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H_2O_2 induces rapid biophysical and permeability changes in the plasma membrane of *Saccharomyces cerevisiae*

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Abstract

In *Saccharomyces cerevisiae*, the diffusion rate of hydrogen peroxide (H_2O_2) through the plasma membrane decreases during adaptation to H_2O_2 by means of a mechanism that is still unknown. Here, evidence is presented that during adaptation to H_2O_2 the anisotropy of the plasma membrane increases. Adaptation to H_2O_2 was studied at several times (15min up to 90min) by applying the steady-state H_2O_2 delivery model. For wild-type cells, the steady-state fluorescence anisotropy increased after 30min, or 60min, when using 2-(9-anthroyloxy) stearic acid (2-AS), or diphenylhexatriene (DPH) membrane probe, respectively. Moreover, a 40% decrease in plasma membrane permeability to H_2O_2 was observed at 15min with a concomitant two-fold increase in catalase activity. Disruption of the ergosterol pathway, by knocking out either *ERG3* or *ERG6*, prevents the changes in anisotropy during H_2O_2 adaptation. H_2O_2 diffusion through the plasma membrane in *S. cerevisiae* cells is not mediated by aquaporins since the H_2O_2 permeability constant is not altered in the presence of the aquaporin inhibitor mercuric chloride. Altogether, these results indicate that the regulation of the plasma membrane permeability towards H_2O_2 is mediated by modulation of the biophysical properties of the plasma membrane.

Keywords: Hydrogen peroxide; H_2O_2 gradient; H_2O_2 adaptation; Catalase; Cytochrome *c* peroxidase; Plasma membrane fluidity

1. Introduction

Hydrogen peroxide (H_2O_2) is the most abundant reactive oxygen species in vivo, being continuously produced intracellularly as a by-product of the metabolism in aerobic organisms, and extracellularly during inflammation in higher organisms [1]. Contrary to the usual assumption that H_2O_2 diffuses freely across biomembranes [1], recently it has been shown that in Jurkat T-cells, a mammalian cell line [2], in *Escherichia coli* [3], and in *Saccharomyces cerevisiae* (*S. cerevisiae*) [4,5], biomembranes form a barrier against H_2O_2 diffusion. So, when cells are exposed to external H_2O_2 the intracellular consumption of H_2O_2 catalyzed by antioxidant enzymes generates a gradient of H_2O_2 across the plasma membrane, which makes the intracellular H_2O_2 concentration lower than the external one. The magnitude of this gradient is dependent on the extent of the intracellular consumption of H_2O_2 and on the permeability properties of cell barriers to H_2O_2 [2]. Recent results obtained in our laboratory also showed that in *S. cerevisiae* cell adaptation to H_2O_2, *i.e.* the induction of resistance to a high level (usually lethal) of H_2O_2 by a preliminary low (adaptive) dose of H_2O_2, involves a decrease in the plasma membrane permeability to H_2O_2 [4]. In fact, in *S. cerevisiae* cells the permeability of the

Abbreviations: 2-AS, 2-(9-anthroyloxy) stearic acid; DMSO, dimethyl sulfoxide; DPH, Diphenylhexatriene; *E. coli*, *Escherichia coli*; H_2O_2, hydrogen peroxide; OD_{600}, optical density at 600nm; ROS, reactive oxygen species; *S. cerevisiae*, *Saccharomyces cerevisiae*; TMA-DPH, trimethylammonium diphenylhexatriene; *wt*, BY4741 wild-type

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plasma membrane towards H_2O_2 is under active regulation both during the response to oxidative stress [4] and during the life cycle [5].

