Methionine-R-sulfoxide Reductase Plays a Defensive Role against t-Butyl hydroperoxide-induced Oxidative Stress in Schizosaccharomyces pombe

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Methionine-R-sulfoxide (Met-R-SO), oxidatively generated from methionine by reactive oxygen species (ROS), is reduced by methionine-R-sulfoxide reductase (MsrB). The Schizosaccharomyces pombe msrB+ gene encoding MsrB was previously cloned into the shuttle vector pRS316 to produce the recombinant plasmid pFMetSO. In the present work, we examined the cellular role of S. pombe MsrB under tert-butyl hydroperoxide (t-BOOH)-induced oxidative stress. When shifted to the fresh rich medium containing t-BOOH, the S. pombe cells harboring pFMetSO displayed better growth than the vector control cells. They contained lower ROS levels in the presence of t-BOOH than the vector control cells. Even in the presence of the antioxidant tert-butylhydroquinone (t-BHQ), they contained further lower ROS level than the vector control cells. However, the nitric oxide (NO) levels of the S. pombe cells harboring pFMetSO and the vector control cells remained unchanged in the absence or presence of t-BOOH and t-BHQ. The total glutathione (GSH) levels of the S. pombe cells harboring pFMetSO appeared to be higher than the vector control cells both in the absence and the presence of t-BOOH. In brief, the S. pombe MsrB plays a defensive role against t-BOOH-induced oxidative stress through the down-regulation of ROS.

Key words: t-Butyl hydroperoxide; Methionine-R-sulfoxide reductase; MsrB; Oxidative stress; Schizosaccharomyces pombe.

Reactive oxygen species (ROS), such as superoxide radical, hydroxy radical and hydrogen peroxide (HP), are produced as byproducts of normal oxidative metabolism primarily in mitochondria, during metabolism of xenobiotics, and by radiation (Riley, 1994). Excess ROS, leading to oxidative stress, directly or indirectly oxidize critical cellular components, such as DNA, proteins, lipids and membranes. These are ultimately implicated in aging and apoptosis and in numerous abnormal cellular states, for instance cancer (Finkel and Holbrook, 2000). On the contrary, ROS at physiological cellular concentrations play a crucial role in the vital signaling pathways for diverse cellular phenomena (Veal et al., 2007). To maintain normal intracellular redox homeostasis via coping with the production of excess ROS, the living cells are equipped with a variety of defense systems, including enzymatic antioxidants catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, thioredoxin, thioredoxin reductase, and nonenzymatic antioxidants vitamin E, vitamin C and glutathione (GSH) (Fekete et al., 2007).

In addition to HP, t-BOOH, which is analogous to naturally generated short chain lipid hydroperoxides, is one of the well-known organic peroxides used to experimentally induce oxidative...
stress in a variety of living cells. Organic hydroperoxides, including \( t\)-BOOH, and HP induce oxidative damage via different mechanisms (Van der Zee et al., 1996). The oxidative species generated from \( t\)-BOOH exposure is alkoxy or peroxy radical but not hydroxyl radical, indicating that the oxidative damages by \( t\)-BOOH is different from those from HP. Although the overall transcriptional responses to the two pro-oxidants HP and \( t\)-BOOH are similar in \( S\). \textit{pombe}, transcription factors Sty1 and Afl1 are less critical for the response to \( t\)-BOOH. Instead, Pmk1, an MAPK, is involved in surviving \( t\)-BOOH (Chen et al., 2008). HP and \( t\)-BOOH can also provide different regulatory responses (Chen et al., 2008). These findings reveal a considerable plasticity and differential control of regulatory pathways in distinct oxidative stress conditions, providing both specificity and backup for protection from oxidative damage. \( t\)-BOOH, an oxidative stress-inducing agent with long-lasting effects (Emri et al., 1999), accelerates lipid peroxidation chain reactions and subsequent oxidative injuries in biological membranes (Greenley and Davies, 1992).

