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OxyR Regulon Controls Lipid Peroxidation-mediated Oxidative Stress in *Escherichia coli*

Seon Joo Yoon, Ji Eun Park, Joon-Hyuck Yang and Jeen-Woo Park*

Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

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Membrane lipid peroxidation processes yield products that may react with DNA and proteins to cause oxidative modifications. The *oxyR* gene product regulates the expression of enzymes and proteins that are needed for cellular protection against oxidative stress. Upon exposure to *tert*-butylhydroperoxide (*t*-BOOH) and 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH), which induce lipid peroxidation in membranes, the *Escherichia coli oxyR* overexpression mutant was much more resistant to lipid peroxidation-mediated cellular damage, when compared to the *oxyR* deletion mutant in regard to growth kinetics, viability, and DNA damage. The deletion of the *oxyR* gene in *E. coli* also resulted in increased susceptibility of superoxide dismutase to lipid peroxidation-mediated inactivation. The results indicate that the peroxidation of lipid is probably one of the important intermediary events in free radical-induced cellular damage. Also, the *oxyR* regulon plays an important protective role in lipid peroxidation-mediated cellular damage.

Keywords: DNA damage, Lipid peroxidation, *OxyR* regulon, Superoxide dismutase

Introduction

Oxidative modification of cellular constituents (including lipids, proteins, and DNA) has been implicated in the etiology of different pathological conditions. These include diabetes, cataracts, pulmonary emphysema, arthritis, cancer, and aging (Halliwell, 1987). In biological membranes, lipid peroxidation is frequently a consequence of free radical attack. The peroxidation of unsaturated fatty acids of cells produces many reactive species, such as free radicals, hydroperoxides, and carbonyl compounds, which may cause damage to proteins and DNA (Cerutti, 1985). It has also been assumed that the decomposition of hydroperoxides that are mediated by catalytic transition metal ions may form much more toxic breakdown products. These include alkoxy radicals ($RO^{\cdot}$), peroxy radicals ($ROO^{\cdot}$), hydroxyl radicals ($^{\cdot}OH$), and reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Slater, 1984; Ueda *et al.*, 1985). It is possible that in complex biological systems, the oxygen free radicals and reactive aldehydes may indirectly cause protein and DNA damage by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation (Fleming *et al.*, 1982).

The presence of a global regulatory mechanism for the coordinate expression of enzymes and proteins that are needed for cellular protection against oxidative stress is well documented (Christman *et al.*, 1985; Morgan *et al.*, 1986; Storz *et al.*, 1987). The induction of the oxidative response regulon is under positive control by the *oxyR* gene product, whose expression or activity is oxidant-inducible (Christman *et al.*, 1985). The *Escherichia coli* mutant, *oxyR2*, carries a dominant mutation that confers resistance to a variety of oxidizing agents. It also causes the overexpression of several enzymes that are involved in defenses against oxidative damage. Deletions of *oxyR*, however, are recessive and highly sensitive to oxidant (Christman *et al.*, 1985).

In the present study, the role of *oxyR* in the cellular defense against lipid peroxidation-mediated damage was investigated using the *oxyR* overexpression mutant strain TA4110 and the *oxyR* deletion mutant strain TA4112. The two strains were expected to exhibit differences in regard to sensitivity to the toxic effects of lipid peroxidation. In order to determine whether or not such differences exist between the two types of cells, growth kinetics, viability, and oxidative damage to DNA were examined upon exposure to *tert*-butylhydroperoxide (*t*-BOOH) and 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). These induce lipid peroxidation in membranes (Fraga *et al.*, 1988; Kristal *et al.*, 1997). The results indicate that the peroxidation of lipid is probably one of the important intermediary events in free radical-induced cellular damage.

*To whom correspondence should be addressed.
Tel: 82-53-950-6352; Fax: 82-53-943-2762
E-mail: parkjw@knu.ac.kr

Also, the *oxyR* regulon plays an important role in the lipid peroxidation-mediated cellular damage.

