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Expression of Thiol-Dependent Protector Protein from Yeast Enhances the Resistance of *Escherichia coli* to Menadione

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Abstract: A soluble protein from *Saccharomyces cerevisiae* specifically provides protection against a thiol-containing oxidation system but not against an oxidation system without thiol. This 25-kDa protein was thus named thiol-dependent protector protein (TPP). The role of TPP in the cellular defense against oxidative stress was investigated in *Escherichia coli* containing an expression vector with a yeast genomic DNA fragment that encodes TPP (strain YP) and a mutant in which the catalytically essential amino acid in the active site of TPP (Cys-47) has been replaced with alanine by site-directed mutagenesis (strain YPC47A). There was a distinct difference between these two strains in regard to viability, modulation of activities of superoxide dismutase and catalase, and the oxidative damage of DNA upon exposure to menadione. These results suggest that TPP may play a direct role in the cellular defense against oxidative stress by functioning as an antioxidant protein.

Key words: antioxidant protein, menadione, oxidative DNA damage, site-specific mutagenesis.

Reactive oxygen species such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) are generated *in vivo* from the incomplete reduction of oxygen during aerobic metabolism or from exposure to environmental agents such as radiation, redox cycling agents, or stimulated host phagocytes (Ames, 1983; Fridovich, 1983; Babior, 1992). These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks (Halliwell and Gutteridge, 1984; Storz *et al.*, 1987). Biological systems have evolved several defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve the 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).

Recently, a 25 kDa antioxidant protein from yeast and mammalian brain tissues has been purified (Kim *et al.*, 1988; Kim *et al.*, 1989). In the presence of thiols such as dithiothreitol or reduced glutathione (GSH), this protein prevents damage induced by metal-catalyzed oxidation systems comprised of Fe^{3+}, O_2, and ascorbate or thiol. The enzyme was thus named thiol-dependent protector protein (TPP; also called thiol-specific antioxidant protein) (Kim *et al.*, 1988; Kwon *et al.*, 1994a). TPP does not possess any activity of known antioxidant enzymes including catalase, glutathione peroxidase, and SOD, or iron chelation activity (Kim *et al.*, 1988). Studies with purified protein revealed that TPP contains neither a heme or a flavin prosthetic group nor tightly bound metal ions, but it does contain two cysteine residues presumably essential for its antioxidant activity (Chae *et al.*, 1994). Although the physiological role of TPP has not been elucidated unequivocally, we and others reported that TPP is a peroxidase that removes H_2O_2 using hydrogen provided by either dithiothreitol or the NADPH-dependent thioredoxin system comprised of thioredoxin, thioredoxin reductase, and NADPH (Chae *et al.*, 1994; Kwon *et al.*, 1994b).

In the present study the role of TPP in the cellular defense against oxidative stress was investigated upon exposure to menadione using *Escherichia coli* containing an expression vect or with either i) a yeast genomic DNA fragment that encodes TPP (strain YP), or ii) a cDNA encoding a nonfunctional form of TPP as a control in which the catalytically essential amino acid in the active site of TPP (Cys-47) has been replaced with alanine by site-specific mutagenesis (strain YPC47 A).

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Menadione (2-methyl-1,4-naphthoquinone) is a synthetic naphthoquinone derivative which is one of the redox-cycling agents that catalyze the flow of reducing equivalents to oxygen; this process generates superoxide ion and other reduced oxygen species (Powis, 1987). The mechanism of action of menadione involves the one-electron reduction of menadione at the expense of NADPH or NADH, followed by one-electron oxidation of the reduced forms by O_2, generating O_2^-. The O_2^- produced by menadione can be converted to H_2O_2 and subsequently to hydroxyl radicals, which are powerful oxidants that inflict damage on lipids, proteins, and nucleic acids through spontaneous disproportion and enzyme reactions. Therefore, menadione has been used extensively to study oxidant stress in both eucaryotes and procaryotes (Thor *et al.*, 1982; Greenberg and Demple, 1989).

In this report, we show that expression of TPP enhances the resistance of *E. coli* to oxidative stress induced by menadione as judged by the effect on viability and oxidative DNA damage.

