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Biphasic modulation of fatty acid synthase by hydrogen peroxide in *Saccharomyces cerevisiae*

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**A B S T R A C T**

Taking into account published contradictory results concerning the regulation of fatty acid synthase (Fas) by H₂O₂, we carried out a systematic study where two methods of H₂O₂ delivery (steady-state and bolus addition) and the effect of a wide range of H₂O₂ concentrations were investigated. A decrease in Fas activity was observed for cells exposed to 100 and 150 μM H₂O₂ in a steady-state, while a bolus addition of the same H₂O₂ concentrations did not alter Fas activity. Similar results were observed for the mRNA levels of FAS1, the gene that encodes Fas subunit b. However, the exposure to a steady-state 50 μM H₂O₂ dose lead to an increase in FAS1 mRNA levels, showing a biphasic modulation of Fas by H₂O₂. The results obtained emphasize that cellular effects of H₂O₂ can vary over a narrow range of concentrations. Therefore, a tight control of H₂O₂ exposure, which can be achieved by exposing H₂O₂ in a steady-state, is important for cellular studies of H₂O₂-dependent redox regulation.

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**Introduction**

Hydrogen peroxide (H₂O₂), the most abundant reactive oxygen species, is continuously produced both intracellularly and extracellularly [4]. When present in low concentrations it acts as a signalling messenger [23] while at high concentrations it can induce cellular damage to membrane lipids, proteins, and DNA, which can lead to cell death [24]. When cells are exposed to an extracellular source of H₂O₂, several protection mechanisms are induced, including H₂O₂ scavenging enzymes and DNA and protein repair systems [10]. In *Saccharomyces cerevisiae* (S. cerevisiae), adaptation by pre-exposure to non-lethal doses of H₂O₂ increases the activities of both catalase and cytochrome c peroxidase, the two major H₂O₂-consuming enzymes in this organism [11], and also decreases plasma membrane permeability to H₂O₂ [5,6], leading to increased cell resistance to a subsequent exposure to lethal doses of H₂O₂. Adaptation to H₂O₂ also changes the plasma membrane biophysical properties [6] and lipid profile, including phospholipid and fatty acid composition and also leads to a reorganization of the plasma membrane ergosterol-rich microdomains [7].

Previous work by our group showed that fatty acid synthase may have an important role in the plasma membrane permeability alterations observed during adaptation to H₂O₂ [15]. Adaptation of *S. cerevisiae* cells by pre-exposure to steady-state 150 μM H₂O₂ leads to a decrease in both FAS1 gene expression and in Fas activity. Moreover, fas1Δ cells, which have decreased Fas activity due to the deletion of one FAS1 allele, have an increased ability to survive to lethal doses of H₂O₂. However, recently, opposing results were obtained by Kelley et al. [14] who found that in *S. cerevisiae* cells exposed for 1 h to 100 μM H₂O₂ FAST mRNA levels increased while for 150 μM H₂O₂ no changes were found in FAST mRNA levels. One major difference between the two works was the method used to expose cells to H₂O₂. Kelley et al. used a bolus addition of H₂O₂ while in our work a steady-state approach was used [5,8]. This prompted us to revisit Fas regulation by H₂O₂ by carrying out a systematic study where two methods of H₂O₂ delivery and the effect of a wide range of H₂O₂ concentrations were investigated.

In this work, the importance of using a calibrated and controlled H₂O₂ delivery system when studying redox regulation is, once again, reinforced, by showing that the results obtained for Fas regulation by H₂O₂ are dependent on the method used to expose *S. cerevisiae* cells to H₂O₂. Moreover, by using H₂O₂ delivered as a steady-state, we were able to demonstrate that there is a narrow range of H₂O₂ concentrations (50–150 μM) whereas H₂O₂ effect on FAS1 transcription changes from inducing to inhibitory.

**Materials and methods**

**Materials**

The *S. cerevisiae* strain used in this work was BY4743 (wild type, genotype: MATα/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0)

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*Abbreviations used:* Fas, fatty acid synthase; H₂O₂, hydrogen peroxide; *S. cerevisiae*, Saccharomyces cerevisiae; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; OD₆₀₀, optical density at 600 nm; wt, wild-type.

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**MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0** and was obtained from EUROSCARF (Frankfurt, Germany).

Yeast extract, bactopeptone, yeast nitrogen base and agar were from Difco (Detroit, MI, USA). Glucose oxidase (Aspergillus niger), digitonin, amino acids and bases were from Aldrich (Steinheim, Germany). Hydrogen peroxide was obtained from Merck (Whitehouse Station, NJ, USA). Bovine liver catalase was from Sigma Chemical Company (St. Louis, MO, USA).