Materials and Methods

Materials TBH, AAPH, pyrogallol, 2-thiobarbiturate, butylated hydroxytoluene (BHT), nuclease P1, *E. coli* alkaline phosphatase, RNase A, RNase T1, lysozyme, and pronase were obtained from the Sigma Chemical Co. (St. Louis, USA). Agarose and the Bio-Rad Protein Assay Kit were from Bio-Rad (Hercules, USA).

Bacterial strains and culture conditions The *E. coli* strains that harbored the *oxyR2* (TA4110, *oxyR*-constitutive) and *oxyΔ3* (TA4112, *oxyR* deletion) genes were a kind gift from Dr. B. N. Ames (University of California, Berkeley). A minimal salt medium that was supplemented with 0.5% glucose, 0.05% yeast extract, and 10 mg/ml each arginine and methionine was used. It is designated as a GY medium. The rich medium, TSY, contained 3% Trypticase soy broth plus 0.5% yeast extract. Overnight cultures, which were grown in the GY medium at 37°C on a rotary shaker at 200 rpm, were used to inoculate the fresh GY and TSY media. Once the optical density (600 nm) reached 0.3-0.4, exposure to *t*-BOOH or AAPH was started. Aliquots of the exposed cells were removed at time intervals and assayed for changes in absorbance at 600 nm. Viability was determined at various times by removing the samples and performing viable cell counts.

Lipid peroxidation Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidation. The samples were evaluated for malondialdehyde (MDA) production using a lipid peroxidation assay kit from Calbiochem (La Jolla, USA).

DNA isolation Chromosomal DNA was isolated from a bacterial pellet that was treated with lysozyme (2 mg/ml) and digestion in the presence of SDS, EDTA, and pronase. This was followed by phenol/chloroform/isoamyl alcohol extraction and differential precipitation by ethanol, as previously described (Park, 1991). Residual RNA was destroyed by incubation at 37°C 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. After extraction with chloroform/isoamyl alcohol (24 : 1, v/v), the DNA was precipitated with 0.1 vol of 3 M sodium acetate, pH 4.8, and 2 vol of cold ethanol.

Agarose gel electrophoresis DNA samples were applied to 0.5% agarose gels in a TAE buffer system. Electrophoresis was performed at 5 V/cm for 2 h at room temperature. Following electrophoresis, the gels were stained with ethidium bromide, irradiated from below with a UV transilluminator box, and photographed.

8-OH-dG levels DNA samples in 0.02 M sodium acetate, pH 4.8, were digested to nucleotides with 20 μg of nuclease P1 at 37°C for 30 min, then treated with 1.3 units of *E. coli* alkaline phosphatase in 0.1 M Tris-HCl, pH 7.4, at 37°C for 1 h in order to liberate the corresponding nucleosides. Levels of 8-OH-dG were 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. An electrochemical detector (applied potential, 600 mV) was used to detect 8-OH-dG. A vacuum-degassed 50 mM phosphate buffer that contained 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.

Superoxide dismutase activity assay The bacterial cells were collected at 10,000 × *g* for 10 min at 4°C. They were washed once with cold 50 mM potassium phosphate/0.1 mM EDTA at pH 7.8 before being resuspended in 1.0 ml of this buffer. The cell-free extracts were prepared by sonication. Cellular debris was removed by a 10-min centrifugation at 15,000 × *g* at 4°C. The supernatant was collected, and the protein levels were determined by the method of Bradford using reagents that were purchased from Bio-Rad. The total superoxide dismutase (SOD) activity in the cell extracts was assayed spectrophotometrically using the pyrogallol assay (Kim *et al.*, 2001), where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%.