Materials and Methods

Materials

Menadione, hydrogen peroxide, nuclease P1, *E. coli* alkaline phosphatase, RNase A, and RNase T1 were obtained from Sigma Chemical Co. (St. Louis, USA). Acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), riboflavin, and nitroblue tetrazolium (NBT) were obtained from Bio-Rad (Hercules, USA). Monospecific rabbit antibodies against TPP purified from yeast and a rabbit antiserum against catalase were kindly provided by Dr. K. Kim (Chonnam National University, Kwangju, Korea) and Dr. Y. S. Lee (Kyungpook National University, Taegu, Korea), respectively. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) was synthesized as described previously (Shigenaga *et al.*, 1990).

Bacterial strains

Bacterial strains were a kind gift of Dr. K. Kim (Chonnam National University, Kwangju, Korea). *E. coli* JM109 derivative strains have plasmid pKK223-3 containing either the 585 nucleotide long structural gene for TPP or a mutated *tpp* gene (Cys47→Ala) that was constructed by site-specific mutagenesis. The purified mutant TPP from YPC47A did not exhibit any detectable antioxidant activity measured by the ability to protect thiol/$Fe^{3+}O_2$-dependent inactivation of glutamine synthetase (Ahn *et al.*, 1996).

Bacterial growth

Cells were grown overnight at 37℃ with shaking in LB (Luria-Bertani) broth containing 4% ampicillin. Overnight cultures were used to inoculate fresh medium. Once the optical density (600 nm) reached 0.3~0.4, exposure to menadione was started. Aliquots of the exposed cells were removed at time intervals and assayed for changes in OD_{600}. Viability was determined at various times by removing samples and performing viable cell counts.

Cell-free extract and enzyme assay

Cell-free extracts were prepared by sonication followed by dialysis (Hassan and Fridovich, 1977). Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard. 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). SOD isozymes, i.e. manganese, iron, and hybrid, were separated by electrophoresis in 10% nondenaturing polyacrylamide gels (Davis, 1984), and were visualized as described by Beauchamp and Fridovich (1971). Briefly, the gel was placed in 2.45 mM nitroblue tetrazolium for 20 min, followed by 15 min in 28 μM riboflavin and 28 mM TEMED. The gel was then exposed to moderately intense light until the bands showed maximum resolution.

Western blotting

Crude extract prepared from YP strains was 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.*, 1978) that were subsequently incubated with Blotto. Blocked nitrocellulose blots were incubated with rabbit antibodies against TPP purified from yeast (1:2000 dilution) or with rabbit antiserum against catalase (1:100 dilution). The detection method used alkaline phosphatase-labeled goat anti-rabbit IgG (CALTAG, 1:2000 dilution) with the BCIP/NBT detecting system (Bio-Rad).

DNA isolation and 8-OH-dG assay

DNA was isolated from bacteria as previously described (Park, 1991). Residual RNA was destroyed by incubation at 37℃ for 30 min with a mixture of RNase T1 (50 U/ml) and RNase A (100 μg/ml) in 0.05 M Tris-HCl, pH 7.4. DNA samples in 0.02 M sodium acetate, pH 4.8, were digested to nucleotides with 20 μg of nuclease P1 at 37℃ for 30 min, and then treated with 1.3 units of *E. coli* alkaline phosphatase in 0.1 M Tris-HCl, pH 7.4, at 37℃ for 1 h to liberate the corresponding nucleosides. Levels of 8-OH-dG were

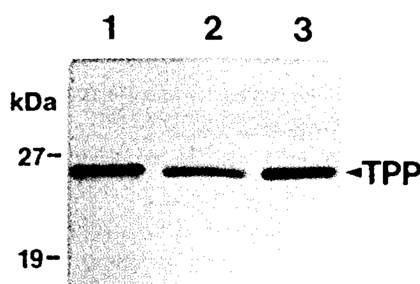

Fig. 1. Immunoblots of cell-free extracts from YP strains. The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a polyclonal yeast TPP antibody. Lane 1 contains purified TPP from yeast (0.2 μg); lanes 2 and 3, lysates (25 μg of protein) from YP and YPC47A, respectively.

determined by HPLC (C18 Ultrasphere, 5 mm; 250× 4.6 mm) with electrochemical detection (Park *et al.*, 1989). A UV detector at 254 nm was used to detect deoxyguanosine and an electrochemical detector (applied potential, 600 mV) was used to detect 8-OH-dG. Vacuum-degassed 50 mM phosphate buffer containing 10% methanol (pH 5.5) was used as a mobile phase. The flow rate was 1.0 ml/min. The molar ratio of 8-OH-dG to deoxyguanosine in each DNA sample was measured based on the peak height of authentic 8-OH-dG with the electrochemical detector and the UV absorbance at 254 nm for deoxyguanosine.

