The Journal of Microbiology, September 2003, p.248-251
Vol. 41, No. 3

NOTE

Glutathione Content and the Activities of Glutathione-Synthesizing Enzymes in Fission Yeast are Modulated by Oxidative Stress

Yuk-Young Lee[1], Su-Jung Kim[1], Eun-Hee Park[2], and Chang-Jin Lim[1,*]

[1]*Division of Life Sciences, Kangwon National University, Chuncheon 200-701, Korea*
[2]*College of Pharmacy, Sookmyung Womens University, Seoul 140-742, Korea*

(Received March 28, 2003 / Accepted June 12, 2003)

Glutathione (GSH) is an important factor in determining tolerance against oxidative stress in living organisms. It is synthesized in two sequential reactions catalyzed by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) in the presence of ATP. In this work, the effects of three different oxidative stresses were examined on GSH content and GSH-related enzyme activities in the fission yeast *Schizosaccharomyces pombe*. GSH content in *S. pombe* was significantly enhanced by treatment with hydrogen peroxide, β-naphthoflavone (BNF) and *tert*-butylhydroquinone (BHQ). Simultaneously, they greatly induced GCS and GS activity. However, they did not have any effects on glutathione reductase activity. These results suggest that GCS and GS activities in *S. pombe* are up-regulated by oxidative stress.

Key words: *tert*-butylhydroquinone, γ-glutamylcysteine synthetase, glutathione, glutathione synthetase, hydrogen peroxide, β-naphthoflavone, *Schizosaccharomyces pombe*

The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH), which is widely distributed in most living cells, is a principal antioxidant and a low-molecular-weight non-proteinous thiol compound. GSH plays an important role in maintaining the intracellular thiol redox state and protecting cells against oxidative damage, xenobiotic organic chemicals, and heavy metals (Meister *et al.*, 1989). GSH is synthesized in the cell cytosol via two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from L-glutamate and L-cysteine, and the formation of GSH from γ-glutamylcysteine and glycine. The first step is catalyzed by γ-glutamylcysteine synthetase (EC 6.3.2.2, GCS), whereas the second step is catalyzed by glutathione synthetase (EC 6.3.2.3, GS). The first step of GSH biosynthesis is generally regarded as the rate-limiting step and is regulated by feed-back competitive inhibition by GSH and the availability of L-cysteine (DeLeve *et al.*, 1991). The regulation of the expression of the GCS genes has been relatively well documented. GCS subunits in higher cells are up-regulated transcriptionally by β-naphthoflavone (Moinova *et al.*, 1998), the commonly used hepatocarcinogen thioacetamide (Lu *et al.*, 1999), cadmium (Dormer *et al.*, 2000; Shukla *et al.*, 2000a; Shukla *et al.*, 2000b), tumor necrosis factor (Morales *et al.*, 1997), butylated hydroxytoluene (Tu *et al.*, 1998), *tert*-butylhydroperoxide (Stover *et al.*, 2000) and nitric oxide (Moellering *et al.*, 1998). Only a few findings have been reported on the regulation of the GS genes. Mouse GS was reported to be induced by 1, 10-phenanthroline, a typical metal chelating agent, although its induction was not sufficient to cause apoptosis (Sun, 1997). In the budding yeast *Sacchromyces cerevisiae*, the expression of the GS gene was found to be increased by heat-shock stress in a Yap1p-dependent fashion and consequently intracellular GSH content was increased (Sugiyama *et al.*, 2000). However, little more is known about the regulatory mechanisms of GSH biosynthesis in yeast cells.

The reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radicals ($OH\cdot$), are produced by the normal aerobic metabolism and by environmental agents, and these can damage intracellular components such as DNA, proteins, and membrane lipids (Ross *et al.*, 2000; Smirnova *et al.*, 2000). β-Naphthoflavone (BNF) and *tert*-butylhydroquinone (BHQ) are known to produce ROS, and are used as detoxifying enzyme inducers in several organisms (Stephensen *et al.*, 2002; Jiang *et al.*, 2003). Before inves-

* To whom correspondence should be addressed.
(Tel) 82-33-250-8514; (Fax) 82-33-242-0459
(E-mail) cjlim@kangwon.ac.kr

tigating the regulatory mechanism of GSH biosynthesis in *Schizosaccharomyces pombe*, we examined the effects of oxidative stresses, such as hydrogen peroxide, β-naphthoflavone and *tert*-butyhydroquinone, on GSH content and GSH-related enzyme activities.

