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Biophysical experiments reveal a protective role of protein phosphatase Z1 against oxidative damage of the cell membrane in *Candida albicans*

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ABSTRACT

Protein phosphatase Z1 (Ppz1) has been shown to take part in important physiological functions in fungi including a contribution to virulence of *Candida albicans*. Although its involvement in the oxidative stress response has also been documented, the exact mechanism of action of its protective effect against oxidative damage remains unknown. By developing a pipeline to analyze the biophysical properties of the cell membrane in fungi, we demonstrate that the plasma membrane of Ppz1-KO *Candida albicans* displays increased sensitivity to tert-butyl-hydroperoxide-induced oxidative damage. In particular, the response to the oxidizing agent, characterized by increased lipid peroxidation, reduced lipid order, and inhibited lateral mobility of plasma membrane components, is significantly more pronounced in the Ppz1-KO *C. albicans* strain than in the wild-type counterpart. Remarkably, membrane constituents became almost completely immobile in the phosphatase deletion mutant exposed to oxidative stress. Furthermore, moderately elevated membrane lipid peroxidation accompanied by the aforementioned changes in the biophysical characteristics of the plasma membrane are already detectable in untreated Ppz1-KO cells indicating latent membrane damage even in the absence of oxidative stress. In conclusion, the hypersensitivity of cells lacking Ppz1 to oxidative damage establishes that potential Ppz1 inhibitors may synergize with oxidizing agents in prospective anti-fungal combination therapies.

1. Introduction

Candida albicans is an innocuous yeast species present in the microbiome of more than half of healthy adults [1]. This opportunistic pathogen can become life-threatening in people with compromised immune system [2]. The most serious health hazard is posed by emerging new strains resistant to multiple medications [3]. The success of any kind of pharmacological intervention hinges upon specificity. Many currently available anti-fungal drugs affect the yeast cell membrane, since its unique sterol component, ergosterol, presents an ideal target [4]. The yeast plasma membrane is highly compartmentalized, a phenomenon that is attributed to the strong separation tendency of ergosterol, the hydroxylation and the long hydrocarbon chains of fatty acids, the lower versatility of lipids and to the partitioning preferences of membrane proteins [5–8]. Consequently, fluid membrane areas are intermixed with several membrane domains, among which membrane compartment of the Can1 arginine permease (MCC) and membrane compartment of the Pma1 H^+-ATPase (MCP) are the most abundant [9,10]. Fungal plasma membranes are also unique in the sense that they contain sphingolipid-enriched gel domains and distinct sterol-enriched domains, possibly corresponding to MCP and MCC, respectively [8]. Conventional ergosterol-directed anti-fungal drugs act not only by undermining the function of the membrane as a permeability barrier, but also by subverting the organizing principle of the aforementioned membrane domain structure required for certain yeast virulence factors [5].

Since resistance against conventional anti-fungal medications

Abbreviations: GP, generalized polarization; KO, Ppz1 deletion mutant strain; PPZ, protein phosphatase Z; Ppz1, *Candida albicans* protein phosphatase Z1; tBOOH, tert-butyl-hydroperoxide; WT, QMY23 control strain.