Single-strand DNA breaks

Isolation of the plasmids from the bacteria was carried out in 1.5-ml tubes, avoiding strongly alkaline conditions. Approximately 2×10^{10} bacteria in 300 μl of STET solution (8% sucrose/5% Triton X-100/50 mM EDTA/50 mM Tris-HCl, pH 8.0) were treated at 0℃ with 200 μg lysozyme for 2 min. After placing the tube in a boiling water bath for 2 min, the undissolved material was removed by centrifugation and 200 μl cold isopropanol was added to the supernatant. After placing at −20℃ for 30 min, the DNA pellet was recovered and redissolved in 50 μl TE buffer. DNA samples were applied to 1% agarose gels in a TAE buffer system, and electrophoresis was performed at 5 V/cm for 2 h at room temperature. Following electrophoresis, gels were stained with ethidium bromide, irradiated from below with a UV transilluminator box, and photographed.

Results

The expression of the *tpp* gene products of YP strains was induced without adding IPTG, and it was confirmed by Western blot analysis using a polyclonal rabbit antibody against TPP. The mutation introduced in a *tpp* gene did not affect TPP expression in bacteria, since both bacterial crude lysates from YP and YPC47A cells contain a protein band of the predicted molecular mass of 25 kDa (Fig. 1).

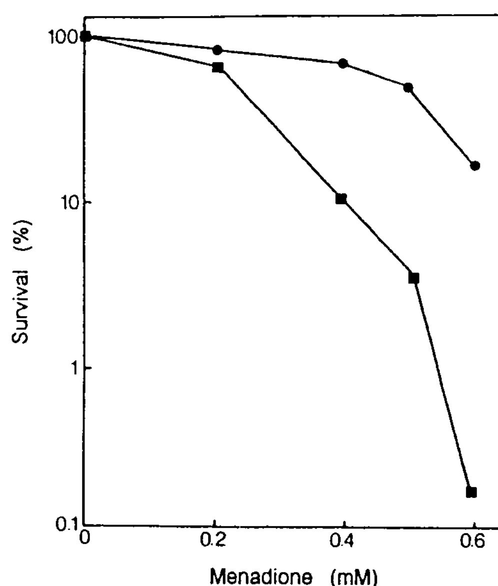

Fig. 2. Effect of menadione on viability of cells. After cells were exposed to several different concentrations of menadione for 1 h, viability was determined as described in the text. ●, YP; ■, YPC47A.

Table 1. Catalase activity of cell-free extracts from YP strains exposed to menadione. Values are mean± S.D. of five determinations

Strain	Catalase activity (U/mg protein)	
	No addition	Menadione (0.5 mM)
YP	8.3± 1.3	7.8± 1.0
YPC47A	5.2± 0.8[a]	4.8± 1.1[b]

[a] Significantly different (p<0.01) from YP cells.
[b] Significantly different (p<0.01) from YP cells treated with menadione.

To determine the rate of viability loss, early exponential phase cells were harvested and exposed to various concentrations of menadione for 1 h. Exponential phase was chosen since cells can become resistant to various forms of stress when they enter a stationary phase. As shown in Fig. 2, YP cells were more resistant to menadione than YPC47A cells. At 0.5 mM menadione the survival fraction was 49% for YP but only 3.5% for YPC47A. he strain exhibiting no TPP activity was killed to a greater extent upon exposure to manadione, indicating that TPP may be involved in protecting cells from oxidative stress.

To determine whether the expression of cellular TPP activity induces concomitant alteration in the activity of other major antioxidant enzymes, the modulation of activities of catalase and SOD upon exposure to

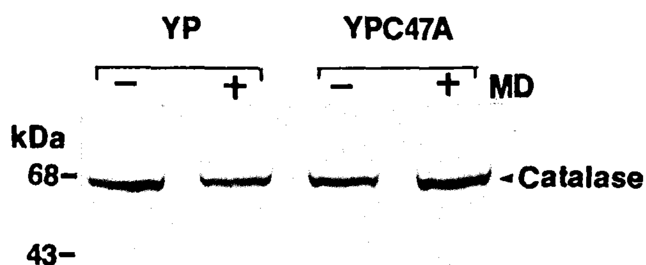

Fig. 3. Immunoblots of cell-free extracts from control and menadione (0.5 mM)-treated YP strains. The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a polyclonal catalase antibody. The blot was probed for catalase, and subsequently alkaline phosphatase-stained as described under "Materials and Methods". Each lane contained 25 μg of protein. The results are representative of three separate experiments.