Total GSH content was determined by measuring absorbance at 412 nm (Sies and Akerboom, 1984) using oxidized GSSG as a standard. In a final volume of 0.5 ml, the reaction mixture contained; 100 mM phosphate buffer (pH 7.0), 1 mM EDTA, 0.24 mM NADPH, 0.0756 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and 0.06 units glutathione reductase (GR). Then, 100 μl of the appropriate GSSG standard or 100 μl of the crude extract was added to each reaction mixture. The absorbances of known concentrations of GSSG were used to construct a standard curve. γ-Glutamylcysteine synthetase (GCS) activity was determined as previously described (Seelig and Meister, 1985). Enzyme activity was determined at 37°C in a reaction mixture (final volume, 1.0 ml) containing 0.1 M Tris-HCl buffer, 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADH, 17 μg of pyruvate kinase and 17 μg of lactate dehydrogenase. The reaction was initiated by the addition of the yeast extract, and absorbance at 340 nm was monitored. Glutathione synthetase (GS) activity was determined by measuring the formation of ADP in the reaction mixtures containing the enzyme and its substrates (Meister, 1985). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.2 at 37°C), 50 mM potassium chloride, 5 mM L-γ-glutamyl-L-α-aminobutyrate, 10 mM ATP, 5 mM glycine, 20 mM magnesium chloride, 2 mM EDTA, and extract in a final volume of 0.1 ml. The assay mixture was incubated for 2.5-30 min at 37°C. To determine ADP, the reaction mixtures were treated with 0.02 ml of 10% sulfosalicylic acid and 0.9 ml of a solution containing 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, pyruvate kinase (1 unit), 40 mM magnesium chloride, 50 mM potassium chloride, and 250 mM potassium phosphate buffer (pH 7.0). The amount of ADP formed was calculated from the change in absorbance at 340 nm induced by adding 0.1 ml (1 unit) of lactate dehydrogenase. Glutathione reductase (GR) activity was spectrophotometrically assayed during the oxidation of NADPH at 340 nm (Carlberg and Mannervik, 1985). The reaction mixture (0.2 ml) contained 0.1 M phosphate buffer (pH 7.0), 1 mM GSSG, and 0.1 mM NADPH. The reaction was initiated by adding the enzyme.

Reactive oxygen species and other harmful compounds are produced during the normal growth of aerobic cells, and these may inhibit cell growth. Defense systems such as antioxidant and redox enzymes are required for the normal growth of the cells. GSH, known as a major antioxidant, is present in high concentrations (up to 10 mM in the liver) in most living cells, from microorganisms to humans, and is known to be involved in cellular responses to various stresses (Penninckx, 2000). Moreover, endogenous GSH concentrations can alter cellular responses to oxidative stress, and increases in GSH have been proposed as a potential mechanism for enhancing cellular antioxidant defense (Mollering *et al.*, 1998). Various agents modulate the transcription of the γ-glutamylcysteine synthetase genes and GSH levels in different cell types.

The wild-type *S. pombe* KP6 (972h$^-$) cells were grown in rich media (Lee *et al.*, 2001), and the *S. pombe* culture was split at the early exponential phase. Hydrogen peroxide (100 μM), β-naphthoflavone (10 μM) and *tert*-butylhydroquinone (50 μM) were added to the yeast cultures. After 6 h shaking, the yeast cultures were harvested and their extracts were used to determine GSH content and GSH-related enzyme activities. Treatment of *S. pombe* cultures with hydrogen peroxide (100 μM), β-naphthoflavone (10 μM) and *tert*-butylhydroquinone (50 μM) did not give rise to changes in growth rates (data not shown). Hydrogen peroxide, which is generated predominantly in mitochondria, is transformed into the highly reactive hydroxyl radical, which damages virtually all macromolecules (Gosslau and Rensing, 2002). Hydrogen peroxide (100 μM) strongly enhanced the levels of GSH and GSH-synthesizing enzyme activity in *S. pombe* (Fig. 1). The GSH level was increased 3.14-fold by hydrogen peroxide (100 μM). GCS and GS activities were also enhanced 3.13 and 3.82-fold by treatment with hydrogen peroxide, respectively, which corresponded with the observed increase in GSH content. However, GR activity was unaffected by hydrogen peroxide treatment. Steady-state levels of the mRNAs corresponding to the heavy and light subunits of GCS, have been reported to be elevated after exposing cells to various xenobiotics (Mulcahy *et*