Originally, the changes in *S. cerevisiae* plasma membrane permeability to H_2O_2 were ascribed to putative changes in lipid composition that would change the biophysical properties of the plasma membrane, decreasing its permeability to species that cross the membrane by simple diffusion. Several observations support this hypothesis: (1) an increase in the rate of H_2O_2 consumption by *S. cerevisiae* intact cells is triggered by disruption of the plasma membrane with digitonin, a detergent that binds to the sterols of the membrane [4]; (2) mutant strains with deletion of genes (*ERG3* or *ERG6*) of the ergosterol biosynthetic pathway (the main sterol in yeast and an analogue of cholesterol), are very sensitive to H_2O_2 [4]; (3) in *S. pombe*, the F-box protein Pof14 plays an essential role in the stress response to H_2O_2 by negatively regulating ergosterol synthesis, most likely by directly binding squalene synthase (Erg9) [6]. However, plasma membrane biophysical alterations in *S. cerevisiae* during adaptation to H_2O_2 have not been investigated so far, and this is the primary aim of the present work. We also investigated the importance of the biophysical changes by measuring the time hierarchy of permeability changes, biophysical changes, and antioxidant enzyme induction. The induction of antioxidant enzymes was used as a benchmark measurement because it is an important and well-characterized mechanism of adaptation against oxidative stress [7,8].

2. Materials and methods

2.1. Materials

The *S. cerevisiae* strains used in this work were Y00000 (wild-type, genotype BY4741 *MATa*; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0), Y02667 (*erg3*Δ, isogenic to BY4741 with *YLR056w::kanMX4*) and Y00568 (*erg6*Δ, isogenic to BY4741 with *YML008c::kanMX4*) and were obtained from EUROSCARF (Frankfurt, Germany).

Yeast extract, bactopeptone, yeast nitrogen base, and agar were from Difco (Detroit, MI, USA). Glucose oxidase (*Aspergillus niger*) and digitonin were from Aldrich (Steinheim, Germany). Bovine liver catalase was from Sigma Chemical Company (St Louis, MO, USA). H_2O_2 was obtained from Merck (Whitehouse Station, NJ, USA), 2-(9-anthroyloxy) stearic acid (2-AS) was from Fluka (Buchs, Switzerland). Diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) were obtained from Molecular Probes (Eugene, OR, USA). For fluorescent measurements all solvents were from Merck with spectroscopic grade.

2.2. Media and growth conditions

S. cerevisiae cells were inoculated at an OD_{600} of 0.05. Growth and all incubations (if not otherwise referred) were made in synthetic complete medium containing 6.8% (w/v) yeast nitrogen base, 2% (w/v) glucose and the amino acids and nitrogen base as indicated in [4,9], at 30°C and with shaking at 160rpm. For all measurements cells were harvested in the exponential phase at OD_{600} = 0.3–0.5 ($1OD_{600}$ = 2–3 × 10^7 cells).

2.3. Exposure to $HgCl_2$

To block plasma membrane water channels, wild-type (*wt*) *S. cerevisiae* cells were pre-incubated with 5μM $HgCl_2$ for 5min. After pre-incubation, cells were harvested, and washed twice with sterile water. Then cells were suspended in 0.1M potassium phosphate buffer, pH 6.5, at 30°C and with shaking at 160rpm for the determination of H_2O_2 consumption and H_2O_2 plasma membrane permeability constant.

2.4. Exposure to H_2O_2 and cell survival

S. cerevisiae cells were exposed to steady-state H_2O_2 concentrations using glucose oxidase as described in [10,4,9]. In brief, steady-state levels of H_2O_2 were obtained by adding an initial amount of H_2O_2 together with glucose oxidase that, by forming H_2O_2, compensated for the consumption of H_2O_2 by the cells. The consumption of H_2O_2 in *S. cerevisiae* cells shows first-order decay kinetics with a rate constant of $0.059 min^{-1} OD_{600}^{-1}$ (see [4]). To induce adaptation to H_2O_2 cells were exposed to a steady-state concentration of 150μM during 90min [4]. H_2O_2 was measured as O_2 release with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) following the addition of catalase [10].

Cell survival to a lethal steady-state dose of H_2O_2 (0.7mM) was monitored by plating diluted culture aliquots on YPD plates (1% [w/v] yeast extract, 2% [w/v] bactopeptone, 2% [w/v] glucose and 2% [w/v] agar) and counting colonies after 48h of incubation at 30°C [11]. The survival rates obtained were normalized against the survival rate of the appropriate control samples (not exposed to a lethal dose of H_2O_2) considered to be 100%.

2.5. Cell permeabilization

Cell membrane permeabilization was achieved by incubating cells in 0.1M potassium phosphate buffer, pH 6.5, with 0.01% (w/v) digitonin dissolved either in dimethylsulfoxide (studies using $HgCl_2$) or in water after purification by recrystallization (determination of permeability constant during adaptation to H_2O_2), for 15min at 30°C with shaking at 160rpm. Permeabilization was checked by measuring lactate dehydrogenase activity, according to [12].