Methionine sulfoxide reductases (Msrs) are oxidoreductases which catalyze thiol-dependent reduction of methionine sulfoxides in the free and protein-bound states back to corresponding methionines. Methionine-S-sulfoxide (Met-S-SO) and methionine-R-sulfoxide (Met-R-SO) are reduced by methionine-S-sulfoxide reductase (MsrA) and methionine-R-sulfoxide reductase (MsrB), respectively. Both MsrA and MsrB play crucial physiological roles in the diverse kinds of prokaryotic and eukaryotic organisms as antioxidant and protein repair enzymes and are an important part of major physiological and pathological processes, including aging, cancer, rheumatoid arthritis, schizophrenia and neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease (De Luca et al., 2010; Kim and Gladyshev, 2007; Walss-Bass et al., 2009). MsrBs were identified in a variety of organisms, such as bacteria (Etienne et al., 2003), budding yeast (Kryukov et al., 2002), and mammals (Moskovitz and Stadtman, 2003). The fission yeast \textit{Schizosaccharomyces pombe} is one of the popular model organisms used to elucidate oxidative stress response mechanisms, most of which exhibit a remarkable resemblance in multicellular eukaryotic organisms (Ikner and Shiozaki, 2005). The \( S\). \textit{pombe} m\( srb\) gene was previously cloned into the shuttle vector pRS316 to generate the recombinant plasmid pFMetSO (Jo et al., 2013). As shown in Fig. 1, the \( S\). \textit{pombe} MsrB has significant homologies with the counterparts from three fungi \textit{Aspergillus terreus} (60% identities, 74% positives), \textit{Magnaporthe grisea} (62% identities, 75% positives) and \textit{Aspergillus clavatus} (61% identities, 78% positives). In the presence of L-methionine-\((R,S)\)-sulfoxide, the \( S\). \textit{pombe} cells harboring pFMetSO could grow normally but the growth of the vector control cells was almost arrested (Jo et al., 2013). The \( S\). \textit{pombe} MsrB was basically identified to play a protective role against stresses possibly through diminishing the intracellular ROS levels. In this communication, we examined the defensive role of the \( S\). \textit{pombe} MsrB against \( t\)-BOOH-induced chronic oxidative stress.

**MATERIALS AND METHODS**

**Chemicals**

tert-Butyl hydroperoxide (\( t\)-BOOH), tert-butylhydroquinone (\( t\)-BHQ), bovine serum albumin (BSA), Bradford reagent, NADPH, 5,5’-dithio-(2-nitrobenzoic acid) (DTNB), Griess reagent, 2’,7’-dichlorodihydrofluorescein-diacetate (DCFH-DA), sodium nitrite, D-glucose, glutathione reductase (GR) and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Yeast extract, peptone and agar were obtained from Amersham Life Science (Cleveland, Ohio, USA). All other chemicals used were of highest grade commercially available.

**Strains and growth conditions**

\( S\). \textit{pombe} KP1 (\( h^+\) \( \text{leu}1\)-32 \( \text{ura}4\)-294), a derivative of \( S\). \textit{pombe} heterothallic haploid strain 975\( h^+\), was used in the present work. The \( S\). \textit{pombe} cells harboring pFMetSO or pRS316 (Myers et al., 1986) were previously constructed (Jo et al., 2013). The yeast cells were grown in yeast extract peptone dextrose (YPD) medium (pH 6.5) which typically contains 1% yeast extract, 2% peptone, and 1% glucose. The yeast cells were grown with shaking at 30°C, and the yeast growth was monitored by measuring the absorbance at the wavelength of 600 nm. The yeast cells used for the treatments were chiefly obtained from the early exponential growth phase.
Preparation of the cellular extracts

The appropriate number of the yeast cells was obtained by centrifugation. They were resuspended in 20 mM Tris buffer (pH 8.0)–2 mM EDTA and disrupted using a glass bead beater. The supernatants obtained after centrifugation were used as the crude extracts for total GSH and protein determinations as detailed below.