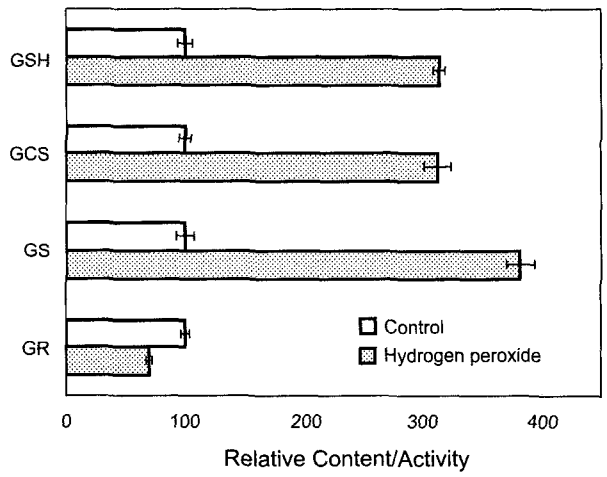

Fig. 1. Effect of hydrogen peroxide (100 μM) on total glutathione content (GSH), γ-glutamylcysteine synthetase (GCS), glutathione synthetase (GS) and glutathione reductase (GR) activities in the fission yeast *Schizosaccharomyces pombe*. Experiments were performed in triplicate.

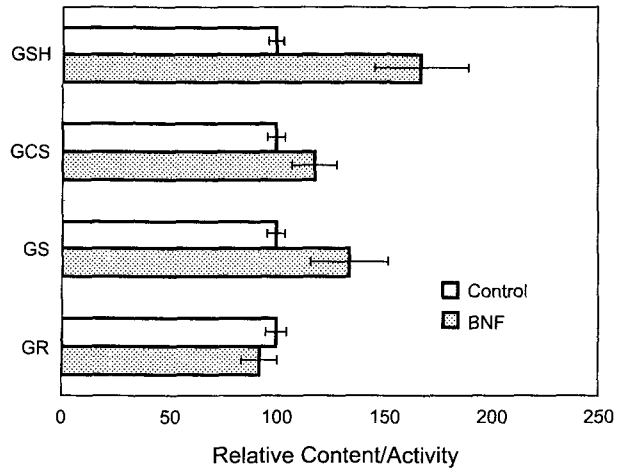

Fig. 2. Effect of β-naphthoflavone (BNF, 10 μM) on total glutathione content (GSH), γ-glutamylcysteine synthetase (GCS), glutathione synthetase (GS) and glutathione reductase (GR) activities in the fission yeast *Schizosaccharomyces pombe*. Experiments were performed in triplicate.

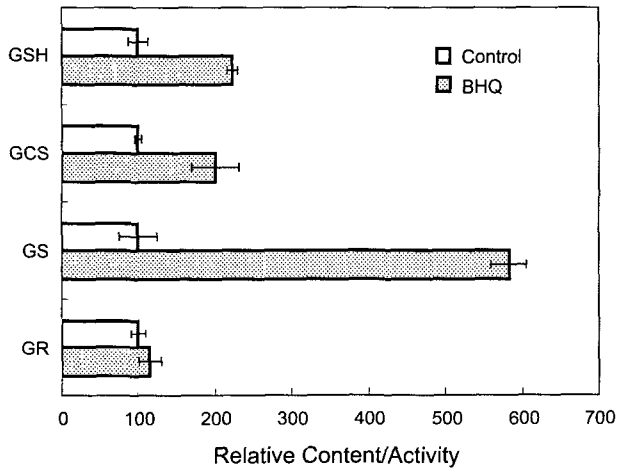

Fig. 3. Effect of *tert*-butylhydroquinone (BHQ, 50 μM) on total glutathione content (GSH), γ-glutamylcysteine synthetase (GCS), glutathione synthetase (GS) and glutathione reductase (GR) activities in the fission yeast *Schizosaccharomyces pombe*. Experiments were performed in triplicate.