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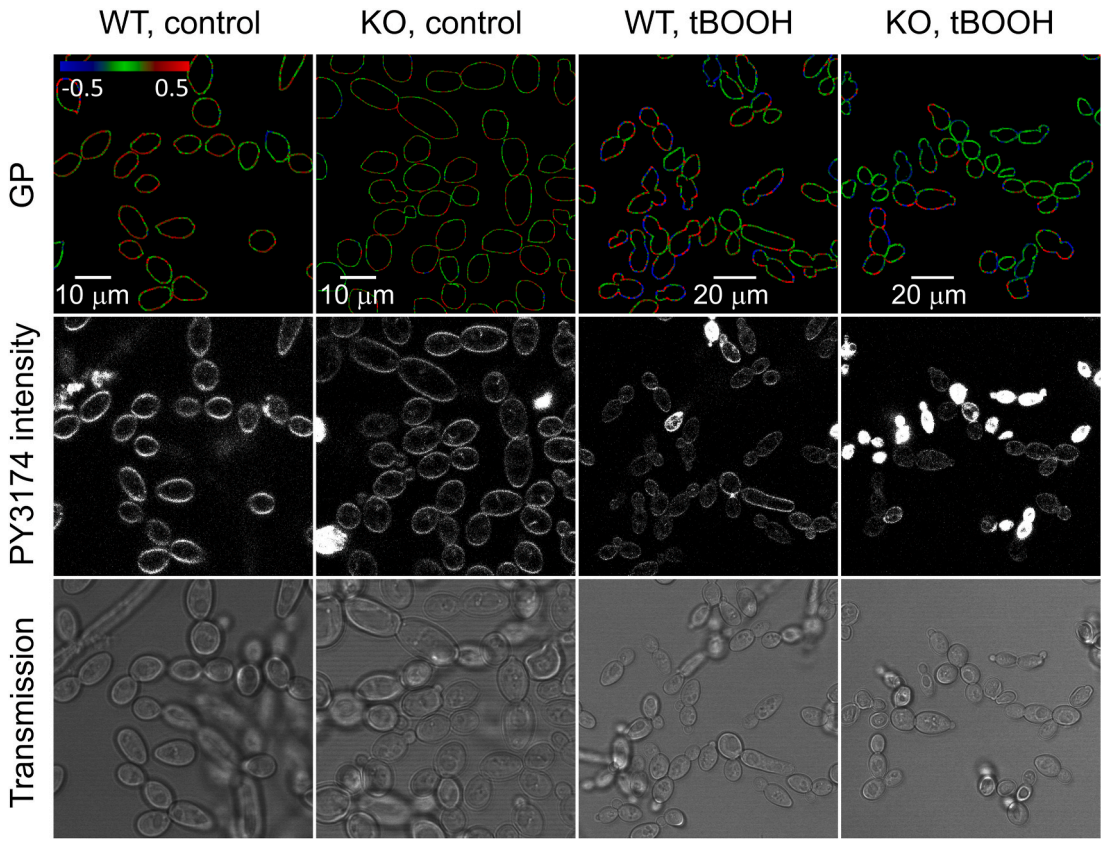

Fig. 1. Representative images of yeast cells labeled by PY3174. WT and KO *C. albicans* cells were treated with tBOOH or left untreated followed by labeling with PY3174 and confocal microscopy to measure the GP of the fluorescent marker. The intensity of PY3174 and the transmission images are shown in the middle and bottom rows, respectively. Images were segmented and the fluorescence intensities were only evaluated in membrane pixels. This feature of the analysis prevented potential unfair bias that could have been the result of the high fluorescence intensity observed inside certain cells that suffered extensive oxidative damage. The GP of the membrane is shown in the top row according to the color scale in the upper left corner. Representative images from five independent experiments are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increases [3,11], there is a pressing need for new drug targets. Protein phosphatases present such a possible target due to their diversity stemming from convergent evolution [12]. Homology searching identified 78 potential protein phosphatases in the *C. albicans* genome [13]. Among these, the serine/threonine-specific PPZ protein phosphatases are fungus-specific enzymes without closely related human orthologues [13–15]. The product of the *C. albicans PPZ1* gene, the Ppz1 protein, is involved in important physiological functions, e.g., maintenance of cell wall integrity, salt tolerance and transmembrane transport [16,17]. Homozygous *PPZ1* deletion mutants exhibited reduced competitive fitness, reduced virulence, and diminished attachment to surfaces [16,18,19]. The role of PPZ in the oxidative stress response of several fungi, including *C. albicans* and *A. fumigatus*, has been reported [20,21]. Upregulation of genes involved in oxidative processes, enhanced activity of several oxidative enzymes, an elevated ratio of oxidized to reduced glutathione and an increase in reactive oxygen species (ROS) were observed in the phosphatase deletion mutant *C. albicans* implying that an oxidative stress response was primed [17,22]. Interestingly, the overexpression of Ppz1 in *S. cerevisiae* also increased the expression of oxidative enzymes and ROS formation [23]. The unique structural features of CaPpz1 [24,25] and the synergistic transcriptional effects of oxidative agents with the Ppz1-null mutation [17,22] suggest that specific combination therapies could be developed. Since Ppz1 affects transmembrane transport [16,17] as well as plasma membrane-related processes [22], and since one of the primary targets of oxidative damage is the cell membrane [26,27], we set out to characterize the effect of the oxidizing agent tert-butyl-hydroperoxide (tBOOH) and the Ppz1 deletion on the biophysical properties of the *Candida* cell membrane. tBOOH was selected as it accelerates lipid peroxidation chain reactions in biological membranes and contributes to the generation of butoxy radicals *via* Fenton-type reactions [28,29]. As an organic peroxide, tBOOH (i) modifies membrane lipid composition [30,31]; (ii) increases ROS concentration [30]; (iii) induces the antioxidative defense system [32–34]; and (iv) stimulates cyanide-sensitive respiration in fungi [33].

