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Protective Role of Thioredoxin Peroxidase Against Ionizing Radiation

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A soluble protein from *Saccharomyces cerevisiae* provides protection against a thiol-containing oxidation system but not against an oxidation system without thiol. This 25-kDa protein acts as a peroxidase but requires the NADPH-dependent thioredoxin system or a thiol-containing intermediate, and was thus named thioredoxin peroxidase. The protective role of thioredoxin peroxidase against ionizing radiation, which generates reactive oxygen species harmful to cellular function, was investigated in wild-type and mutant yeast strains in which the *tsa* gene encoding thioredoxin peroxidase was disrupted by homologous recombination. Upon exposure to ionizing radiation, there was a distinct difference between these two strains in regard to viability and the level of protein carbonyl content, which is the indicative marker of oxidative damage to protein. Activities of other antioxidant enzymes, such as catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase were increased at 200–600 Gy of irradiation in wild-type cells. However, the activities of antioxidant enzymes were not significantly changed by ionizing radiation in thioredoxin peroxidase-deficient mutant cells. These results suggest that thioredoxin peroxidase acts as an antioxidant enzyme in cellular defense against ionizing radiation through the removal of reactive oxygen species as well as in the protection of antioxidant enzymes.

Keywords: Ionizing radiation, Reactive oxygen species, Thioredoxin peroxidase.

Introduction

The damaging effect of ionizing radiation on living cells is predominantly due to reactive oxygen species generated by the decomposition of water. The major damaging species is the hydroxyl radical; the secondary radicals formed by interaction of ·OH with organic molecules may also be of importance (von Sonntag, 1987). Biological systems have evolved an effective and intricate network of defense mechanisms which enable cells to cope with cytotoxicity involving reactive oxygen species. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2 (McCord and Fridovich, 1969), catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides (Chance *et al.*, 1979). Glucose-6-phosphate dehydrogenase, which is a key enzyme for the generation of NADPH, and glutathione reductase, which is involved in the regeneration of reduced glutathione (GSH), are also considered as essential antioxidant enzymes (Cerutti, 1985).

Recently, a 25-kDa antioxidant protein from yeast and mammalian brain tissues has been purified (Kim *et al.*, 1988; 1989). Although the physiological role of this protein has not been elucidated unequivocally, it was reported as a peroxidase that removes H_2O_2 using hydrogen provided by either thiols or the NADPH-dependent thioredoxin system composed of thioredoxin, thioredoxin reductase, and NADPH (Kwon *et al.*, 1994a; 1994b; Chae *et al.*, 1994). The protein was thus named thioredoxin peroxidase (Chae *et al.*, 1994). The yeast *Saccharomyces cerevisiae* thioredoxin peroxidase has been demonstrated by Western blot determination to increase 1.5–3 fold when *S. cerevisiae* cells undergo oxidative stress, suggesting that it is of physiological importance (Kim *et al.*, 1989; Lee and Park, 1998). Furthermore, it has been shown that thioredoxin peroxidase-deficient cells are more sensitive than wild-type cells to oxidative stress induced by *tert*-butylhydroperoxide, hydrogen peroxide, cumene hydroperoxide, and a redox-cycling agent, menadione, strongly suggesting an antioxidant role for thioredoxin peroxidase (Chae *et al.*, 1993; Park *et al.*, 1996; Lee and Park, 1998).

In the present study, the role of thioredoxin peroxidase

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in cellular defense against ionizing radiation was investigated using the wild-type strain and a thioredoxin peroxidase-deficient mutant strain, in which the gene encoding thioredoxin peroxidase (*tsa*) was disrupted. The results revealed that intracellular thioredoxin peroxidase protected yeast cells from the damage induced by ionizing radiation.

Materials and Methods

Materials Hydrogen peroxide, β-NADPH, β-$NADP^+$, glucose-6-phosphate, oxidized glutathione (GSSG), 2,4-dinitrophenylhydrazine (DNPH), pyrogallol, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, USA). Acrylamide, agarose, ammonium persulfate, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), riboflavin, bromochloroindolyl phosphate (BCIP), and nitroblue tetrazolium (NBT) were from Bio-Rad (Hercules, USA). Monospecific rabbit antibody against thioredoxin peroxidase purified from yeast was kindly provided by Dr. K. Kim (Chonnam National University, Kwangju, Korea).