Determination of intracellular ROS

For analysis of intracellular ROS, the redox-sensitive fluorescent probe DCFH-DA was used as previously described (Royall and Ischiropoulos, 1993). DCFH-DA enters into the cells, and the diacetate group is removed by cellular esterase to generate non-fluorescent DCFH which is oxidized to fluorescent dichlorofluorescein (DCF) in the presence of ROS such as hydrogen peroxide (Kiani-Esfahani et al., 2012). Cells were incubated with 5 µmol/l DCFH-DA for 30 min at 30°C. The treated cells were immediately analyzed by flow cytometry.

Determination of nitrite in culture supernatants

Accumulated nitrite (NO$_2^-$), as an index of nitric oxide (NO), in the culture supernatants was determined using a colorimetric assay based on the Griess reaction (Sherman et al., 1993). The supernatant fractions (100 µl) were reacted with 100 µl Griess reagent (6 mg/ml) at room temperature for 10 min, and then NO$_2^-$ concentration was determined by measuring the absorbance at the wavelength of 540 nm. The standard curve was constructed using the known concentrations (0 - 160 µmol/l) of sodium nitrite.

Determination of total glutathione (GSH)

As previously described (Nakagawa et al., 1990), the total GSH content in cellular extracts was determined using an enzymatic recycling assay based on GR. The reaction mixture (200 µl) contained 175 mmol/l KH$_2$PO$_4$, 6.3 mmol/l EDTA, 0.21 mmol/l NADPH, 0.6 mmol/l DTNB, 0.5 units/ml GR, and cellular extract at 25°C. The absorbance at 412 nm was monitored using a microplate reader. The total GSH content was represented as µg/mg protein. Protein content in cellular extracts was determined according to the procedure of Bradford (1976) using BSA as a standard.

Statistical analysis

The results were expressed as mean ± SD. Statistical comparisons between experimental groups were performed using Kruskal-Wallis test, followed by Dunn’s post hoc test for pairwise individual comparison. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Cellular growth under t-BOOH

In order to test the yeast growth in the presence of t-BOOH, the medium-shift experiments were performed. The S. pombe cells harboring pFMetSO and the vector control cells, exponentially grown in the rich medium, were shifted into the fresh rich medium containing no t-BOOH, 0.02 mM and 0.05 mM t-BOOH, and the yeast growth was monitored by measuring absorbance at the wavelength of 600 nm. As previously shown (Jo et al., 2003), the S. pombe cells harboring pYFMetSO exhibited better growth under no t-BOOH than the vector control cells. As shown in Fig. 2, t-BOOH induced a delay in the growth of both the S. pombe cells harboring pFMetSO and the vector control cells. However, the growth of the vector control cells was almost arrested under 0.02 mM and 0.05 mM t-BOOH, whereas the S. pombe cells harboring pFMetSO could grow, although delayed, under the same growth conditions (Fig. 2 A and B). Taken together, the S. pombe MsrB is involved in the cellular growth under t-BOOH-induced oxidative stress.

Reactive oxygen species (ROS) under t-BOOH and t-BHQ

ROS at physiological concentrations are implicated in normal cellular function such as intracellular signaling and redox regulation (Nordberg and Arnér, 2001). On the other hand, excessive ROS, produced during abnormal metabolic reactions, cause a variety of damages to macromolecules. This results in genetic mutation and physiological dysfunction and eventually cell death. The intracellular ROS level is markedly enhanced in presence of oxidative stress-inducing agents, which sequentially threatens the integrity of various biomolecules. Cells have powerful defense mechanisms, which includes antioxidant enzymes and free radical scavengers, to protect against oxidative damages. However, when the cellular defense systems do not sufficiently cope with stressful agents, the cells undergo oxidative stress. Since the intracellular ROS level increases under a variety of stresses, especially oxidative...
stress, it is considered to be one of cellular markers which are closely linked with the stress level inside cells.

