that MDA is toxic to yeast, the ability of cells to mount an adaptive response was examined. This was performed by administering sublethal doses of MDA, followed by a recovery period in a rich medium to allow the acquisition of any adaptive response and a further treatment in mild-to-lethal concentrations of MDA (see Materials and Methods). The wild-type strain exhibited greater viability following a le-
FIG. 4. Induction of MDA resistance in strains CY7 (gtr1), CY9 (gsh1), CY29 (yap1), and CY4p (petite mutant). Cells received either no MDA pretreatment or a 1 mM pretreatment prior to MDA challenge. Conditions were as described in the legend to Fig. 3. Samples were diluted and plated on YEPD medium to monitor viability. Data are the means of triplicates of a representative experiment.
that dose (5 mM) of MDA if it had been previously treated with sublethal doses (0.5 to 1.5 mM) (Fig. 3a). Greater viability following mildly lethal treatment (4 mM) was also observed. A sublethal pretreatment of 1 mM MDA resulted in optimum survival, although other doses (0.5 and 1.5 mM) conferred greater viability than no pretreatment. This indicates that wild-type yeast cells can acquire resistance to MDA.

To test whether protein synthesis was required for adaptation, wild-type yeast cells were treated with cycloheximide during both pretreatment with MDA and the subsequent recovery period. The inhibition of cytoplasmic protein synthesis abolished the ability of wild-type yeast to acquire MDA resistance from a prior exposure. It also rendered them more susceptible to MDA than nonpretreated cells (Fig. 3b). Cells receiving cycloheximide alone exhibited a higher viability than those pretreated with MDA in the presence of cycloheximide, following subsequent lethal treatment. This higher viability indicated that the results of pretreatment and treatment are cumulative when protein synthesis is inhibited; e.g., the MDA-pretreated cells received a larger cumulative dose, accounting for reduced viability. These results imply the need for protein synthesis and, in all likelihood, gene expression for adaptation. In order to examine the systems activated during adaptation, mutants were tested for their ability to adapt to MDA.

**yap1** is required for adaptation. The petite, gsh1, glr1, and yap1 mutants were tested for their ability to mount an adaptive response like that of the wild-type strain. All strains, with the exception of the yap1 mutant, acquired resistance to lethal doses of MDA if previously treated with a nonlethal dose (Fig. 4a to d). Additionally, greater resistance to mildly lethal doses (3 to 4 mM) was observed.

Following pretreatment, the glr1 and gsh1 mutants survived as well as the wild type (compare Fig. 3a with 4a and b). This result is in contrast to the variation in their sensitivities exhibited to MDA prior to pretreatment (Fig. 2). This finding implies that although GSH is necessary for maximal survival in nonpretreated cells, it is not required for the mechanism of adaptation, nor is MDA resistance acquired through adaptation dependent on GSH. In addition, the adaptive response mounted by the petite strain was comparable to that of the wild-type strain, despite increased sensitivity to MDA. Similarly, petite mutants were sensitive to H$_2$O$_2$ and the superoxide anion but were still able to mount an adaptive response (2, 7). This demonstrates that mitochondria confer some protection against both oxidative and MDA stress but are not required for adaptation to either. Finally, the yap1 mutant did not appear to exhibit any significant adaptive response at higher MDA concentrations, indicating that the adaptive response is mediated by yap1-1-regulated transcriptional control. The yap1-1 transcriptional activator protein has been implicated in the response to oxidative stress, and target genes for the yap1-1 protein include gsh1 and glr1 (11, 34, 38). Since glr1 and gsh1 are not involved in adaption to MDA, other activities regulated by yap1 must be required. Genes known to be under yap1-1 control include trx2, which encodes thioredoxin, and ycf1, which encodes a vacuolar pump specific for GSH-toxin conjugates (23, 25). The latter gene represents an obvious candidate, and thioredoxin has antioxidant activity although its exact physiological role is unknown. Accordingly, both represent possible MDA detoxification systems.