Yeast strains and culture conditions The wild-type *S. cerevisiae* strain JD7-7C (*mat*α, *ura 3-52, leu2, trpA,* K^+) and the *tsa* mutant strain, which was created by the integrative disruption method as described by Chae *et al.* (1993), were kindly provided by Dr. I. H. Kim (Pai Chai University, Taejon, Korea). Overnight cultures were grown in a shaker at 30°C in YPD medium consisting of 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. Once the optical density (650 nm) reached 0.3–0.4, the cultures were irradiated at ambient temperature by X-rays at different doses. Survival was determined by plating suitable cell dilutions on YPD agar plates. Cell viability was defined by colony growth at 30°C on plates counted after 48 h.

Quantitation of free radical detoxifying systems The yeast cells were harvested by centrifugation at 3000 × *g* and resuspended in 600 μl of lysis buffer (20 mM HEPES/1 mM EDTA/2 mM PMSF). To disrupt cells, 1/2 vol of ice-chilled 0.5 mm-diameter glass beads were added and the microfuge tubes were vortexed for five 2-min intervals interspersed with periods of cooling in an ice bath (Lee and Park, 1998). Cellular debris was removed by a 10-min centrifugation at 15,000 × g. The supernatant was collected and protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad (Hercules, USA). Catalase activity was measured in terms of the decomposition of hydrogen peroxide, which was followed directly by the decrease in absorbance at 240 nm (Beers and Sizer, 1952). Total SOD activity in cell extracts was assayed spectrophotometrically using the pyrogallol assay (Marklund and Marklund, 1974), where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%. Glutathione reductase activity was quantified by the GSSG-dependent loss of NADPH (Pinto and Bartley, 1969) as measured absorbance at 340 nm (ε = 6.67 $mM^{-1}cm^{-1}$). Incubations were conducted with 0.1 mM NADPH, yeast cell-free extract, 1 mM GSSG, 1 mM EDTA, and 0.1 M potassium phosphate, pH 7.4, to a final volume of 1.5 ml. Glucose-6-phosphate dehydrogenase activity was measured by following the rate of $NADP^+$ reduction using the procedure described (Beutler, 1971).

SDS-PAGE and Western blotting Crude extracts prepared from yeast strains were first subjected to SDS-PAGE using 12.5% polyacrylamide gels (Laemmli, 1970). Proteins on the slab gels were electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979) that were subsequently incubated with Blotto. Blocked nitrocellulose blots were incubated with a monospecific rabbit antibody against thioredoxin peroxidase purified from yeast (1:2000 dilution). The detection method used alkaline phosphatase-labeled goat anti-rabbit IgG (CALTAG, 1:2000 dilution) with the BCIP/NBT detection system (Bio-Rad).

Protein carbonyl content The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure as previously described (Levine *et al.*, 1990). The crude extract (~1 mg protein) was incubated with 0.4 ml 0.2% DNPH in 2 M HCl for 1 h at 37°C. The protein hydrazone derivatives were sequentially extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ethyl acetate, 1:1 (v/v), and reextracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride, and the difference spectrum of the sample treated with DNPH in HCl was examined versus a sample treated with HCl alone. Results are expressed as nmol of DNPH incorporated per mg of protein calculated from an absorbtivity of 21.0 $mM^{-1}cm^{-1}$ at 360 nm for aliphatic hydrazones.

Replicates Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

The lack of expression of the *tsa* gene product in the deletion mutant was confirmed by Western blot analysis using a polyclonal rabbit antibody against thioredoxin peroxidase (Lee and Park, 1998). An exposure of ionizing radiation in the range of 6–45 Gy to a culture of aerobically grown wild-type cells did not significantly change the expression of thioredoxin peroxidase (Fig. 1, left). At 600 Gy irradiation, fragmentation of thioredoxin peroxidase was observed (Fig. 1, right), indicating that the higher dose of X-rays led to a partial inactivation of the enzyme.