2.6. Determination of H_2O_2 consumption, cellular H_2O_2 gradient and permeability constant to H_2O_2

The assays performed to measure H_2O_2 consumption rate in permeabilized and in intact cells, were as follows: (*a*) H_2O_2 consumption (initial concentration, 100μM) by permeabilized cells suspended in the permeabilization buffer at 30°C with shaking at 160rpm was followed using the oxygen electrode. This assay can also be considered a measurement of catalase activity *in situ*, since the overall H_2O_2 consumption rate in permeabilized cells under these experimental conditions is only due to catalase [4]. (*b*) For H_2O_2 consumption rate in intact cells a similar approach to the first assay was used but no digitonin was added to the buffer, and the cells were incubated with 150μM H_2O_2 for 15min before starting the measurements of H_2O_2 consumption, to oxidize internal pools of reducing equivalents. This catalase activity in intact cells, should be called apparent because it is partially limited by the plasma membrane, being thus lower than the real activity [4].

H_2O_2 concentrations were plotted semi-logarithmically against time, and catalase activity was calculated as the slope of the linear fitting (*i.e.* as a first-order rate constant). The H_2O_2 gradient generated by catalase was determined based on the principle of enzyme latency [2]. The gradient was obtained as $1/R$, where R, is the ratio between apparent catalase activity in intact cells and catalase activity in permeabilized cells (see [4,5]). This enzymatic latency is due to an apparent lower activity shown by enzymes entrapped in compartments when compared with enzymes free in solution [13,14]. This lower activity is a consequence of the permeability barrier imposed by the compartment entrapping the enzyme, which limits the diffusion of the substrate to the enzyme. k_{perm}, the first-order rate constant for the permeation of H_2O_2 into the cell was calculated using the equation $k_{perm} = [R/(1 - R) \times k_{catabolism}]$ where $k_{catabolism}$ is the rate constant for the intracellular catabolism of H_2O_2 [4,5].

2.7. Plasma membrane fluidity measurement

Steady-state anisotropy of the fluorescent probes DPH, 2-AS, and TMA-DPH in whole cells was used to study yeast plasma membrane properties. Measurements were made according to [15] with some modifications. Yeast cultures were grown overnight in synthetic complete medium at 30°C and then reinoculated into synthetic complete medium at an OD_{600} of approximately

0.05. After incubation for 5–6h, cells were harvested, washed twice with sterile water and then suspended in buffer 1 (100mM sodium phosphate, 100mM sodium chloride, 1mM EDTA, pH 7.4) to a density of 2mg wet weight ml^{-1}. The cells were incubated at 20°C for 5min and the fluorescent probe, dissolved in dimethyl sulfoxide (DMSO), was added to either a final concentration of 2μM (DPH) or 5μM (2-AS and TMA-DPH). DMSO volume added was kept in a range which does not affect membrane properties. The same DMSO volume used to dissolve the probes was added to the cells as a control. Incubation was continued for 20min at 20°C, after which the cells were immediately washed twice with buffer 1 and then suspended in the same volume of buffer 1.

Steady-state fluorescence anisotropy was measured on a SLM Aminco 8100 spectrofluorometer, equipped with 450W Xe lamp, Glan-Thompson polarizers, and double monochromators. For DPH and TMA-DPH, the excitation and emission wavelengths were 360nm and 450nm, respectively; for 2-AS the excitation and emission wavelengths were 365nm and 455nm, respectively. The measured fluorescence intensities (I) were corrected for background fluorescence and light scattering from the unlabelled sample, *i.e.* the sample treated with the solvent alone. Fluorescence intensities were measured parallel and perpendicular to the direction of the polarized excitation light. The degree of steady-state anisotropy (r) was calculated according to the equation:

$$r = (I_{VV} - G \times I_{VH})/(I_{VV} + 2G \times I_{VH}),$$

in which G is the instrumental correction factor given by the observed ratio, I_{HV}/I_{HH} [16]. "V" and "H" in the subscripts represent the vertical and horizontal directions.