Since the *S. pombe* MsrB was verified to be involved in the yeast growth in the presence of t-BOOH (Fig. 2), the effect of MsrB on the intracellular ROS level under t-BOOH was examined after shifting the same numbers of the exponentially grown cells to fresh liquid medium with or without stress-inducing agents (Fig. 3A). The *S. pombe* cells harboring pFMetSO and the vector control cells were subjected to 0.02 and 0.05 mM t-BOOH for 6 h. As previously identified (Jo et al., 2013), the *S. pombe* cells harboring pFMetSO contained a lower ROS level than the vector control cells in the absence of t-BOOH (Fig. 3A). This might be explained by the capability of the *S. pombe* MsrB to diminish the ROS generation or to scavenge ROS generated even in the absence of exogenously added stress-inducing agents. In the

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**Fig. 1.** Sequence alignment of the *S. pombe* MsrB and other similar sequences. The putative amino acid sequence encoded from the *S. pombe* msrB+ gene is aligned with the counterparts from *Aspergillus terreus* (peptide MsrB), *Magnaporthe grisea* (hypothetical protein) and *Aspergillus clavatus* (MsrB). The asterisks, colons and periods indicate identical residues, conserved substitutions and semi-substitutions, respectively. Gaps, shown as short bars, were introduced for optimal alignment.

**Fig. 2.** Growth of the *S. pombe* cells harboring pRS316 or pFMetSO in the presence of t-butyl hydroperoxide (t-BOOH). In the medium-shift experiments, the yeast cells exponentially grown in rich medium were shifted to the fresh medium containing 0.02 mM t-BOOH (A) and 0.05 mM t-BOOH (B). The yeast growth was monitored at the wavelength of 600 nm at 3 and 6 h after the shifts. Representatives of the three separate experiments are shown.
presence of \( t\)-BOOH, the vector control cells were found to contain significantly higher ROS levels, when compared with the untreated cells (Fig. 3A). However, the \textit{S. pombe} cells harboring \textit{pFMetSO} exhibited much lower ROS levels in the presence of \( t\)-BOOH than the vector control cells (Fig. 3A). This finding might be explained assuming that MsrB plays a role in diminishing the ROS level enhanced by exogenously added \( t\)-BOOH. Collectively, the \textit{S. pombe} MsrB plays a suppressive role on the intracellular ROS level under \( t\)-BOOH-induced oxidative stress.

\( t\)-BHQ, a lipophilic sterically hindered hydroquinone commonly known as an antioxidant in food, has also been used as an antioxidant component for experimental purposes. \( t\)-BHQ, a phase II enzyme inducer, is known to induce drug metabolizing enzymes such as glutathione-S-transferase A1/2 and NAD(P)H:quinone oxidoreductase (Pinkus et al., 1995; Pinkus et al., 1996). Thioredoxin reductase and glutathione synthesis are also up-regulated by \( t\)-BHQ in astrocytes, the star-shaped glial cells (Eftekharpour et al., 2000). \( t\)-BHQ at 0.1 and 0.3 mM decreased...

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**Fig. 3.** Changes in reactive oxygen species (ROS, A) and nitric oxide (NO, B) levels of the \textit{S. pombe} cells harboring \textit{pRS316} or \textit{pFMetSO} under \( t\)-BOOH (0.02 mM, 0.05 mM) for 6 h. In A, the intracellular ROS levels were detected by cytometry, and represented as DCF fluorescence, a relative arbitrary unit. In B, the levels of nitrite, as an index of NO, in the supernatant fractions were determined based upon Griess reaction. *, \( P<0.05\); **, \( P<0.01\) versus the corresponding \textit{pRS316}-containing cells.