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

Results

The growth kinetics of the TA4110 and TA4112 strains were compared following exposure to agent-induced lipid peroxidation. Under aerobic conditions, the growth of both of the mutant strains were inhibited by *t*-BOOH or AAPH; however, the growth inhibition was more pronounced in TA4112, when compared to that in TA4110 (Fig. 1). Growth inhibition was also accompanied by a loss of cell viability. To determine the rate of viability loss, early exponential phase cells were incubated in liquid culture in the presence of various concentrations of *t*-BOOH or AAPH, and subsequently plated in order to determine their colony forming ability. The exponential phase was chosen since cells can become resistant to various forms of stress when they enter the stationary phase. As shown in Fig. 2, the TA4110 cells were much more resistant to exposure of *t*-BOOH or AAPH than the TA4112 cells. The TA4110 cells showed a survival of 42% or 60% after exposure to 0.2 mM *t*-BOOH or 20 mM AAPH for 1 h, compared with the TA4112 mutant cells, which showed a survival of 16% or 24%, respectively. The *oxyR*-deleted strain was killed to a greater extent upon exposure to *t*-BOOH or AAPH, indicating that *oxyR* may be involved in protecting cells from lipid peroxidation-mediated cellular damage.

To evaluate the induction of lipid peroxidation in membranes upon exposure to *t*-BOOH or AAPH, the TBARS formation was measured. As shown in Fig. 3, lipid

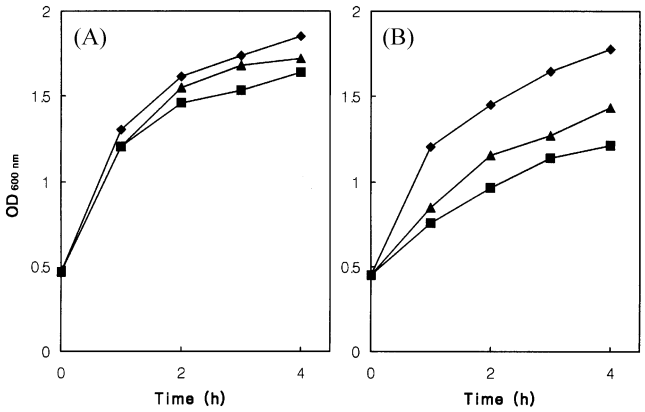

Fig. 1. Growth of TA4110 (A) and TA4112 (B) upon exposure to *t*-BOOH or AAPH. Exponentially growing bacteria were cultured at 37°C with shaking, and 0.2 mM *t*-BOOH or 20 mM AAPH was added. Cell growth was monitored by increasing the optical densities of the cultures. ◆, No addition; ▲, AAPH; ■, *t*-BOOH.

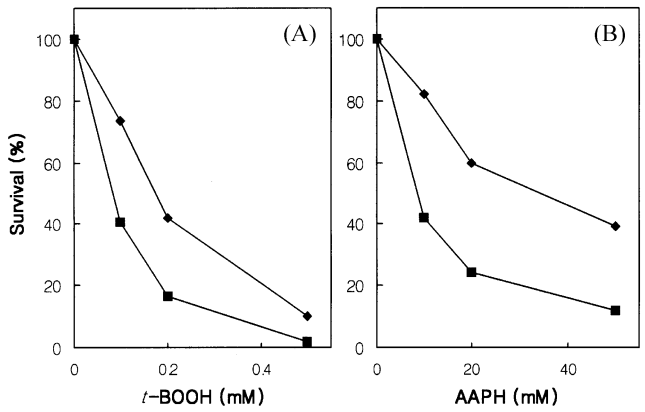

Fig. 2. Effect of *t*-BOOH (A) or AAPH (B) on the viability of *oxyR* cells. After the cells were exposed to various concentrations of *t*-BOOH or AAPH for 1 h, viability was determined, as described in the text. ◆, TA4110; ■, TA4112.

peroxidation was significantly increased in both strains of incubation with 4 mM *t*-BOOH or 50 mM AAPH for 1 h.