2.8. Statistical analysis

The results presented are the means ± standard deviations of independent experiments. Data statistical analysis was undertaken using either a two-tailed Student t test for comparison between means of two different groups or by using analysis of variance and the Tukey-Kramer multiple comparisons test for comparison of more than two different groups.

3. Results

3.1. Plasma membrane biophysical properties are altered during adaptation to H_2O_2 in wt S. cerevisiae cells

Steady-state fluorescence anisotropy of the fluorescent probes DPH, 2-AS and TMA-DPH incorporated into intact cells was used to study the effect of adaptation to H_2O_2 on plasma membrane biophysical properties. Changes in fluorescence anisotropy in cellular membranes reflect alterations in probe rotational mobility and the degree of membrane order [17]. An inverse relationship exists between membrane fluidity and anisotropy.

Exposure to 150μM H_2O_2 for 90min, a dose that induces both adaptation to H_2O_2 and the associated decrease of the plasma membrane permeability constant to H_2O_2 [4], causes a significant increase in fluorescence anisotropy for both DPH (Fig. 1A) and 2-AS (Fig. 1B) in *S. cerevisiae wt* cells. No changes were observed in fluorescence anisotropy when TMA-DPH was used (results not shown). The failure to see biophysical changes induced by H_2O_2 when using TMA-DPH is in agreement with previous studies with *S. cerevisiae*, where TMA-DPH failed to reveal changes in fluorescence anisotropy in cells of the *pdr16*Δ*pdr17*Δ mutant strain. In spite of that, this strain shows both an increased uptake of rhodamine-6-G and marked changes in plasma membrane lipid composition [18].

Overall, these are the first observations that associate alterations in the plasma membrane biophysical properties with adaptation to H_2O_2.

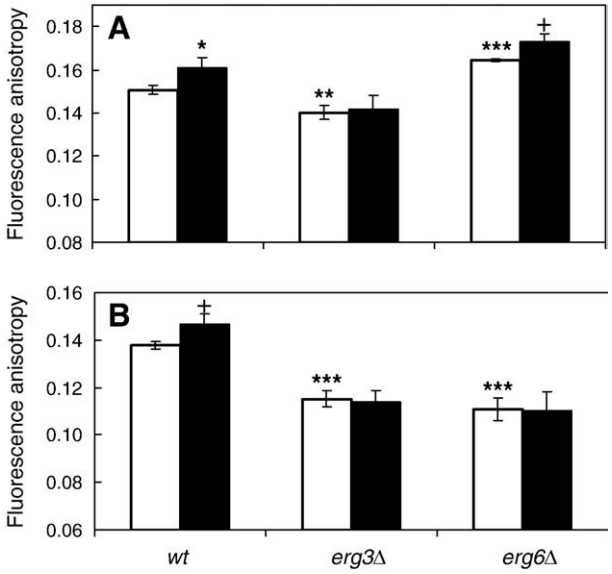

Fig. 1. Adaptation of *S. cerevisiae* cells to H_2O_2 alters plasma membrane biophysical properties and decreases membrane fluidity. For adaptation to H_2O_2, *S. cerevisiae wt*, *erg3*Δ or *erg6*Δ cells in the exponential phase were pre-exposed to a 150 μM H_2O_2 steady-state concentration for 90 min. Fluorescence anisotropy values were calculated as described in Materials and methods in whole cells for both control cells (empty bars) and cells adapted to H_2O_2 (filled bars) using (A) DPH and (B) 2-AS. The values are the mean ± standard deviation of at least five independent experiments. $^{*}P<0.001$ vs control cells; $^{+}P<0.01$ vs control cells; $^{**}P<0.01$ vs *wt* control cells; $^{***}P<0.001$ vs *wt* control cells.