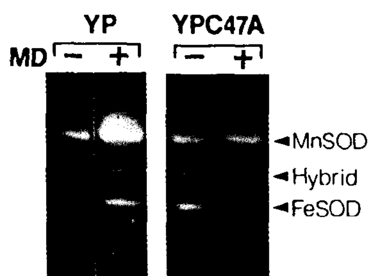

Fig. 4. Nondenaturing polyacrylamide gel of SOD from YP strains exposed and not exposed to 0.5 mM menadione. Aliquots of lysates containing 50 μg of protein were loaded onto a 10% polyacrylamide gel, which was developed and stained for activity as described under "Materials and Methods". The results are representative of four separate experiments.

menadione was examined. Table 1 compares the catalase activity of strains exposed to 0.5 mM menadione. Although the induction of catalase activity was not observed in both strains, YP cells exhibited a higher activity in cells either treated or untreated with menadione than that of YPC47A cells. However, no significant differences in the catalase expression for the YP and YPC-47A cells were discemible as shown in Fig. 3. Non-denaturing gels stained for SOD activity in bacterial lysates-both exposed and unexposed to menadione - are shown in Fig. 4. Examination of the isozyme forms of SOD from menadione-treated cells showed that the manganese SOD (MnSOD) activity was induced in YP cells; however, no significant increase of activity was detected in YPC47A cells. We also found no evidence for significant induction of iron SOD (FeSOD) by oxidative stress in either strain.

DNA lesions resulting from exposure to reactive oxygen species are modified bases and strand breaks. The modified base 8-OH-dG is considered to be one of

Table 2. Level of 8-OH-dG in DNA from YP strains exposed to menadione. Values are mean± S.D. of eight determinations

Strain	fmol 8-OH-dG/mg DNA	
	No addition	Menadione (0.5 mM)
YP	6.2± 1.6	8.4± 0.9
YPC47A	7.6± 0.8	12.9± 1.2[a]

[a] Significantly different ($p<0.01$) from YP cells treated with menadione.

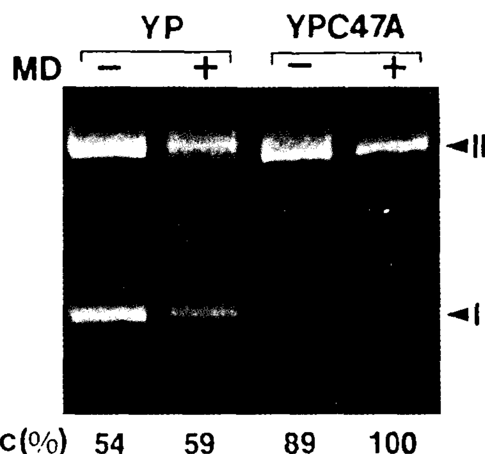

Fig. 5. Agarose gel electrophoresis of DNA from YP strains. Bacteria were treated with menadione (0.5 mM) for 1 h and DNA isolated from the bacteria was analyzed as described in the text. The relative fluorescence intensities of the open circular forms of plasmid were quantitated by measuring areas of densitometer tracing. Closed circular supercoiled DNA is labeled I; nicked, circular DNA, II.

the oxidative DNA products induced by oxygen radicals which can be easily measured by HPLC with electrochemical detection. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* (Shigenaga *et al.*, 1990). As shown in Table 2, subjecting YP and YPC47A cells to menadione for 1 h increased the level of 8-OH-dG. However, the increase of 8-OH-dG was more significant in YPC47A than in YP cells. Control strains contain considerable amounts of endogenous 8-OH-dG which reflects a certain level of oxidative damage in *E. coli* cells caused by normal metabolism. In order to estimate single strand breaks, DNA from cells unexposed and exposed to menadione was extracted and analyzed by electrophoresis on a 0.8% agarose gel. The ethidium bromide staining pattern of plasmid DNA revealed the conversion of closed circular super-coiled DNA (form I) to open circular DNA (form II). As shown in Fig. 5, extensive DNA breakage in YPC47A was found in cells both exposed and unexposed to menadione, and DNA breakage was reduced in cells expressing active TPP.