2. Materials and methods

2.1. Cells

Two strains of *Candida albicans* were applied in the experiments: the control QMY23 (WT) with the genotype *his1Δ/his1Δ, leu2Δ:C. dubliniensis HIS1/leu2Δ:C. maltose LEU2, URA3/ura3Δ:imm^{434}, IRO1/iro1Δ:imm^{434}* [35] and the protein phosphatase-knockout (KO) with the genotype *ura3Δ-iro1Δ:imm^{434}/URA3-IRO1, his1Δ/his1Δ, leu2Δ/leu2Δ, ppz1Δ:C. dubliniensis HIS1/ppz1Δ:C. maltosa LEU2* [16]. Both strains were cultured as previously described [17].

2.2. Treatment and biophysical investigation of cells

Oxidative stress was induced by a 1-h treatment with 0.4 mM of tBOOH [17]. Membrane hydration and membrane lipid peroxidation were measured with PY3174 [36–39] or with the Image-iT® Lipid peroxidation sensor (C10445, ThermoFisher Scientific, Waltham, MA), respectively. Image analysis strategies and representative image segmentation results are summarized in Suppl. Figs. 1-2. Mobility of the fluorescent marker FAST-DiI (D3899, ThermoFisher Scientific) was

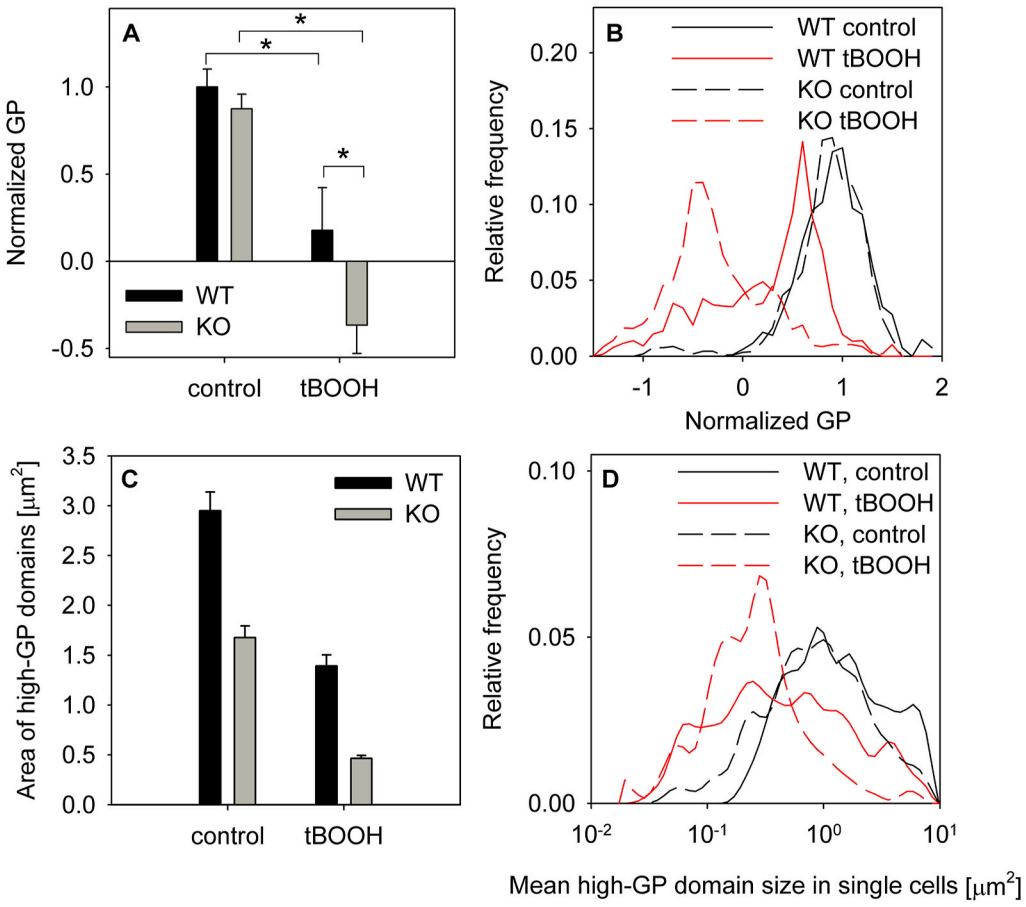

Fig. 2. Measurement of membrane hydration. WT and KO cells were grown in the presence or absence of tBOOH for 1 h that was followed by labeling with PY3174, whose fluorescence was measured in the blue- and red-edge of its emission spectrum. The GP, characterizing the extent of water penetration into the membrane, was calculated for membrane pixels only. (A) The mean GP of each experimental condition was normalized to the untreated, WT cells in each experiment. The mean of 100–200 cells, recorded in five biological replicates, along with the standard deviation, is shown. Asterisks indicate significant difference obtained with Tukey's HSD test after significant F values were calculated in two-way ANOVA ($p < 0.05$). (B) The distribution of GP values of individual cells is shown by the histograms. (C) Pixelwise GP values were calculated for the membrane of control and tBOOH-treated WT and KO cells. A pixel was considered to have high GP if its GP was above zero. The total area of such high-GP domains was determined for every individual cell, and the mean ± standard deviation of high-GP membrane areas is shown. Both the effects of the genotype (WT vs. KO) and of the treatment (control vs. tBOOH) were significant ($p < 0.05$). (D) The area of individual membrane domains containing contiguous pixels with high-GP values was determined, and the distribution of these domain sizes is displayed. Both the effect of the phenotype (KO vs. QM) and of the treatment (control vs. tBOOH) were significant ($p < 0.05$). Image analysis algorithms for pixelwise analysis (panels C and D) are summarized in Suppl. Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