3.2. Plasma membrane biophysical properties are not altered during adaptation to H_2O_2 in erg3Δ and erg6Δ S. cerevisiae cells

Besides *wt* cells, we also studied two strains (*erg3*Δ and *erg6*Δ) that have a defect in the ergosterol biosynthesis pathway: *erg3*Δ mutants lack a C-5 desaturase producing ergosta-7,22-dienol instead of ergosterol [19], and *erg6*Δ mutants lack a C-24 methyltransferase producing zymosterol and colesta-5,7,24-trienol instead of ergosterol [20]. It has been shown that these mutant strains have a lower fluorescence anisotropy [15,21] than *wt* cells, and therefore a higher fluidity which results in an increased permeability to lipophilic compounds [21] including H_2O_2 [4]. These cells are not able to form a catalase-driven H_2O_2 gradient across the plasma membrane [4] and, therefore, the plasma membrane should not play any role in the defense against external H_2O_2. To test this hypothesis, *erg3*Δ and *erg6*Δ were subjected to an adaptation protocol similar to that of *wt* cells. As can be seen in Fig. 2, both *erg3*Δ and *erg6*Δ cells were successfully adapted showing a higher resistance to H_2O_2 when subjected to lethal doses. But contrary to what was observed in *wt* cells, adaptation of *erg3*Δ and *erg6*Δ cells to H_2O_2 was not associated with a decrease of membrane fluidity, since no significant differences were found in fluorescence anisotropy with both DPH and 2-AS for *erg3*Δ, and 2-AS for *erg6*Δ (Fig. 1A and B). The result obtained with DPH for the *erg6*Δ strain, which indicates an increased anisotropy, may be anomalous. In fact, published steady-state fluorescence anisotropies using DPH for *erg3*Δ and *erg6*Δ cells are lower than those observed

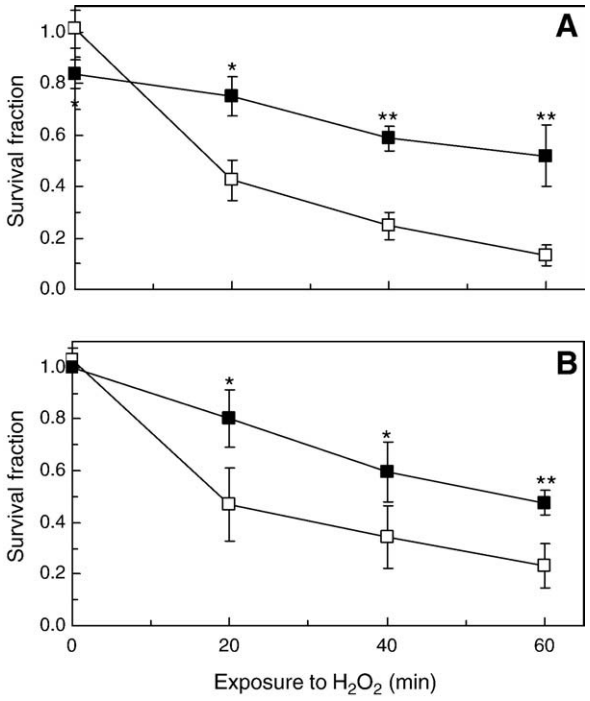

Fig. 2. *Saccharomyces cerevisiae erg3*Δ and *erg6*Δ strains adapt to H_2O_2. The survival fraction was determined for control cells (open symbols), and H_2O_2-adapted cells (closed symbols), after exposure to a steady-state of 0.7 mM H_2O_2 (lethal dose). Adapted cells were pre-exposed a 150 μM H_2O_2 steady-state concentration for 90 min. (A), *erg3*Δ strain; (B), *erg6*Δ strain. The values are the mean±standard deviation of three independent experiments. *$P<0.05$, **$P<0.01$ vs control cells.

in *wt* cells [15,21], a result that we were able to reproduce in *erg3*Δ cells with both probes and in *erg6*Δ cells with 2-AS, but not with DPH (Fig. 1).

Overall, we conclude that most likely *erg3*Δ and *erg6*Δ mutant strains do not undergo biophysical changes in the plasma membrane upon adaptation to H_2O_2.

3.3. Changes in plasma membrane biophysical properties and permeability to H_2O_2 occur early on during adaptation of wt S. cerevisiae cells to H_2O_2

To find out whether the changes in plasma membrane biophysical properties and permeability were an early event during *S. cerevisiae* cellular adaptation to H_2O_2, fluorescence anisotropy and plasma membrane permeability to H_2O_2 were determined in *wt* cells for shorter times of exposure to an adaptive dose of H_2O_2.