al., 1997). It was recently found that the expression of the *S. cerevisiae* GCS gene is regulated at the level of transcription by oxidants such as hydrogen peroxide and heavy metals (Dormer *et al.*, 2002). The up-regulation of the GCS gene could be mediated by Yap1p, a critical transcription factor of oxidative stress response in the budding yeast (Sugiyama *et al.*, 2000). However, the involvement of the Pap1 protein, an *S. pombe* analogue of Yap1p, in the up-regulation of the GCS and GS genes remains to be elucidated. The GCS transcript in *S. cerevisiae* was greatly induced after exposure to cadmium, which was shown using a DNA microarray (Momose and Iwahashi, 2001). Treatment of the fission yeast *S. pombe* with BNF (10 μM) increased the GSH content and GSH-synthesizing enzyme activities (Fig. 2). The GSH content was increased 1.67-fold, and GCS and GS activities were increased 1.18- and 1.34-fold, respectively. Their enhancement also corresponded with the increase in GSH content. In other words, increases in the activities of glutathione-synthesizing enzyme result in a higher GSH content in *S. pombe*. BHQ is a monofunctional Phase II enzyme inducer and one of the major metabolites of butylated hydroxyanisole, a synthetic antioxidant. Pretreatments of rat lung epithelial L2 cells with sublethal concentrations of BHQ were found to increase the intracellular GSH content in a concentration- and time -dependent manner, due to the up-regulation of both β-glutamyltranspeptidase and GCS (Tian *et al.*, 1997). Treatment of BHQ (50 μM) was found to elevate the GSH content and GSH-synthesizing enzyme activities in *S. pombe* (Fig. 3), the GSH content was increased 2.22-fold, and the GCS and GS activities were enhanced 2.01 and 5.83-fold, respectively. Interestingly, GS activity was dramatically increased by BHQ treatment. The regulation of the GCS and GS genes might be largely dependent on the oxidant species and concentration, which will be future examined. Treatment with BNF and BHQ did not alter GR activity (Fig. 2, Fig. 3). However, the expression of the *S. cerevisiae* GR gene was significantly induced by exposure to menadione (Cyrne *et al.*, 2003). This suggests that the GR genes in *S. cerevisiae* and *S. pombe* are subject to different regulatory processes. Collectively, the levels of GSH and GSH-synthesizing enzyme activities are up-regulated by hydrogen peroxide, BNF and BHQ, which indicates that GCS and GS are regulated by oxidative stress in the fission yeast *S. pombe*. These results encourage gene-level studies to elucidate the precise regulatory mechanisms of GCS and GS in *S. pombe*.

This work was supported by the Korea Research Foundation Grant (KRF-2002-070-C00062)

References

Carlberg, I. and B. Mannervik. 1985. Glutathione reductase. *Methods Enzymol.* 113, 484-490.

Cyrne, L., L. Martins, L. Fernandes, and H.S. Marinho. 2003. Regulation of antioxidant enzymes gene expression in the yeast *Saccharomyces cerevisiae* during stationary phase. *Free Radic. Biol. Med.* 34, 385-393.

DeLeve, L. and N. Kaplowitz. 1991. Glutathione metabolism and its role in hepatotoxicity. *Pharmacol. Ther.* 52, 287-305.

Dormer, U.H., J. Westwater, N.F. McLaren, N.A. Kent, J. Mellor, and D.J. Jamieson. 2000. Cadmium-inducible expression of the yeast *GSH1* gene requires a functional sulfur-amino acid regulatory network. *J. Biol. Chem.* 275, 32611-32616.

Dormer, U.H., J. Westwater, D.W.S. Stephen, and D.J. Jamieson. 2002. Oxidant regulation of the *Saccharomyces cerevisiae GSH1* gene. *Biochim. Biophys. Acta* 1576, 23-29.

Gosslau, A. and L. Rensing. 2002. Oxidative stress, age-dependent [correction of age-related] cell damage and antioxidative mech-

anisms. *Z. Gerontol. Geriatr.* 35(2), 139-150.

Jiang, Z.-Q., C. Chen, B. Yang, V. Hebbar, and A.-N.T. Kong. 2003. Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers. *Life Sci.* 72, 2243-2253.

Lee, Y.-J., K. Kim, E.-H. Park, K.-S. Ahn, D. Kim, and C.-J. Lim. 2001. Cloning and regulation of *Schizosaccharomyces pombe* gene encoding ribosomal protein S20. *J. Microbiol.* 39, 31-36.

Lu, S.C., Z.-Z. Huang, H. Yang, and H. Tsukamoto. 1999. Effect of thioacetamide on the hepatic expression of γ-glutamylcysteine synthetase subunits in the rat. *Toxicol. Appl. Pharmacol.* 159, 161-168.