**Media and growth conditions**

For all experiments cells were grown overnight, in synthetic complete medium (SC) containing 6.8% (w/v) yeast nitrogen base, 2% (w/v) glucose and the amino acids and nitrogen base as indicated in [5], at 30 °C with shaking at 160 rpm. Cells were re-inoculated at OD₆₀₀ of 0.075 OD/mL in fresh medium and grown for 2–3 generations before harvesting. Cells were harvested in mid-exponential phase at OD₆₀₀ of 0.6 OD/mL (1 OD₆₀₀ = 2–3 × 10⁷ cells).

**Cell exposure to H₂O₂ and measurements of cellular H₂O₂ consumption**

Cells in mid-exponential phase were exposed either to bolus additions of H₂O₂ (100 and 150 μM) or to steady-state concentrations of H₂O₂ (25–150 μM) for 60 min as described in [5]. This latter method guarantees that cells are exposed to the desired H₂O₂ concentration, which stays constant along time. Briefly, the desired concentration of H₂O₂ was added to the cell culture together with an adequate amount of glucose oxidase so that the rate of conversion of glucose into H₂O₂ catalysed by glucose oxidase compensates for H₂O₂ consumption by the cells. H₂O₂ concentration in the cell cultures was measured along time by following the O₂ release after the addition of catalase (typically 50 U as defined by the manufacturer) [1] with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK).

**Cell survival studies**

Cell survival to lethal bolus doses of H₂O₂ 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 48 h of incubation at 30 °C [13]. The survival rates obtained for H₂O₂-exposed cells were normalized against the survival rate of the appropriate control samples (not exposed to a lethal dose of H₂O₂ delivered as 750 μM bolus addition) which were considered as having a 100% survival rate.

**Northern blot**

Total RNA was extracted from control cells and H₂O₂-exposed cells by using the Pure-yeast Ambion kit according to the instructions provided by the manufacturer. The synthesis of probes for FAS1 gene product detection was performed by random priming PCR with a digoxigenin-dUTP labelling mixture as described by the manufacturer. Samples of total RNA for northern blot analysis were processed (RNA sample preparation, electrophoresis and blotting to positively-charged nylon membranes) as described in [3]. UV-crosslinked membranes were washed twice in washing buffer (1% (w/v) SDS, 20 mM Na₂HPO₄ pH 7.2, 1 mM EDTA) at 65 °C, pre-hybridized for 1 h at 65 °C in 20% (w/v) SDS, 0.5% blocking reagent (Boehringer), 250 mM Na₂HPO₄ pH 7.2, 1 mM EDTA, and hybridized overnight in the same conditions with a labelled probe at 2 ng/ml. Membranes were washed twice at 65 °C with washing buffer, and immunodetection steps were carried out as described by the manufacturer (Boehringer) using CDP (Tropix) as chemiluminescent substrate. Signals were detected with a Lumi-Imager equipment (Boehringer) and quantified by ImageJ [21].

**Fas activity**

Fas activity was determined at pH 6.5 and 25 °C in crude protein extracts prepared by glass bead lysis [8] as described in [15]. Briefly, potassium phosphate buffer 0.1 M, pH 6.5, 2.5 mM EDTA, 10 mM cysteine, 0.3 mg/ml BSA, 0.24 mM acetyl-coenzyme A, 0.15 mM NADPH and 20 μl of total protein extract, were mixed in a 1 ml cuvette. The decrease of absorbance at 340 nm due to NADPH oxidation (ε = 6220 M⁻¹ cm⁻¹) was measured for 1 min for blank determination, prior to the addition of 0.28 mM of malonyl-coenzyme A to start the enzymatic reaction, which was also followed for 1 min. One unit of Fas activity is the quantity of enzyme that catalyses the oxidation of 1 μmol of NADPH/min at 25 °C and pH 6.5. Protein quantification was done according to Peterson [19].

**Statistical analysis**

Results presented are the mean ± standard deviation of at least three independent experiments. Statistical analysis was undertaken using two-tailed Student’s t-test for comparison between two different groups.

**Results**

**Bolus addition versus steady-state H₂O₂ delivery**

The method of H₂O₂ delivery to cells can have profound effects in the results obtained when studying the effects of H₂O₂ in gene expression. For example, we have shown that in the activation of NF-κB by H₂O₂ in MCF7 and HeLa cells, opposite conclusions are obtained when H₂O₂ is delivered either as a bolus addition or as a continuous steady-state [16]. In the bolus addition approach, an initial dose of H₂O₂ is given, which is rapidly consumed by the cellular antioxidant systems. In the present case, more than 50% of the H₂O₂ added was consumed by S. cerevisiae cells during the first 20 min of incubation, and after 60 min the H₂O₂ concentration was negligible (Fig. 1). The profile of H₂O₂ consumption by cells will depend on the particular conditions of the experiments and, consequently, reproducibility of the observations between different laboratories is problematic [17]. On the contrary, in the steady-state approach the H₂O₂ concentration is kept constant during the whole experiment, by adding a continuous source of H₂O₂. In the present case, the oxidation of the glucose in cell medium catalysed by glucose oxidase (see “Methods”) (Fig. 1). It is also clear that for a given reported H₂O₂ concentration, cells are exposed to a higher H₂O₂ load when H₂O₂ is delivered as a steady-state than when H₂O₂ is delivered as a bolus addition and that this difference becomes more and more noteworthy the longer the time of incubation chosen. In conclusion, the steady-state delivery is much more rigorous and controllable and, taking in account the existence of continuous sources of H₂O₂ in vivo, it also simulates better the cellular environment as compared with the bolus addition approach [17].