In order to calculate the plasma membrane permeability constant to H_2O_2 (k_{perm}), H_2O_2 consumption rates in intact and in permeabilized *wt* cells were determined (Fig. 3A). After exposure to H_2O_2 for 15min the plasma membrane permeability constant to H_2O_2 (k_{perm}) is already about 40% lower in *wt* cells treated with H_2O_2 when compared to control cells (Fig. 3B). This decrease was maintained up to 30min and is approximately the same as that previously found for an exposure of 90min to H_2O_2 [4]. So, it can be concluded that the adaptation-induced changes in the plasma membrane are very fast. Concerning fluorescence anisotropy, the fastest increase was observed with 2-AS after 30min exposure of *wt* cells to H_2O_2 (Fig. 4B; a 15-min exposure – results not shown – did not cause any significant changes). With DPH a 60-min exposure was needed to increase the anisotropy (Fig. 4A). As before, the probe TMA-DPH failed to detect any changes in fluorescence anisotropy for TMA-DPH (results not shown).

Catalase induction is a well-known mechanism by which cells adapt to H_2O_2 [7,8]. Thus, to evaluate whether membrane changes are an early event during adaptation, we also measured the kinetics of catalase induction during adaptation. We have previously shown that the H_2O_2 consumption rate in permeabilized cells ($k_{catabolism}$) reflects catalase activity [4]. Therefore, the results in Fig. 3A indicate that catalase activity increased 2- and 3-fold in *wt* cells treated with H_2O_2 for 15 min and 30 min respectively, which is similar to the changes observed in the plasma membrane permeability. The occurrence of both catalase induction and decrease of the H_2O_2 permeability rate constant in the same time window were reflected in a significant increase of the H_2O_2 gradient across the plasma membrane (Fig. 3B). A steeper gradient indicates that for the same extracellular H_2O_2 concentration there is a lower intracellular H_2O_2 concentration, and thus cells are more resistant against H_2O_2.

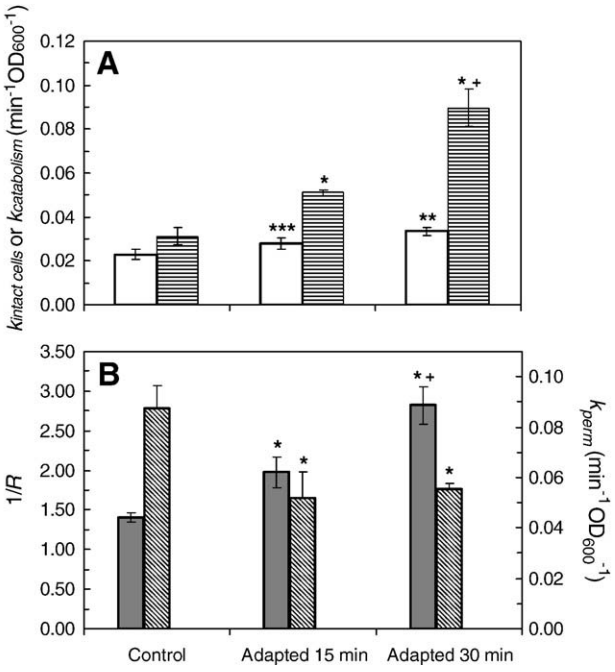

Fig. 3. H_2O_2 induces rapid changes of both its plasma membrane permeability and its gradient. Measurements of H_2O_2 consumption were carried out at 15 min and 30 min in intact and permeabilized *S. cerevisiae wt* cells, as described in Materials and methods. (A). $k_{intact\ cells}$, H_2O_2 consumption rate constant in intact cells (open bars); $k_{catabolism}$, H_2O_2 consumption rate constant in permeabilized cells (striped bars). (B). 1/*R*, H_2O_2 gradient across the plasma membrane (gray bars); k_{perm}, first-order rate constant for the permeation of H_2O_2 into the cell (slashed bars), and is calculated as described in Materials and methods. The values are the mean±standard deviations of 3–5 independent experiments. *$P<0.001$ vs control cells; **$P<0.01$ vs control cells; ***$P<0.05$ vs control cells $^{+}P<0.001$ vs adapted 15 min.