Meister, A. 1985. Glutathione synthetase from rat kidney. *Methods Enzymol.* 113, 393-399.

Meister, A. 1989. Metabolism and function of glutathione, p. 367-474. *In* D. Dolphin, R. Poulson and R. Auramovic (eds.), Glutathione: chemical, biochemical, and medical aspects, John Wiley and Sons, New York, NY.

Moellering, D., J. McAndrew, R.P. Patel, T. Cornwell, T. Lincoln, X. Cao, J. L. Messina, H.J. Forman, H. Jo, and V.M. Darley-Usmar. 1998. Nitric oxide-dependent induction of glutathione synthesis through increased expression of γ-glutamylcysteine synthetase. *Arch. Biochem. Biophys.* 358, 74-82.

Moinova, H.R. and R.T. Mulcahy. 1998. An electrophile responsive element (EpRE) regulates β-naphthoflavone induction of the human γ-glutamylcysteine synthetase regulatory subunit gene. *J. Biol. Chem.* 273, 14683-14689.

Momose, Y. and H. Iwahashi. 2001. Bioassay of cadmium using a DNA microassay: genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. *Environ. Toxicol. Chem.* 20, 2353-2360.

Morales, A., C. Garcia-Ruiz, M. Miranda, M. Mari, A. Colell, E. Ardite, and J.C. Fernandez-Checa. 1997. Tumor necrosis factor increases hepatocellular glutathione by transcriptional regulation of the heavy subunit chain of γ-glutamylcysteine synthetase. *J. Biol. Chem.* 272, 30371-30379.

Mulcahy, R.T., M.A. Wartman, H.H. Bailey, and J.J. Gipp. 1997. Constitutive and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J. Biol. Chem.* 272, 7445-7454.

Penninckx, M. 2000. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. *Enzyme Microb. Technol.* 26, 737-742.

Ross, S.J., V.J. Findlay, P. Malakasi, and B.A. Morgan. 2000. Thioredoxin peroxidase is required for the transcriptional response to oxidative stress in budding yeast. *Mol. Biol. Cell* 11, 2631-2642.

Seelig, G.F. and A. Meister. 1985. Glutathione biosynthesis; γ-Glutamylcysteine synthetase from rat kidney. *Methods Enzymol.* 113, 379-390.

Shukla, G.S., J.-F. Chiu, and B.A. Hart. 2000a. Enhanced expression of pulmonary γ-glutamylcysteine synthetase heavy subunit in rats exposed to cadmium aerosols. *Toxicol. Appl. Pharmacol.* 163, 249-259.

Shukla, G.S., J-F. Chiu, and B.A. Hart. 2000b. Cadmium-induced elevations in the gene expression of the regulatory subunit of γ-glutamylcysteine synthetase in rat lung and alveolar epithelial cells. *Toxicol.* 151, 45-54.

Sies, H. and T.P. Akerboom. 1984. Glutathione disulfide (GSSG) efflux from cells and tissues. *Methods Enzymol.* 105, 445-451.

Smirnova, G.V., N.G. Muzyka, M.N. Glukhovchenko, and U.N. Oktyabrsky. 2000. Effects of menadione and hydrogen peroxide on glutathione status in growing *Escherichia coli*. *Free Radic. Biol. Med.* 28, 1009-1016.

Stephensen, E., J. Sturve, and L. Förlin. 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. *Comp. Biochem. Physiol. Part C* 133, 435-442.

Stover, S.K., G.A. Gushansky, J.J. Salmen, and C.S. Gardiner. 2000. Regulation of γ-glutamate-cysteine ligase expression by oxidative stress in the mouse preimplantation embryo. *Toxicol. Appl. Pharmacol.* 168, 153-159.

Sugiyama, K., S. Izawa, and Y. Inoue. 2000. The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 275, 15535-15540.

Sun, Y. 1997. Induction of glutathione synthetase by 1,10-phenanthroline. *FEBS Letters* 408, 16-20.

Tian, L., M.M Shin, and H.J. Forman. 1997. Increased transiption of the regulatory subunit of gamma-glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. *Arch. Biochem. Biophys.* 342(1), 126-133.

Tu, Z. and M.W. Anders. 1998. Up-regulation of glutamate-cysteine ligase gene expression by butylated hydroxytoluene is mediated by transcription factor AP-1. *Biochem. Biophys. Res. Commun.* 244, 801-805.