**Fig. 4.** Changes in reactive oxygen species (ROS, A) and nitric oxide (NO, B) levels of the \textit{S. pombe} cells harboring \textit{pRS316} or \textit{pFMetSO} under \( t\)-butylhydroquinone (\( t\)-BHQ; 0.1 mM, 0.3 mM) for 6 h. In A, the intracellular ROS levels were detected by cytometry, and represented as DCF fluorescence, a relative arbitrary unit. In B, the levels of nitrite, as an index of NO, in the supernatant fractions were determined based upon Griess reaction. *, \( P<0.05\) versus the corresponding \textit{pRS316}-containing cells.
Fig. 5. Changes in the total glutathione (GSH) levels of the *S. pombe* cells harboring pRS316 or pFMetSO under t-BOOH. The *S. pombe* cells, exponentially grown in the normal rich medium, were shifted to the fresh rich medium containing 0.02 mM and 0.05 mM t-BOOH for 6 h. The total GSH level was represented as μg/mg protein. **, *P* < 0.01 versus the corresponding pRS316-containing cells.

Fig. 6. Comparison of the putative three-dimensional structure of the *S. pombe* MsrB (A) with the counterparts from *A. terreus* (B), *M. grisea* (C) and *A. clavatus* (D).

Nitric oxide (NO) under t-BOOH and t-BHQ

Nitric oxide (NO•, NO) has physiological effects when it is produced in minute quantities by constitutive nitric oxide synthases (NOSs), while it has pathologic effects when it is produced in higher quantities by inducible NOSs. Its direct effects occur from directly reacting with its biological target, while its indirect effects are mediated by reactive nitrogen species (RNS) which undergo further reactions with their respective biological targets such as protein, lipid and DNA.
RNS play important roles in cellular signaling, but when produced at high concentrations they can subject cells to nitrosative stress, which may ultimately lead to cell death. The effect of MsrB on the NO levels of the yeast cells under t-BOOH and t-BHQ was tested. When the S. pombe cells harboring pFMetSO and the vector control cells were subjected to 0.02 and 0.05 mM t-BOOH for 6 h, both of them displayed similar NO levels which appeared to be irrespective of t-BOOH (Fig. 3B). Similarly, the NO levels of the S. pombe cells harboring pFMetSO and the vector control cells were also found to remain unchanged when treated with 0.1 and 0.3 mM t-BHQ (Fig. 4B). These findings imply that t-BOOH and t-BHQ are not capable of modulating the NO level and that MsrB plays a cellular role without the involvement of NO mediation.

**Total glutathione (GSH) under t-BOOH**

Living cells have powerful defense mechanisms, such as antioxidant enzymes and free radical scavengers, to protect against oxidative/nitrosative damages. GSH, one of principal non-enzymatic antioxidants, plays a major role in the protection against many kinds of oxidative stresses. Its main protective function against oxidative stresses is based on its role as a cofactor of several detoxifying enzymes such as glutathione peroxidase and glutathione transferase and its role in regenerating the crucial antioxidants vitamin C and E back to their active forms (Valko et al., 2006). It was examined whether the S. pombe MsrB is involved in the total GSH level in the absence and the presence of t-BOOH or not. Consistent with the previous finding (Jo et al., 2013), the total GSH level in the S. pombe cells harboring pFMetSO was slightly higher than that in the vector control cells in the absence of t-BOOH (Fig. 5). The total GSH level in the vector control cells decreased in proportion to t-BOOH concentration (Fig. 5). Even in the presence of varying concentrations of t-BOOH, the total GSH level remained higher in the S. pombe cells harboring pFMetSO than in the vector control cells (Fig. 5). In brief, MsrB directly or indirectly plays a role in up-regulating the total GSH level in S. pombe.