**Fatty acid synthase regulation by H₂O₂ is biphasic**

In order to explain the opposing results concerning the regulation of FAS1 expression by H₂O₂ observed by us (decreased FAS1 mRNA levels) and by Kelley et al. [14] (increased FAS1 mRNA levels), FAS1 mRNA levels were measured in control cells and in cells exposed to different steady-state and bolus concentrations of H₂O₂ (Fig. 2).

The most striking feature in Fig. 2 is the biphasic effect exerted by H₂O₂ on FAS1 mRNA levels. S. cerevisiae cells exposed to a steady-state 50 μM H₂O₂ showed a 35% increase in FAS1 gene
expression, while for a 150 \( \mu M \) steady-state there was a 26\% decrease in \( \text{FAS1} \) mRNA levels. We were unable to reproduce the stimulatory effect obtained by Kelley et al. [14] by delivering \( \text{H}_2\text{O}_2 \) as a bolus addition, although a small non-significant increase in \( \text{FAS1} \) mRNA levels was observed for a 150 \( \mu M \) \( \text{H}_2\text{O}_2 \) bolus addition.

In conclusion, the apparent contradictory results are reconciled and were most probably due to the different doses of \( \text{H}_2\text{O}_2 \) that cells were exposed to. Low levels of \( \text{H}_2\text{O}_2 \), such as 50 \( \mu M \) steady-state or the 100 \( \mu M \) bolus addition under the experimental conditions of Kelley et al. [14] lead to an increase in \( \text{FAS1} \) mRNA levels, while higher \( \text{H}_2\text{O}_2 \) concentrations (150 \( \mu M \) steady-state) decrease \( \text{FAS1} \) mRNA levels.

Next, we investigated how the changes in \( \text{FAS1} \) mRNA levels induced by \( \text{H}_2\text{O}_2 \) were reflected in Fas activity (Fig. 3). Fas activity decreased 15\% and 11\% when cells were exposed to steady-state 100 and 150 \( \mu M \) \( \text{H}_2\text{O}_2 \), respectively, which is in agreement with the \( \text{H}_2\text{O}_2 \)-dependent decrease in Fas activity found in our previous work [15]. On the contrary, Fas activity did not change when cells were exposed to lower \( \text{H}_2\text{O}_2 \) concentrations during the experiment (either 25 and 50 \( \mu M \) steady-state \( \text{H}_2\text{O}_2 \) or \( \text{H}_2\text{O}_2 \) bolus additions up to 150 \( \mu M \)). Thus, there is a lack of correlation between mRNA \( \text{FAS1} \) levels and Fas activity, indicating that \( \text{H}_2\text{O}_2 \) is exerting regulatory roles also at the protein level. This complex behaviour is not unexpected when using an oxidative stimulus, and we have studied it in detail in \( S. \text{cerevisiae} \) cells using menadione as the oxidant agent [8]. The precise molecular mechanisms underlying the observed behaviour are outside the scope of this article, but either post-transcriptional modifications, mRNA stability and ribosomal transit, translation inhibition or increased protein turnover can explain the observed results. In fact, stationary-phase \( S. \text{cerevisiae} \) cells exposed to menadione also showed increases in Mn-superoxide dismutase, Cu,Zn-superoxide dismutase, and glutathione reductase mRNA levels which were not paralleled by increased protein levels and enzymatic activities due to regulation not only at the transcription level but also at the post-transcriptional, translational, and post-translational levels [8]. Also, studies on translational responses of \( S. \text{cerevisiae} \) cells to \( \text{H}_2\text{O}_2 \) showed that many of the mRNAs translationally up-regulated in response to \( \text{H}_2\text{O}_2 \) did not show concomitant increases in transcript levels but there was an increase of ribosomes associated to certain mRNAs [22]. This phenomenon allows cells to keep a source of ribosome-associated
mRNAs that can be rapidly translated when needed, showing that the response to oxidative stress requires both translational and transcriptional reprogramming. This accumulation of ribosome-associated mRNAs can also explain the increase of FAS1 gene expression without increase in Fas activity observed for an exposure to steady-state 50 μM H2O2.