To determine the viability loss, early exponential phase cells were harvested and exposed to ionizing radiation at different doses (6–600 Gy). The exponential phase was chosen since cells can become resistant to various forms of stress when they enter stationary phase. As shown in Fig. 2, wild-type cells were significantly more resistant to ionizing radiation than *tsa* mutant cells. Wild-type cells showed a survival of 70% after exposure to 200 Gy of irradiation, whereas *tsa* mutant cells showed a survival of 34%.

Because antioxidant systems work as a team, the question of whether the lack of cellular thioredoxin peroxidase activity induced concomitant alterations in the

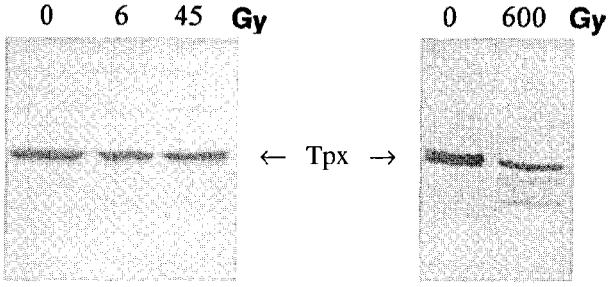

Fig. 1. Immunoblots of cell-free extract (25 μg of protein) from wild-type cells after exposure to irradiation at different doses. The samples were analyzed by SDS-PAGE followed by immunoblotting with a polyclonal yeast thioredoxin peroxidase (Tpx) antibody.

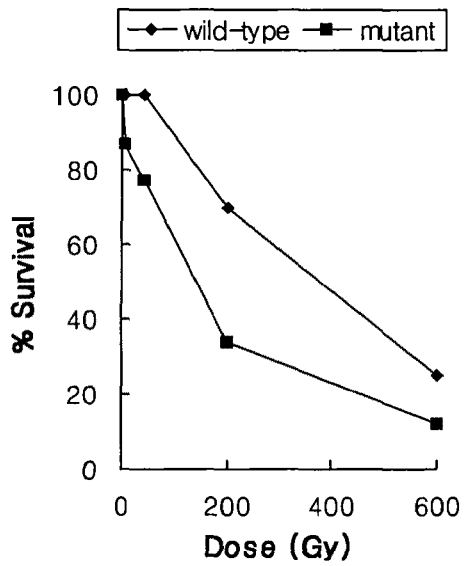

Fig. 2. Resistance of wild-type and *tsa* mutant cells upon exposure to ionizing radiation. After cells were exposed to ionizing radiation at different doses, survival was determined by plating suitable cell dilutions on YPD agar plates. Cell viability was defined by colony growth at 30°C on plates which were counted after 48 h. Percent survival is determined as the viable cell number at each dose divided by the viable cell number before exposure to ionizing radiation.

detoxifying systems against reactive oxygen species was investigated. We therefore measured the activities of SOD, catalase, glucose-6-phosphate dehydrogenase, and glutathione reductase in wild-type and *tsa* mutant cells. As shown in Fig. 3, no major difference in the activities of these antioxidant enzymes occurred in *tsa* mutant cells upon exposure to ionizing radiation in the range of 6–600 Gy. However, activities of antioxidant enzymes increased approximately 2 times by 200–600 Gy of irradiation in the wild-type cells.

We performed carbonyl content measurements for protein oxidation after cellular exposure to ionizing radiation to determine whether thioredoxin peroxidase expression decreased sensitivity to protein damage. Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins (Levine *et al.*, 1990). While in wild-type cells no significant increase was observed after exposure to ionizing radiation, *tsa* mutant cells elicited an approximately 2-fold increase of carbonyl groups compared to unirradiated cells when 200 Gy of ionizing radiation was used (Fig. 4)

Discussion

Recent evidence suggests a growing consensus that reactive oxygen species such as hydroxyl radicals, superoxide anions, and organic hydroperoxides play an important role in cellular damage caused by ionizing radiation (Ewing and Jones, 1987). These reactive oxygen species cause DNA strand breaks, lipid peroxidation, and protein modification (Cerutti, 1985). Limited work has indicated that radiation resistance of many cells is presumably associated with the antioxidant enzyme system which removes reactive oxygen species. Radiation resistance of various mammalian cell lines was shown to correlate with their SOD activity (Yamaguchi *et al.*, 1994) or glutathione peroxidase activity (Marklund *et al.*, 1984), suggesting that ionizing radiation and oxidative stress have common cellular effects.