determined by fluorescence recovery after photobleaching (FRAP) [40] (Suppl. Fig. 3). tBOOH had negligible effects on the fluorescence characteristics of FAST-DiI and PY3174 (Suppl. Fig. 4). More detailed description of the methods is available in the Supplementary Information.

3. Results and discussion

3.1. Methodological innovations

Since yeast cells are rarely analyzed by quantitative microscopy, we first attempted to measure membrane fluidity using fluorimetry of intact cells by fluorescence anisotropy measurements. However, we noted a significant contribution of light scattering to the measured signal at any reasonable density of *C. albicans* in the suspensions (data not shown). As an alternative approach, protoplasts were generated to reduce light scattering. However, removal of the cell wall initiates stress signaling in yeast rendering such experiments for characterizing oxidative damage somewhat questionable [41]. Additionally, the protoplastation efficiency of the WT and KO strains was significantly different, and the oxidative treatment reduced the rate of protoplast formation in both samples (Suppl. Fig. 5) casting further doubt on the interpretation of the anisotropy results. Therefore, fluorescently labeled, intact yeast cells were attached to the surface of poly-lysine-coated coverslips for microscopic investigations in which light scattering is not an issue. As opposed to previous attempts, in which the fluorescence signal was evaluated in all pixels in single cells [42], we wanted to limit the analysis to the cell membrane to eliminate the effect of intracellular dye molecules. Even though most cells displayed typical membrane fluorescence, image segmentation was necessary to achieve this aim. Fluorescence intensities reporting membrane compactness or lipid peroxidation were only evaluated in membrane masks determined by a convolutional neural network (Fig. 1, Suppl. Fig. 2). Such analysis permitted cell-by-cell and pixel-by-pixel analysis of membrane biophysical properties that will be described in the following sections.