DNA, with its central role in the control of cell function, is of fundamental importance. Damage to DNA that is induced by free radicals appears to play an important role in mutagenesis and carcinogenesis. DNA lesions, resulting from the exposure to reactive oxygen species, include modified bases and strand breaks. We investigated the extent of lipid peroxidation-induced DNA breakage in relation to the cellular levels of *oxyR*. In order to estimate single-strand breaks, DNA from cells that were unexposed and exposed to *t*-BOOH was extracted and analyzed by electrophoresis on a 0.5% agarose gel. The significant increase in strand breaks that are represented by a smear was clearly observed in the TA4112 mutant cells that were treated with 4 mM *t*-BOOH for 1 h (Fig. 4A). DNA strand breaks that were induced by *t*-BOOH were reduced in the *oxyR*-expressing cells. The modified base

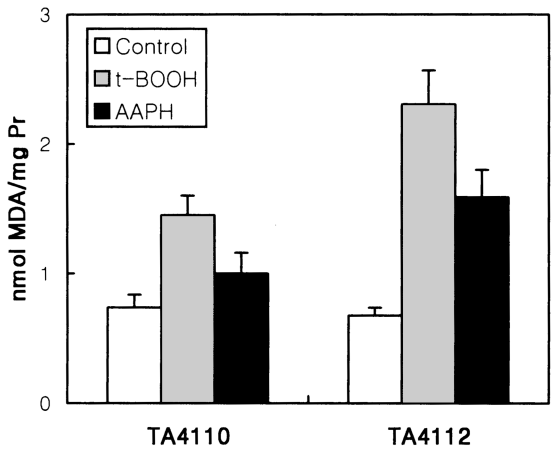

Fig. 3. Lipid peroxidation of *oxyR* strains after exposure to 4 mM *t*-BOOH or 50 mM AAPH for 1 h. Results are the means ± S.D. of three determinations.

8-OH-dG is considered to be one of the oxidative DNA products that are induced by oxygen radicals, which can be easily measured by HPLC and electrochemical detection. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* (Park and Floyd, 1994; Lee and Park, 1995). As shown in Fig. 4B, the endogenous levels of 8-OH-dG in DNA from TA4112 cells were similar to that in TA4110. However, the increase of 8-OH-dG was significantly higher ($P<0.01$) in the TA4112 cells than in TA4110 when the bacterial cells were exposed to either 4 mM *t*-BOOH or 50 mM AAPH for 1 h. The addition of 1 mM BHT, a lipid-soluble chain-breaking antioxidant, diminished the DNA damage that was induced by *t*-BOOH or AAPH to the control level.

Previously, we showed that lipid peroxidation products inactivate SOD, one of the key antioxidant enzymes (Lee and Park, 1995). The effect of lipid peroxidation on the activity of SOD was examined in *E. coli oxyR* strains. Fig. 5 demonstrates that SOD was inactivated in both strains upon exposure to 4 mM *t*-BOOH or 50 mM AAPH for 1 h. However, the inactivation of SOD was more pronounced in the *oxyR*-deletion mutant strain.

Discussion

The following has been proposed: lipid peroxidation is a continual process in living aerobic cells; it is maintained at a low level; and it can be prevented from entering into the autocatalytic phase by protective enzymes and antioxidants (Munkres, 1976). Chemical and physical agents that enhance membrane free radical reactions may accelerate this process beyond the capabilities of the protective systems, thereby causing widespread lipid peroxidation (Cerutti, 1985).

The DNA-damaging action of peroxidized lipids may contribute to disorders in genetic information transfer; therefore, peroxidized lipids act as etiological agents in

