Free Radical Biology and Medicine 67 (2014) 103–114

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contributions

# Glutathione is essential to preserve nuclear function and cell survival under oxidative stress

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ARTICLE INFO

*Article history:*
Received 10 July 2013
Received in revised form
8 October 2013
Accepted 12 October 2013
Available online 18 October 2013

*Keywords:*
GSH
Nuclear function
Oxidative stress response
Protein carbonylation
Starvation
$H_2O_2$
Free radicals

ABSTRACT

Glutathione (GSH) is considered the most important redox buffer of the cell. To better characterize its essential function during oxidative stress conditions, we studied the physiological response of $H_2O_2$-treated yeast cells containing various amounts of GSH. We showed that the transcriptional response of GSH-depleted cells is severely impaired, despite an efficient nuclear accumulation of the transcription factor Yap1. Moreover, oxidative stress generates high genome instability in GSH-depleted cells, but does not activate the checkpoint kinase Rad53. Surprisingly, scarce amounts of intracellular GSH are sufficient to preserve cell viability under $H_2O_2$ treatment. In these cells, oxidative stress still causes the accumulation of oxidized proteins and the inactivation of the translational activity, but nuclear components and activities are protected against oxidative injury. We conclude that the essential role of GSH is to preserve nuclear function, allowing cell survival and growth resumption after oxidative stress release. We propose that cytosolic proteins are part of a protective machinery that shields the nucleus by scavenging reactive oxygen species before they can cross the nuclear membrane.



## Introduction

Organisms growing in an aerobic environment must cope with reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($HO^{\bullet}$). In particular, mitochondria generate $O_2^{\bullet -}$ that is dismutated to $H_2O_2$ by the manganese superoxide dismutase [1–3]. In a process known as the Fenton reaction, $H_2O_2$ reacts with $Fe^{2+}$ and produces $HO^{\bullet}$, one of the most reactive oxidants in nature [4]. ROS damage all the cellular macromolecules, especially proteins, because they can introduce modifications in the side chain of amino acids. These modifications can be irreversible, such as the introduction of carbonyl groups into the side chain of particular amino acids (i.e., arginine, lysine, proline, and threonine). The so-called carbonylation process causes protein dysfunction [5] and protein aggregation, leading to their accumulation during oxidative stress [6,7]. Because carbonylated amino acids are now easily detectable, protein carbonylation has emerged as a general biomarker of protein oxidation [8,9]. The consequences of in vivo protein carbonylation have been studied in many different organisms, and it is well established that their accumulation is tightly associated with numerous diseases (e.g., Alzheimer and Parkinson diseases, diabetes, and cancer) and is linked to aging processes [10–12]. Importantly, recent reports have suggested that protein carbonylation could be a cause, rather than a consequence, of cellular death [13]. In particular, it was proposed that γ and UV irradiation would induce cellular mortality of bacteria through the carbonylation of enzymes involved in DNA repair [14].

ROS also play a central role in a range of biological processes, including signaling [15], which requires a tight control of their abundance. Redox homeostasis is achieved by antioxidant systems that scavenge or degrade the ROS produced endogenously at low levels during cell growth [16]. In the yeast *Saccharomyces cerevisiae*, $H_2O_2$ homeostasis involves a large collection of enzymes [4]: two catalases (Ctt1 and Cta1), five peroxiredoxins (Tsa1, Tsa2, Ahp1, Dot5, and Prx1), and three glutathione peroxidases (Gpx1, Gpx2, and Gpx3). However, these constitutive antioxidant machineries can be rapidly overwhelmed by exogenous oxidative conditions, and cells have developed mechanisms of response that lead to the induction of oxidative stress-responsive enzymes. In *S. cerevisiae*, oxidative stress sensing triggers the nuclear accumulation of transcription factors, some being stress specific (e.g., Yap1 [17]), others being activated under a wide variety of stress conditions (e.g., Msn2/4 [18]). The physiological response to

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.10.807

$H_2O_2$ treatment changes the expression of at least 167 proteins [19]: antioxidant enzymes are highly induced, whereas expression of the majority of other proteins is strongly downregulated. This deep reorganization of genomic expression allows a dedicated response to rapid changes in the cellular environment.

In addition to the enzymatic protection against ROS, cells also contain small antioxidant molecules, such as glutathione (GSH). With an intracellular concentration between 1 and 10 mM, GSH is the most abundant nonprotein thiol in the cell and is considered the major redox buffer of the cell [20–22]. This tripeptide is synthesized via a two-step process [23]: the γ-glutamylcysteine synthetase (Gsh1) generates γ-glutamylcysteine (γ-GC) from cysteine and glutamate, and the glutathione synthetase adds a glycine on the γ-GC to form a molecule of GSH [24]. We have shown that, in *S. cerevisiae,* the intracellular concentration of GSH is not exclusively controlled at the synthesis level, because GSH degradation also plays a major role. In particular, during sulfur starvation, degradation of GSH into cysteine, from which are synthesized the sulfur-containing compounds required for cellular growth, is strongly increased [25]. However, a high concentration of GSH is not strictly essential for yeast cell division. For instance, Δ*gsh1* mutant cells, unable to synthesize GSH, can grow in the absence of additional GSH in the culture medium for 8 to 10 generations [26,27]. This demonstrates that a very low concentration of GSH is sufficient for cell viability. It is noteworthy that the essential role of GSH is not to buffer the redox equilibrium of the cell. Indeed, anaerobia does not improve the growth of Δ*gsh1* mutant cells depleted of GSH [27]. Sipos et al. [28] provided the first evidence that GSH is involved in the maturation of cytoplasmic Fe/S-containing proteins. More recently, it has been shown that GSH is required for the maintenance of mitochondrial DNA and that GSH depletion in Δ*gsh1* cells triggers an iron starvation-like response [29]. This observation was confirmed by Kumar et al. [30], who suggested that a high concentration of GSH serves as a backup to the thioredoxin pathway, whereas scarce amounts of GSH are required to ensure its vital function in iron metabolism.