3.2. Oxidative stress-induced increase in the hydration of the plasma membrane is exacerbated in Ppz1-null Candida cells

Intact *C. albicans* cells were labeled with the environment sensitive dye PY3174, and the generalized polarization (GP) of the indicator was compared in untreated WT and KO cells. Although the mean GP of the KO strain was only non-significantly lower than that of WT cells (Fig. 2A), cell-by-cell analysis revealed that there was a fraction of untreated KO cells with significantly reduced GP (Fig. 1and Fig. 2B). Due to the low number of these cells, their contribution to the mean was minimal leading to the nonsignificant difference between the population averages. tBOOH-induced oxidative stress decreased GP in both WT and KO cells with the reduction in the latter being significantly larger (Fig. 1 and Fig. 2A). Cell-by-cell analysis proved to be invaluable since it

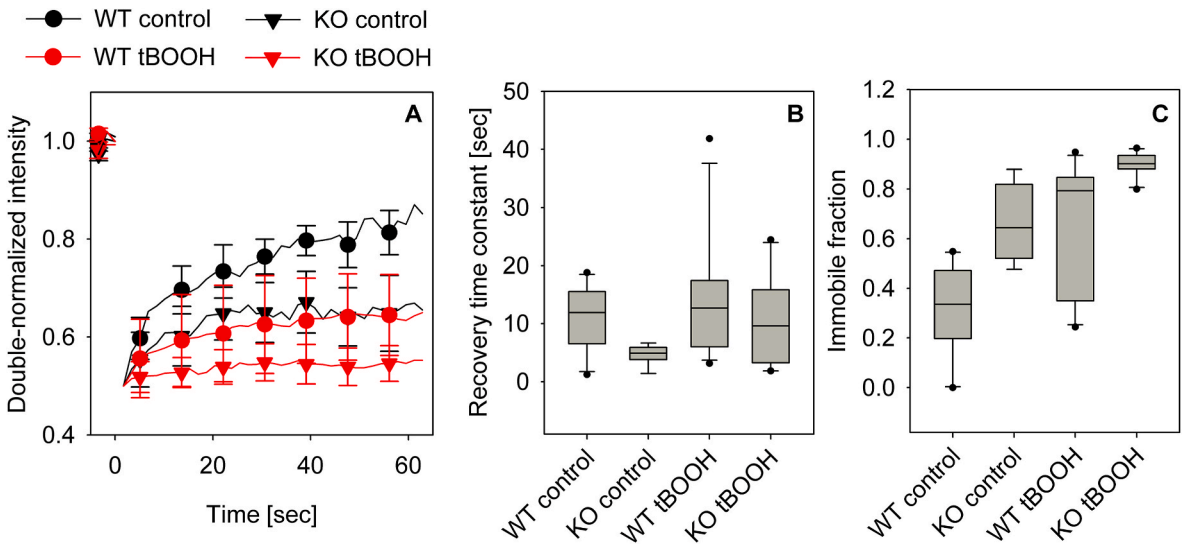

Fig. 3. Results of fluorescence recovery after photobleaching experiments. tBOOH-treated and control WT and KO cells were labeled by the fluorescent membrane marker FAST-DiI. The fluorescence intensity in a membrane spot was measured before photobleaching and during the recovery period. The fluorescence intensity in the membrane spot was double-normalized, and the curves were averaged for five biological replicates. These average curves are shown in part A. The standard deviation is only shown for every 6th data point for clarity. Equation S3 was fitted to individual recovery curves providing the recovery time constant and the immobile fraction as described in the Supplementary Materials and Methods. Box plots showing their distributions are presented in panels B and C. Due to the very high immobile fraction of tBOOH-treated KO cells, the recovery time constants are not reliable in this condition. As far as the recovery time constants are concerned, only untreated KO cells revealed significant difference from all other samples in pairwise comparisons. Regarding the immobile fractions, all pairwise comparisons other that the KO-control vs. WT-tBOOH revealed significant differences. Post-hoc comparisons were carried out after significant F values obtained in ANOVA, and were considered significant if $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