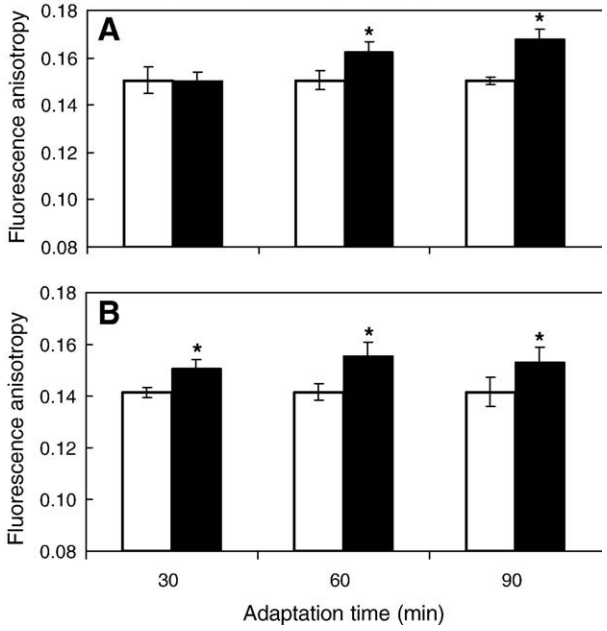

Fig. 4. H_2O_2 induces rapid anisotropy increases during adaptation of *S. cerevisiae wt* cells to H_2O_2. For adaptation to H_2O_2, *S. cerevisiae wt* cells in the exponential phase were pre-exposed to a 150 μM H_2O_2 steady-state concentration for 30–90 min. Steady-state fluorescence anisotropy was measured as in Fig. 1 for both control cells (empty bars) and cells adapted to H_2O_2 (filled bars) using (A) DPH and (B) 2-AS, as described in Materials and methods. The values are the mean±standard deviation of at least five independent experiments. *$P<0.01$ vs control cells.

3.4. Aquaporins are not involved in H_2O_2 transport across the plasma membrane in S. cerevisiae

Two possible causes could explain the absence of observed biophysical changes at 15 min, when the permeability rate constant was already decreased by 40%: either the three probes used are not sensitive enough for the changes observed, or the increase in the permeability is due to protein channels that transport H_2O_2 into the cell. In particular, aquaporins, which transport water, glycerol and other small molecules [22], have been shown to transport H_2O_2 [23]. To investigate the involvement of these aquaporins in H_2O_2 transport across the plasma membrane in *S. cerevisiae* cells, we determined the H_2O_2 permeability constant in the absence and in the presence of mercuric chloride ($HgCl_2$) an aquaporin inhibitor [24]. This pre-treatment with 5 μM $HgCl_2$ did not lead to alterations in the H_2O_2 permeability constant since $k_{perm}=0.244\pm0.034$ min^{-1} OD_{600}^{-1} for control *wt* cells and 0.253 ± 0.041 min^{-1} OD_{600}^{-1} for $HgCl_2$-treated *wt* cells. This indicates that in *S. cerevisiae*, aquaporins are not involved in H_2O_2 transport across the plasma membrane.

4. Discussion

The results obtained in this work highlight the important role of the plasma membrane in the adaptation of *S. cerevisiae* cells to H_2O_2. In fact, the alterations in plasma membrane permeability and biophysical properties occur rapidly, within minutes after exposure to H_2O_2. These alterations are accompanied by an increase in catalase activity which allows cells to create steeper H_2O_2 concentration gradients across the plasma membrane, thus becoming better suited to survive lethal doses of H_2O_2.