GSH-depleted Δ*gsh1* cells are hypersensitive to $H_2O_2$ treatment [20,31], but the consequence of GSH depletion during oxidative stress has never been clarified. To address this question, we used *S. cerevisiae* to characterize the physiological response to $H_2O_2$ treatment both in wild-type (WT) cells grown under sulfur starvation and in Δ*gsh1* cells grown under GSH depletion. We observed that a very low concentration of GSH led to the accumulation of carbonylated proteins and cell lethality during oxidative stress. We identified a minimal GSH concentration that preserved cells from $H_2O_2$-induced death without protecting proteins against oxidative damage. In these cells, the translational activity was strongly repressed by $H_2O_2$ treatment, suggesting that the induction of stress-responsive enzymes was not strictly required to maintain cell viability. In contrast, further analyses revealed that this minimal concentration of GSH was sufficient to protect nuclear DNA and nuclear function against oxidative damage. This protection appeared to be an essential parameter of cell survival during $H_2O_2$ treatment.

## Materials and methods

### *Strains and growth conditions*

Yeast strains used in this study derived from the BY4742 background (Euroscarf) (Supplementary Table SI). Primers used to construct the YAP1-GFP chimeric chromosomic gene were designed according to Longtine et al. [32] (Supplementary Table SII). BY4742 (WT) cells were grown at 30 °C to $A_{600\ nm}=0.4$ (midexponential phase, $1.7\times10^7$ cells/ml) in an S100 minimal medium containing 100 μM $(NH_4)_2SO_4$ [25] and supplemented with the appropriate amino acids. To induce sulfur starvation, yeast cells were grown in S100 medium, centrifuged (4000 rpm, 5 min, 30 °C), and the pellet was suspended for an extra hour in S0 medium, which is S100 medium without $(NH_4)_2SO_4$ [25]. Δ*gsh1* cells were grown in S100 medium, supplemented with the appropriate amino acids + 100 μM GSH (Sigma–Aldrich) to $A_{600\ nm}=0.4$ (midexponential phase, $1.7\times10^7$ cells/ml). For GSH depletion, Δ*gsh1* cells were grown in S100 + 100 μM GSH medium to $A_{600\ nm}=0.4$. After centrifugation (4000 rpm, 5 min, 30 °C), the pellet was diluted in S100 medium and the growth was resumed for three to eight generations.

For all experiments, oxidative stress was induced by adding 400 μM $H_2O_2$ (Sigma–Aldrich) to the culture medium.

For survival assays, cells were grown under normal or deprived conditions before addition of 400 μM $H_2O_2$. At $t=0$, 20, and 60 min, cells were harvested and diluted samples were plated onto S100 + 100 μM GSH + 0.8% agarose solid medium. Viability was assessed by counting the colony-forming units after 3 days at 30 °C.

### *Fluorescence microscopy experiments*

Yeast cells ($8.5\times10^7$), grown under the various conditions, were harvested by centrifugation and fixed for 5 min at room temperature in 5 ml of 4% formaldehyde in phosphate-buffered saline (PBS). Cells were washed with PBS and nuclei were stained by adding 4,6-diamidino-2-phenylindole (DAPI) to a final concentration of 50 μg/ml. Microscopy observations were done using a DMIRE2 Leica microscope (63 × /1.4 oil immersion objective).

### *Iron and GSH quantification, $H_2O_2$ consumption rate*

Iron quantification were performed on $42.5\times10^7$ yeast cells as previously described [33].

For estimation of the labile iron pool, calcein was used as described [34]. Cell fluorescence was assessed using a DMIRE2 Leica microscope (63 × /1.4 oil immersion objective) and the fluorescence level was measured by image quantification using the ImageJ public software.

For total GSH quantification, $8.5\times10^7$ yeast cells grown under the various conditions were harvested, washed with ice-cold water, suspended in 0.1% perchloric acid, and boiled for 5 min at 95 °C. After centrifugation (13,000 rpm, 1 min, room temperature), 7 μl of the supernatant was diluted three times in 0.1 M potassium phosphate buffer, pH 7.5, 5 mM EDTA. GSH was then quantified according to Rahman et al. [35]. GSH concentration was calculated by assuming that the volume of an individual cell is $4.5\times10^{-14}$ L [30].

Hydrogen peroxide degradation rate was assessed as described [36]. The first-order rate constants were deduced from values of $H_2O_2$ remaining in the culture medium fitted with exponential curves. Results are given in $min^{-1}$ for $1.7\times10^7$ cells/ml.

### *RNA extraction and RT-PCR*

Total RNA extraction and RT-PCR were performed as described [37] using iQ5 real-time PCR detection systems and MESA Green qPCR Master Mix Plus for SYBR Assay (Eurogentec). Gene expression was calculated as described [38]. See Supplementary Table SII for primer sequences.

### *Protein analyses*

For carbonylated protein detection, $25.5\times10^7$ yeast cells were harvested from the various growth conditions. They were washed twice in water and suspended in 200 μl lysis buffer (50 mM Hepes, 100 mM KCl, 10% glycerol, 1.25 mM phenylmethanesulfonyl