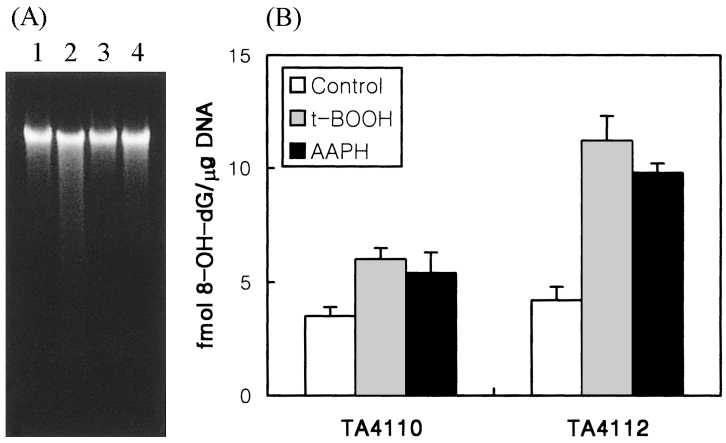

Fig. 4. (A) Effect of *t*-BOOH on DNA strand breaks in *oxyR* strains. Exponentially-growing cells were treated with 4 mM t-BOOH for 1 h. DNA was then extracted, analyzed by electrophoresis on a 0.5% agarose gel, and stained with ethidium bromide. Lane 1, TA4112; lane 2, TA4112 + *t*-BOOH; lane 3, TA4110; lane 4, TA4110 + *t*-BOOH. (B) Levels of 8-OH-dG in DNA from *oxyR* strains exposed to 50 mM AAPH or 4 mM *t*-BOOH for 1 h. Results are the means ± S.D. of three determinations.

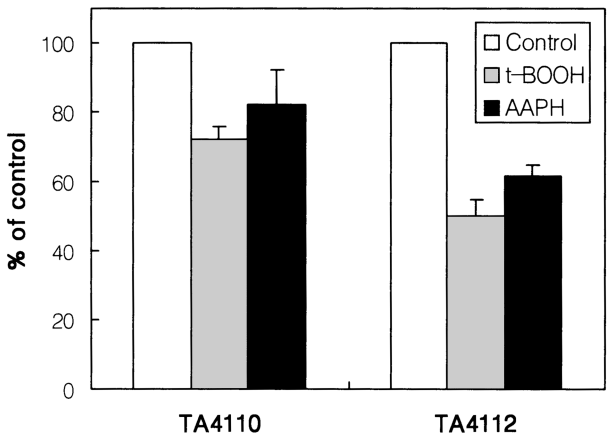

Fig. 5. SOD activity of *oxyR* strains exposed to 4 mM *t*-BOOH or 50 mM AAPH for 1 h. Activity of untreated cells is expressed as 100%. Results are the means ± S.D. of three determinations.

biological aging and tumorigenesis (Ueda *et al.*, 1985). While observation of lipid peroxidation-induced damage to DNA is limited and indirect, it has been proposed that lipid peroxidation is a major mode of membrane-mediated chromosomal damage (Cerutti, 1985). Several reports demonstrate that hydroperoxides can cause DNA strand breaks (Inouye, 1984; Hruszkewycz, 1988). Others have demonstrated that peroxidizing arachidonic acid causes structural changes in isolated DNA (Reiss and Tappel, 1973). We reported that lipid peroxidation products that are capable of reacting with DNA caused the formation of 8-OH-dG (Park and Floyd, 1992). These results indicate that lipid peroxidation represents an important intermediary step in the process of oxygen radical-induced genetic damage.

Despite their role in the cellular defense mechanism, the antioxidant enzymes are susceptible to inactivation by reactive oxygen species. Previous studies demonstrated that oxidative processes result in the loss of key antioxidant enzymes (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 the inactivation of antioxidant enzymes by an enhanced expression of SOD, catalase, and other antioxidant enzymes. The induction of antioxidant proteins in response to oxidative stress in *E. coli* and *Salmonella typhimurium*, as well as mammalian cells, is well known (Christman *et al.*, 1985; Greenberg *et al.*, 1990). Our results show that SOD activity was not induced, but that it decreased in both *oxyR* strains. Also, the inactivation of SOD was significantly more pronounced in the *oxyR*-deletion mutant. It is known that the SOD activity is not regulated by *oxyR* (Demple and Halbrook, 1983; Bowen and Hassan, 1988). However, the *oxyR* overexpressed mutant constitutively produces a very high level of catalase and, presumably, other antioxidant proteins. These results indicate that a higher level of some antioxidant enzymes may protect SOD from damage that is mediated by lipid peroxidation. It may eventually prevent cells from progressing to the pro-oxidant state. This protection may be a mechanism that increases survival and decreases DNA damage in the *oxyR* overexpression mutant.