The distinct results obtained for the three fluorescent membrane probes used to study membrane fluidity properties is a reflection of its different inherent characteristics. During adaptation to H_2O_2, the fluorescence anisotropy was increased when measured with DPH but not with TMA-DPH. Because DPH locates deeply in the hydrophobic bilayer [25], while TMA-DPH, due to its charged group, is anchored at the membrane/water interface [25,26], this could indicate that the alterations in the membrane biophysical properties are potentially occurring deeper inside the membrane. However, fluorescence anisotropy is also increased with 2-AS, which is located at about the same depth in the membrane as TMA-DPH, indicating that changes in the plasma membrane closer to the aqueous interface also occur. So, the different results in fluorescence anisotropy obtained for TMA-DPH and DPH may be explained by a direct effect of anchoring on motion [25] rather than by their positioning within the membrane. Of the three probes used to measure plasma membrane biophysical changes induced by H_2O_2 in intact *S. cerevisiae* cells, 2-AS seems to be the more adequate. In fact, 2-AS was the probe that gave a better correlation between fluorescence anisotropy increases and the changes in the plasma membrane H_2O_2 permeability constant. These results obtained for 2-AS are consistent with the observation made in liposomes that there is a steeper relationship between anisotropy of surface probes, such as 2-AS, and permeation of H_2O_2, which could indicate that there is a larger role for the interface as the rate limiting step in H_2O_2 diffusion through the membrane [27]. However, for shorter times of treatment with H_2O_2 (15 min), even when using 2-AS as probe for measurements of fluorescence anisotropy, no alterations in the membrane biophysical properties were detected, whereas there was already a decrease in the plasma membrane H_2O_2 permeability constant. At these short times, the membrane alterations may be localized and not detected by the overall bulk measurements of anisotropy applied in this work. This interpretation is compatible with the recent observation that reactive oxygen species are essential for the formation of lipid rafts [28,29], which are membrane microdomains, enriched in sterols, sphingolipids, and specific proteins with putative signaling and regulatory functions [30].

The existence of biophysical changes during H_2O_2 adaptation does not imply that the mechanism by which H_2O_2 permeability occurs is simple diffusion across the lipid bilayer. In fact, biophysical properties can affect the activity of membrane proteins [31] and therefore, H_2O_2 channels could be a possible mechanism. In cells with active aquaporins, the facilitated diffusion of H_2O_2 can also contribute for the permeability of H_2O_2 [9,10], and it has been suggested that H_2O_2 could modulate plant aquaporins via possible redox gating [32]. However, we did not find any evidence of the involvement of aquaporins during adaptation in yeast, which may be explained by the absence of a functional *AQY1* in some laboratory yeast strains [33,34] and the absence of a functional *AQY2* in most strains

[34]. Also, recently it was found that wild-type *S. cerevisiae* cells and the *fps1*Δ,*yfl054c*Δ mutant strain, which is devoid of functional aquaporins, have a similar sensitivity to H_2O_2 [35]. These last results indicate that both *fps1* and *yfl054c* are not involved in H_2O_2 transport and support our conclusion that in yeast H_2O_2 diffusion across the plasma membrane does not involve aquaporins.

Thus, in *S. cerevisiae* cells the change in the membrane permeability and the associate changes in the biophysical properties probably involve changes in lipid composition. Peroxidation of lipids is not a likely mechanism, because peroxidation of membrane lipids increases the permeability of the membrane [36], contrary to what is observed during adaptation to H_2O_2. When we first observed that H_2O_2 changed its own plasma membrane permeability, we proposed that the ergosterol pathway was involved [4], and more recently this was supported by the implication of F-box protein Pof14 in the stress response to H_2O_2, by negatively regulating ergosterol synthesis [6]. The results reported here further support the involvement of this pathway, because the biophysical changes induced in the plasma membrane by H_2O_2 were absent when ergosterol is replaced by other sterols as shown by the results with the two mutants of the ergosterol biosynthesis pathway. The ergosterol pathway probably is not the sole component involved, and we recently provided evidence that the sensitivity of yeast cells to H_2O_2 inversely correlates with the decrease in Fas activity, and that this activity is repressed by H_2O_2 during adaptation [9]. We are thus in the presence of a multifactorial process by which H_2O_2 modulates lipid composition of plasma membrane and thus membrane permeability to H_2O_2 in *S. cerevisiae*. Whether changes in membrane biophysical properties in response to H_2O_2 are a common mechanism for adaptation to H_2O_2 in all organisms remains an open question. In mammalian cell lines such as Jurkat T-cells [2], MCF-7 [37], and HeLa (Oliveira-Marques et al., unpublished data), H_2O_2 gradients across the membrane occur which are disrupted by the addition of the detergent digitonin, further supporting a role for membrane lipids in the defense against H_2O_2. In cells with active aquaporins, changes in the transport capacity of aquaporins may provide an additional mechanism for cellular adaptation against H_2O_2.

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