Do cells exposed to 150 μM H2O2 adapt?

To further illustrate the importance of the H2O2 delivery method, adaptation triggered by a steady-state exposure or a bolus addition was compared. In Fig. 4 it can be seen that when the pre-exposure to 150 μM H2O2 is made using a bolus addition, adaptation does not occur, as pre-exposed cells present the same survival fraction as control cells. However, with 150 μM steady-state H2O2 a significant increase in survival fraction was measured, as observed previously for the equivalent BY4741 haploid strain [5].

A survey of the literature, shows that a large range of concentrations has been used to induce adaptation to H2O2 in S. cerevisiae. Initial works indicated that a challenge of 400 μM of H2O2 (bolus addition) for 45 min was the most effective treatment to observe adaptation to H2O2 in S. cerevisiae cells [9], while others exposed cells to doses as high as 700 μM of H2O2 for 1 h in order to see the protective effect to a subsequent challenge with a lethal concentration [12]. In more recent studies on Yap1 activation cells were exposed to a bolus addition of 300 μM H2O2 for 1 h in order to see an adaptive response [18]. The point we want to highlight is that stating that a given H2O2 concentration elicits some biological response is not sufficient if reproducibility and comparison between different published works is at stake. The delivery methods should be stated and, if H2O2 is given as a bolus addition a consumption profile should also be shown, because profiles of consumption of bolus additions are strongly dependent on the experimental conditions including, for example, the cell density used in the experiment or, for attached growing-cells, the volume of the growth media [17]. A different profile of H2O2 consumption implies that cells are subjected to different doses for different periods of time, and thus cell response may change accordingly.

Discussion and conclusion

Recently, in a critical review on redox control Brigelius-Flohé and Flohé [6] expressed the opinion that studies in the field of redox regulation by H2O2 have been deeply affected by the lack of quantitative and controlled conditions. Here we show that possible contradictory published results on H2O2 regulation of Fas expression can be explained by the application of different H2O2-delivery methods – bolus versus steady-state delivery – that impact the actual H2O2 levels experienced by cells. We have previously reviewed in detail the basic biology of H2O2 and concluded that the rigorous and reproducible investigation of the effects of H2O2 is made easy by the application of the steady-state delivery [17].

The main result of this study is the observation of a H2O2-induced biphasic effect on the levels of FAS1 mRNA. In a relative narrow range of H2O2 extracellular concentrations the stimulatory effect of H2O2 turns into an inhibitory effect. This result further supports a concept in which cellular effects of H2O2 are complex. It is an oversimplification stating that H2O2 activates or inhibits a cellular process. For example, under well-controlled conditions of H2O2 delivery, we have observed in MCF7 and HeLa cells that a steady-state of 25 μM H2O2 stimulated TNF-α-dependent NF-κB activation when added simultaneously with TNF-α, but if H2O2 is added before TNF-α, NF-κB activity is decreased [16]. Likewise, the stimulatory effect of H2O2 by the simultaneous addition of H2O2 and TNF-α is only observed for levels of TNF-α lower than 2 ng mL⁻¹ for higher TNF-α levels an inhibition is observed [17].

The contrasting effects of H2O2 on FAS1 mRNA levels observed here for a narrow range of H2O2 concentrations is not unexpected. We have observed a similar diverse response for a narrow H2O2 concentration range when titrating Jurkat-T cells with steady-state H2O2 concentrations: intracellular concentrations below 0.7 μM are regulatory, between 0.7 and 3 μM induce apoptosis, while higher than 3 μM induce necrosis [2]. Also, studies in Schizosaccharomyces pombe showed that some signaling mechanisms are highly dose dependent and some individual transcription factors were also found to be activated only within a limited range of H2O2 concentrations [20]. In fact, H2O2, depending on its concentration, can cause different levels of oxidation in sulfhydryl groups of reactive cysteine residues in target proteins, leading to changes with direct consequences to the resulting biological activity [7,25]. The occurrence of Fenton-type chemistry adds another layer of complexity.

In conclusion, when working in vitro, controlling H2O2 dosage along time is crucial, since small differences in H2O2 concentration can lead to variable, even opposing, results, such as we observed here for the expression of FAS1. Contrary to the commonly used bolus addition, the H2O2 steady-state delivery shows to be perfectly suited for a controlled constant delivery of H2O2 to cells simulating in vivo conditions.

Acknowledgments


References


Fig. 4. S. cerevisiae cells adapt when exposed to steady-state 150 μM H2O2 but not to a bolus addition of 300 μM H2O2. Two experiments were performed with cells in wet control cells and in wet cells at a cell density of 0.6 OD/mL exposed to steady-state (ss) or bolus (b) 150 μM of H2O2 for 90 min (n = 3). **p < 0.01 versus control.