Since thioredoxin peroxidase exhibits its antioxidant activity only in the thiol-containing metal-catalyzed oxidation system, the function of thioredoxin peroxidase was initially suggested as a sulfur radical scavenger (Kim *et al.*, 1988). However, in the presence of thiol, thioredoxin peroxidase acts as an antioxidant protein in an ascorbate-containing oxidation system (Kwon *et al.*, 1994a; 1994b). This result suggested that thioredoxin peroxidase may require reduced thiol as a reducing equivalent to remove reactive oxygen species, presumably hydrogen peroxide. It has been reported that a thiol group of cysteine in thioredoxin peroxidase is involved in catalysis (Chae *et al.*, 1993) and the chemical modification with thiol-specific reagents such as N-ethylmaleimide inhibits the antioxidant activity of thioredoxin peroxidase (Lim *et al.*, 1993). This effect is presumably mediated through the modification of Cys47-SH, which is the primary site of thioredoxin peroxidase catalysis. The antioxidant activity of thioredoxin peroxidase is restored by a cellular reducing catalyst, such as the NADPH-dependent thioredoxin system, through a redox control mechanism.

Despite their role in the cellular defense mechanism, the antioxidant enzymes are susceptible to inactivation by reactive oxygen species. Previous studies have demonstrated that oxidative processes result in the loss of key antioxidant enzymes (Hodgson and Fridovich, 1975;