revealed that (i) there were two kinds of cells considering all treatment conditions with one of them having high GP values and the other, oxidatively damaged cells displaying low GP whose range overlapped in all samples in which they were present; and (ii) all treated KO cells exhibited low GP values while only a fraction of WT cells responded to tBOOH in the same manner. Since decreased GP indicates higher penetration of water into the membrane [36,39,42], our data suggest that oxidative damage led to lower membrane compactness and that KO cells, exhibiting latent oxidative damage even without tBOOH-treatment, responded more vigorously to the oxidizing agent.

3.3. *Pixel-by-pixel analysis of GP reveals alterations in the plasma membrane of Ppz1-null Candida cells*

Since cell-by-cell GP values implied an altered plasma membrane structure in KO cells, we undertook a more detailed investigation of the pixelwise distribution of GP as described in Suppl. Fig. 1. The membrane of tBOOH-treated cells exhibited a larger standard deviation of GP implying that the plasma membrane became more heterogeneous (Suppl. Fig. 6). Next, we divided the plasma membrane into areas characterized by high or low GP values. High-GP domains, arbitrarily defined as pixels with GP above zero, correspond to more compact membrane regions. The total area of high-GP domains was reduced by tBOOH treatment in both WT and KO cells (Fig. 1 and Fig. 2C). Remarkably, the high-GP domains practically disappeared in oxidatively stressed KO cells. The area of high-GP domains was lower in untreated KO cells than in the WT control indicating that the plasma membrane of untreated KO cells was already altered. Calculating the distribution of the size of high-GP domains revealed that all individual high-GP domains shrank in tBOOH-treated KO cells, while only a fraction of them did so in treated WT cells (Fig. 2D). This analysis also confirmed that the membrane of untreated KO cells exhibited changes seen in oxidatively damaged membranes in that a small fraction of high-GP domains was reduced in size. Consequently, the biophysical properties of the plasma membrane suggest that KO cells are not only more sensitive to tBOOH, but their membrane already displays signs of preliminary oxidative damage.

3.4. *Lateral diffusion of lipids is significantly reduced by the loss of Ppz1 and by oxidative stress*

Since membrane fluidity strongly correlates with lipid mobility, we measured the lateral diffusion of the membrane marker FAST-DiI partitioning preferentially in disordered membrane domains [43]. Compared to untreated WT cells, both the KO and tBOOH treatment significantly increased the immobile fraction while their combined effect essentially immobilized the marker (Fig. 3). The KO mutation induced a significant decrease in the recovery time constant implying faster diffusion that may be the consequence of a less ordered membrane. The KO phenotype and tBOOH synergize according to the FRAP and GP experiments. However, both the oxidative stress and the absence of Ppz1 decrease lipid order that is usually associated with higher freedom of lateral mobility contrasting with the drastically lower mobile fraction induced by both experimental conditions. This apparent contradiction can be resolved by the following arguments. (i) Oxidizing agents leading to lipid peroxidation introduce hydrophilic groups in the hydrocarbon chain, especially in unsaturated lipids present in copious amounts in the plasma membrane of yeasts [44,45]. Such hydrophilic groups significantly subvert the orderedness of the membrane, potentially leading to “whiskers” of oxidized fatty acids sticking out of the bilayer [46], and “invite” water molecules deep into the hydrophobic core according to molecular dynamics simulations [47]. (ii) Although extensive peroxidation leads to increased membrane fluidity [48], less subversive oxidation applied in this study causes decreased fluidity due to crosslinking of proteins and lipids [26,49–51] resulting in increased density of obstacles that hinder lateral diffusion of lipids [52]. Note that peroxidation-induced crosslinking in the cell wall may also account for the decreased protoplastation efficiency observed in the KO strain and in tBOOH-treated cells (Suppl. Fig. 5). In some publications, moderate lipid peroxidation has been reported to increase membrane fluidity, but the interpretation of these experiments is complicated by the confounding effect of protoplastation and by the confusion of decreased membrane