Discussion

The aim of the present work was to evaluate the effect of TPP on the protection of bacterial cells from oxidative stress, which was introduced by exposure to menadione during aerobic growth. The cytotoxic effects of menadione are thought to be mediated through one-electron reduction to semiquinone radicals which can rapidly reduce O_2 to O_2 and regenerate the quinone (Thor *et al.*, 1982), or react with thiol-containing compounds such as glutathione. In addition, autoxidation of glutathionyl-hydroquinones and generation of O_2^- also occur (Powis, 1987). Either type of redox cycling may thus result in the intracellular formation of large amounts of O_2 , and H_2O_2 by O_2 dismutation. A large part of the cytotoxic effects of these oxidants can be mediated by the formation of hydroxyl radicals via Fenton-type reactions. The reduced toxicity of such oxidants on cells can be achieved by removal of a potential precursor of hydroxyl radicals, namely O_2 or H_2O_2.

Since TPP exhibits its antioxidant activity only in the thiol-containing metal-catalyzed oxidation system, the function of TPP was initially suggested as a sulfur radical scavenger (Kim *et al.*, 1988). However, in the presence of thiol, TPP acts as an antioxidant protein in an ascorbate-containing oxidation system (Kwon *et al.*, 1994a). This result suggested that TPP may require reduced thiol as a reducing equivalent to remove reactive oxygen species. Recently, we and others provided evidence suggesting that, when coupled with either thiols or a NADPH-dependent thioredoxin system, the key function of this protein is the removal of hydrogen peroxide, thus acting as a peroxidase (Chae *et al.*, 1994; Kwon *et al.*, 1994b). It has been reported that a thiol group of cysteine in TPP is involved in catalysis (Chae *et al.*, 1993). Chemical modification with thiol-specific reagents such as N-ethylmaleimide inhibits the antioxidant activity of TPP (Lim *et al.*, 1993). The antioxidant activity of TPP is presumably restored by the cellular reducing catalyst through a redox control mechanism. Because our results suggest that TPP can act as an antioxidant protein in bacterial cells, it is implied that either GSH plays a role as a direct hydrogen donor for the enzyme or bacterial thioredoxin and thioredoxin reductase provide a reducing equivalent for eucaryotic TPP.

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; Kono and Fridovich, 1982), which may exacerbate oxidative stress-mediated cytotoxicity. However, it is also possible that procaryotes 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 *E. coli* and *Salmonella typhimurium* is well known (Christman *et al.*, 1985; Greenberg *et al.*, 1990). Our results show that catalase activity was not increased in either YP strain, and that MnSOD activity was induced in YP cells exposed to menadione. However, catalase activity was lower and MnSOD activity was not significantly enhanced in YPC47A cells in either culture exposed or unexposed to menadione. We interpret these results to be due to the inactivation of enzymes by oxidative stress. The antioxidant enzymes may be undergoing concomitant inactivation along with induction in response to menadione exposure. TPP presumably provides protection of antioxidant enzymes against oxidative inactivation. Therefore, the antioxidant role of TPP may result via the direct elimination of hydrogen peroxide as well as the protection of other antioxidant enzymes.

Oxidant-induced DNA damage may be one mechanism underlying the genomic instability associated with the loss of biological activity and with mutagenicity (Wallace, 1987). DNA lesions resulting from exposure to reactive oxygen species have been shown to be modified bases and strand breaks. We found that short time exposure to moderately toxic concentrations of menadione induced extensive DNA breakage and increase of oxidative DNA adduct 8-OH-dG in YPC47A cells. Because the menadione genotoxic effect is mediated by ·OH radicals it can be implicated that the removal of a precursor of ·OH radicals, namely H_2O_2, by TPP may alleviate sensitivity of bacterial cells to menadione.

In summary, our results demonstrate distinct differences between cells expressing TPP and cells expressing nonfunctional TPP in regard to the number of surviving cells, activities of SOD and catalase, and the accumulation of oxidized DNA adducts as well as strand breaks in DNA upon exposure to menadione. Our results clearly suggest that TPP is an important antioxidant protein.

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