**H$_2$O$_2$ treatment produces MDA but fails to elicit cross-adaptation to MDA.** MDA levels have previously been shown to reflect the extent of intracellular lipid peroxidation (20, 27), although other evidence, including this study (see below), indicates that MDA levels correspond to the overall cellular oxidation state (4, 6). In order to test this hypothesis with S. cerevisiae, levels of MDA, before and after oxidative stress, were determined by the TBA test and subsequent HPLC analysis. HPLC improved the specificity of the TBA test, such that the absorbance attributable to the MDA-TBA adduct could be distinguished from that of other TBA-reactive substances. HPLC confirmed the presence of other absorbances at the maximum wavelength of the MDA-TBA adduct (532 nm), many of which varied independently of the level of MDA (data not shown). This result indicated that the nonspecific measurement of TBA-reactive substances is inadequate for quantifying MDA levels in the cell. Low levels of intracellular MDA of about 100 μM were detected in unstressed cells grown to exponential phase in YEPD medium. Moreover, the level of MDA adduct was found to increase following hydrogen peroxide treatment (Table 1), indicating that peroxidation of cellular components led to the formation of MDA. After significant H$_2$O$_2$ stress (8 mM for 1 h), intracellular MDA levels increased to 0.5 mM, although even a low H$_2$O$_2$ dose (1 mM for 1 h) was sufficient to result in cells containing 0.4 mM MDA. The main precursors of MDA are polyunsaturated lipid hydroperoxides (20, 21, 30, 31), particularly those of linolenic and arachidonic acids. Linoleic hydroperoxides have also been shown to produce small quantities of MDA (6). No Δ$_2$-desaturase activity has been isolated from S. cerevisiae, nor have any fatty acids with more than monounsaturation (12, 32), although yeast will incorporate polyunsaturated fatty acids present in the medium. The contribution of oxidizable MDA precursors in the medium to the level of MDA detected in cells following H$_2$O$_2$ stress was determined by testing YEPD medium alone under conditions similar to those described above (without the antioxidant BHT). Insignificant levels of MDA were detected (data not shown), indicating that MDA was generated predominantly from components synthesized by the yeast cells, not by constituents of the medium. As yeast cells are unable to synthesize polyunsaturated fatty acids, significant levels of MDA are generated from other sources.

The close relationship between MDA formation and oxidative stress implies that the adaptive response to MDA may be related to those to other oxidents. To test this premise, cells were treated with a nonlethal dose of either MDA (1 mM) or H$_2$O$_2$ (1 mM) followed by a reciprocal lethal treatment. Pretreatment with hydrogen peroxide did not induce any adaptive response to subsequent MDA treatment (data not shown). Considering that the level of MDA produced following H$_2$O$_2$-induced oxidative stress was comparable to the lowest level of MDA used in treatments shown to elicit an adaptive response (Fig. 3), the above-described result appears inconsistent. It should be noted, however, that during MDA treatment cells accumulated MDA in excess of the external concentration (data not shown). Hence, the level of intracellular MDA corresponding to the 0.5 mM external dose necessary to induce

**TABLE 1. Formation of MDA following H$_2$O$_2$ treatment**

<table>
<thead>
<tr>
<th>H$_2$O$_2$ concn (mM)</th>
<th>Mean intracellular [MDA] ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.10 ± 0.04</td>
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<tr>
<td>1.0</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>2.0</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>4.0</td>
<td>0.42 ± 0.17</td>
</tr>
<tr>
<td>8.0</td>
<td>0.51 ± 0.20</td>
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* Wild-type (CY4) cells were grown to exponential phase and treated with the concentrations of H$_2$O$_2$ indicated for 1 h. Lysates were tested for increased levels of intracellular MDA by reversed-phase HPLC. The data are from a representative experiment.
the adaptive response is significantly higher than the intracellular dose detected following H$_2$O$_2$ treatment. The H$_2$O$_2$ pretreatment concentration (1 mM) was, however, sufficient to induce the adaptive response to H$_2$O$_2$ (2), indicating that this response differs from that to MDA. Furthermore, pretreatment with MDA did not confer any resistance to H$_2$O$_2$ further confirming the independence of the two adaptive responses.

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REFERENCES