**DISCUSSION**

In this work, it is demonstrated that MsrB is one of the major antioxidant enzymes which is responsible for the defense against t-BOOH-induced oxidative stress. First of all, the overexpressed MsrB could make the fission yeast cells grow better in the presence of t-BOOH. The enhanced yeast growth would be possible by the diminished ROS level in the MsrB-overexpressed yeast cells, which might be based upon MsrB’s capability of keeping the intracellular ROS levels not to be excessive in the yeast cells. The protective roles of Msr against oxidative damage or stress have been suggested in a variety of cells. In the microaerophilic food-borne pathogen Campylobacter jejuni, the msrA, msrB and msrA/msrB mutants exhibited increased sensitivity to HP, organic peroxide, superoxide, and nitrosative and disulfide stress, and the msrA/msrB double mutant was markedly more sensitive to both oxidative and nitrosative stress, which suggested that MsrA and MsrB significantly protected the cells against these stress conditions (Attack and Kelly, 2008). Both msrA and msrB mutants of Enterococcus faecalis, an important nosocomial pathogen, were more sensitive to exposure to HP and had a reduced level of virulence in a systemic and urinary tract infection model than the wild-type parents (Zhao et al., 2010). RMrS, an S. cerevisiae enzyme specific for free Met-R-SO, was shown to be mainly responsible for the reduction of free Met-R-SO by proving that it is closely linked with growth of yeast cells on Met-R-SO and that its deletion increased sensitivity to oxidative stress and decreased life span (Le et al., 2009). In mouse embryonic stem cells, knockdown of the MsrA expression by siRNA showed less resistance than control cells to HP treatment and, contrastingly, its overexpression improved survivability of the cells to hydrogen peroxide treatment (Zhang et al., 2010). MsrA was also shown to regulate sensitivity to oxidative stress induced by paraquat in mice, but it had no effect on life span (Salmon et al., 2009). The deficiency of MsrA in cardiac myocytes diminished the myocardial cell’s capability against stress stimulations resulting in cellular dysfunction in the heart (Nan et al., 2010). Dimethyl sulfoxide, which competitively inhibits methionine sulfoxide reduction activity of MsrA, is able to enhance HP-mediated S. cerevisiae cell death and markedly accumulate protein-carbonyl under oxidative stress (Kwak et al., 2010). However, the differences in the defensive roles of MsrA and MsrB under various
stress conditions currently remain unclear.

This communication also demonstrates that MsrB participates in maintaining a high level of total GSH pool in the fission yeast even in the presence of t-BOOH. Considering the previous finding that t-BOOH caused oxidative stress through the depletion of intracellular GSH pools, the S. pombe MsrB might exhibit its protective activity against t-BOOH through up-regulating the total GSH pool. However, how MsrB maintains the high intracellular GSH pool currently remains unknown. One possibility is that MsrB would enhance the GSH synthetic enzyme activities on a protein or activity level, which sequentially diminish the ROS level. Another possibility is that high intracellular GSH pool would be preserved due to a sparing effect caused by MsrB’s potent power to diminish the ROS level irrespective of GSH. Whether either mechanism actually works or not, MsrB is deeply responsible for rescuing the yeast cells exposed to t-BOOH through the down-regulation of ROS level although the precise mechanism should be elucidated in the future approaches.

Other defensive proteins against t-BOOH have been also suggested in yeasts. The fission yeast thioredoxin-like 1 protein (Txl1), a two-domain protein comprising an N-terminal thioredoxin-like domain and a C-terminal domain of unknown function, plays a role in the cellular detoxification of t-BOOH through a constitutive transcription (Jiménez et al., 2007). In Saccharomyces cerevisiae, cytosolic thioredoxin peroxidase I and II play a key role in the defense against organic hydroperoxide, while catalases and cytosolic thioredoxin peroxidase II cooperate with cytosolic thioredoxin peroxidase I in the defense against HP (Munhoz and Netto, 2004). Although Prx1, a thioredoxin-linked peroxidase localized in the cytosol of Candida albicans, is capable of reducing t-BOOH and HP, its peroxidase activity is specified to t-BOOH only (Srinivasa et al., 2012). As shown in Fig. 1, the amino acid sequence of the S. pombe MsrB is homologous with those of the counterparts of A. terreus, M. grisea and A. clavatus. Among the four homologous fungal MsrBs, the putative three-dimensional structures of MsrBs from S. pombe, A. terreus and M. grisea are very similar (Fig. 6). However, the putative three-dimensional structure of the A. clavatus MsrB is different from those of the others (Fig. 6). This example shows that a family of homologous proteins can have different three-dimensional structures.

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