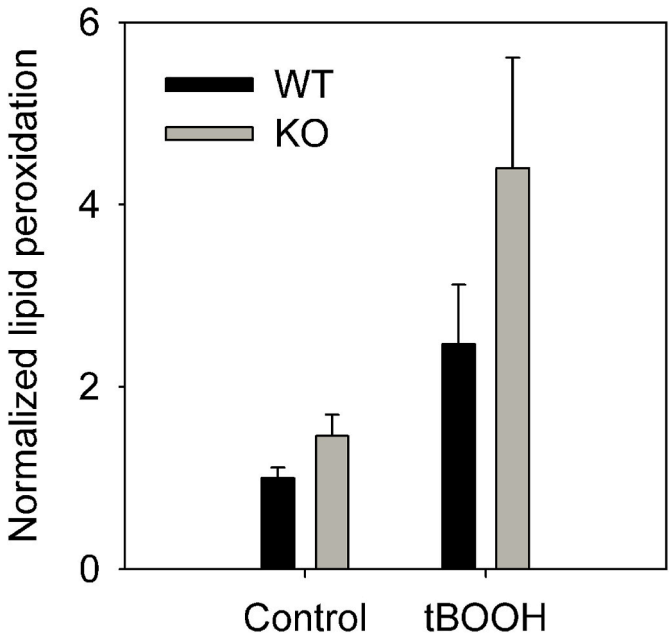

Fig. 4. Measurement of membrane lipid peroxidation. WT and KO cells were left untreated or were cultured in the presence of tBOOH followed by labeling with the Image-iT® Lipid peroxidation sensor. Cells were investigated by confocal microscopy, and the fluorescence emission ratio of the dye, proportional to the extent of membrane lipid peroxidation, was measured in membrane pixels. The mean ± standard deviation calculated from five biological replicates is shown in the figure. Measurements in each replicate were normalized to untreated WT cells. All pairwise comparisons between the samples, evaluated after obtaining significant F values in two-way ANOVA, were significant ($p < 0.05$).

order with increased fluidity [31,53]. In conclusion, latent oxidative damage in the membrane of KO cells restricts lateral diffusion in the membrane and its exacerbation by tBOOH leads to such an extent of immobilization that seems to be incompatible with proper membrane function.

3.5. Membrane lipid peroxidation reveals more pronounced oxidative damage induced by tBOOH in Ppz1-null cells

We directly tested the assumption that both tBOOH and the Ppz1 deletion induce lipid peroxidation and revealed that KO cells exhibited a 50% higher level of membrane lipid peroxidation than the WT (Fig. 4). This finding not only provides an explanation for the changes in the biophysical properties of the membrane, but it is also in accordance with a two-times higher free radical generation in KO cells measured by dichlorofluorescein-diacetate assay [22]. Furthermore, the tBOOH-induced lipid peroxidation was augmented significantly by the absence of Ppz1. Thus, Ppz1 seems to be involved in protecting *Candida* cells from oxidative stress, and the loss of its expression leads to oxidative membrane damage among which the most significant is the almost complete immobilization of membrane components after mild oxidative challenge. Although putative substrates of Ppz1 have been identified [23,54,55], neither their involvement in the oxidative stress response, nor the role of their phosphorylation is known necessitating further research to reveal the molecular target of Ppz1 in this process. However, based on the synergism between the Ppz1-KO mutation and oxidative damage, combination of prospective Ppz1 inhibitors with mild oxidizing agents may efficiently tackle fungal infections caused by drug-resistant *Candida* species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.09.020.

Declaration of competing interest

The authors declare no competing interests.

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