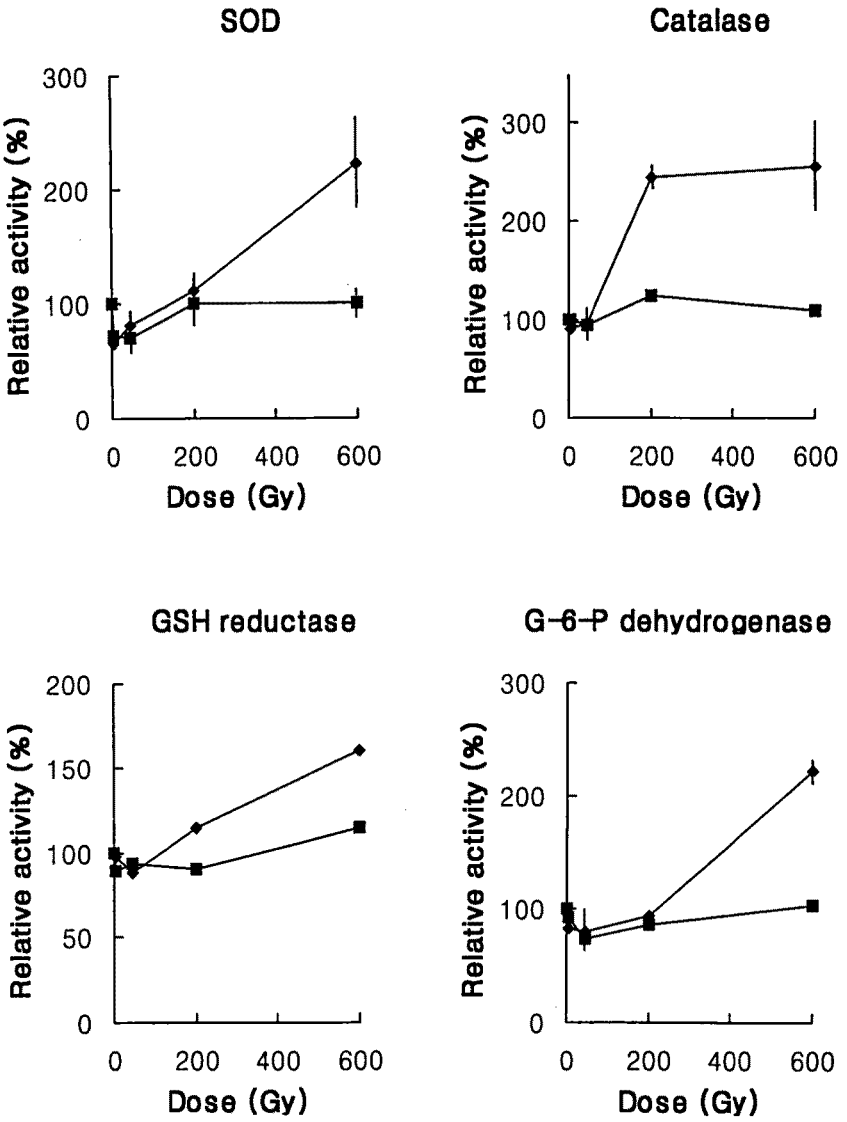

Fig. 3. Activities of antioxidant enzymes in wild-type cells (◆) and *tsa* mutant cells (■) exposed to irradiation in a range of 6–600 Gy. Activity of unirradiated cells is expressed as 100%. Results are mean ± SD of three to four determinations.

Kono and Fridovich, 1982; Tabatabaie and Floyd, 1994), which may exacerbate oxidative stress-mediated cytotoxicity. However, it is also possible that prokaryotes and eukaryotes compensate for inactivation of antioxidant enzymes by an enhanced expression of SOD, catalase, and other antioxidant enzymes. Induction of antioxidant proteins in response to oxidative stress in *Escherichia coli*, *Salmonella typhimurium*, (Christman *et al.*, 1985; Greenberg *et al.*, 1990), yeast (Davies *et al.*, 1995), and mammalian cells (Wiese *et al.*, 1995) is well known. Our results show that antioxidant enzyme activities were not increased in mutant cells upon exposure to ionizing radiation. In contrast, activities of antioxidant enzymes were increased approximately 2-fold at 200–600 Gy of irradiation in wild-type cells. The inactivation of enzymes by ionizing radiation may be one of the several possibilities to interpret these results. The antioxidant enzymes may be undergoing concomitant inactivation along with induction in response to ionizing radiation. Thioredoxin peroxidase presumably provides protection for antioxidant enzymes against oxidative inactivation. Therefore, the antioxidant role of thioredoxin peroxidase in ionizing radiation may result from the direct elimination of hydrogen peroxide as well as the protection of other antioxidant enzymes.

In summary, our results demonstrate distinct differences between cells expressing thioredoxin peroxidase and cells lacking this enzyme in regard to the number of surviving cells, activities of other antioxidant enzymes, and the accumulation of oxidized protein upon exposure to

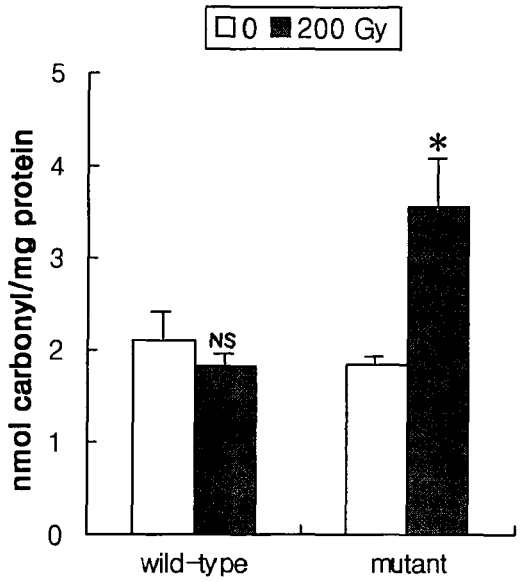

Fig. 4. Protein carbonyl content of yeast strains exposed to ionizing radiation of 200 Gy. Protein carbonyls were measured in cell-free extracts by the method of Levine and co-workers (Levine *et al.*, 1990) with the use of 2,4-dinitrophenyl hydrazine. Results are mean ± SD of three determinations. The asterisk denotes significant difference ($p < 0.05$) when compared to unirradiated cells. NS indicates not significantly different from unirradiated cells.

ionizing radiation. Our results suggest that thioredoxin peroxidase is an important antioxidant protein in the protection of yeast cells against ionizing radiation.

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