In conclusion, results of this study indicate that a deficiency of *oxyR* increased the sensitivity of *E. coli* to the agent that induces lipid peroxidation, such as *t*-BOOH or AAPH. These results provide support for the role of *oxyR* as an important regulator to protect cells against lipid-peroxidation-mediated oxidative stress.

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References

Bowen, S. W. and Hassan, H. M. (1988) Induction of the

manganese-containing superoxide dismutase in *Escherichia coli* is independent of the oxidative stress (*oxyR*-controlled) regulon. *J. Biol. Chem.* **263**, 14808-14811.

Cerutti, P. A. (1985) Prooxidant states and tumor promotion. *Science* **227**, 375-381.

Christman, M. F., Morgan, R. W., Jacobson, F. S. and Ames, B. N. (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**, 753-762.

Demple, B. and Halbrook, J. (1983) Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* **304**, 466-468.

Fleming, J. E., Miguel, J., Cottrell, S. F., Yengoyan, L. S. and Economos, A. C. (1982) Is cell aging caused by respiration-dependent injury to the mitochondrial genome? *Gerontology* **28**, 44-53.

Fraga, C. G. and Tappel, A. L. (1988) Damage to DNA concurrent with lipid peroxidation in rat liver slices. *Biochem. J.* **252**, 893-896.

Greenberg, J. T., Monach, P., Chou, J. H., Josephy, P. D. and Demple, B. (1990) Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **87**, 6181-6185.

Halliwell, B. (1987) Oxidants and human disease: some new concepts. *FASEB J.* **1**, 358-364.

Hruszkewycz, A. M. (1988) Evidence for mitochondrial DNA damage by lipid peroxidation. *Biochem. Biophys. Res. Commun.* **153**, 191-197.

Inouye, S. (1984) Site-specific cleavage of double-strand DNA by hydroperoxide of linoleic acid. *FEBS Lett.* **172**, 231-232.

Kim, S. Y., Kim, R. H., Huh, T.-L. and Park, J.-W. (2001) α-Phenyl-*N*-*t*-butylnitrone protects oxidative damage to HepG2 cells. *J. Biochem. Mol. Biol.* **34**, 43-46.

Kono, Y. and Fridovich, I. (1982) Superoxide radical inhibits catalase. *J. Biol. Chem.* **257**, 5751-5754.

Kristal, B. S., Koopmans, S. J., Jackson, C. T., Ikeno, Y., Park, B. J. and Yu, B. P. (1997) Oxidant-mediated repression of mitochondrial transcription in diabetic rats. *Free Radic. Biol. Med.* **22**, 813-822.

Lee, M. H. and Park, J.-W. (1995) Lipid peroxidation products mediate damage of superoxide dismutase. *Biochem. Mol. Biol. Int.* **35**, 1093-1102.

Lee, S. M. and Park, J.-W. (1998) A yeast mutant lacking thiol-dependent protector protein is hypersensitive to menadione. *Biochim. Biophys. Acta* **1382**, 167-175.

Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G. and Ames, B. N. (1986) Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proc. Natl. Acad. Sci. USA* **83**, 8059-8063.

Munkres, K. D. (1976) Aging of *Neurospora crassa* IV. *Mech. Ageing Dev.* **5**, 171-191.

Park, J.-W., Cundy, K. C. and Ames, B. N. (1989) Detection of DNA adducts by high-performance lipid chromatography with electrochemical detection. *Carcinogenesis* **10**, 827-832.

Park, J.-W. (1991) A correlation between oxygen radical-induced mutations and DNA adducts formation in *Salmonella typhimurium*. *Korean Biochem. J.* **24**, 387-393.

Park, J.-W. and Floyd, R. A. (1992) Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Radic. Biol. Med.* **12**, 245-250.

Park, J.-W. and Floyd, R. A. (1994) Generation of strand breaks and formation of 8-hydroxy-2-deoxyguanosine in DNA by a thiol/Fe^{3+}/O_2-catalyzed oxidation system. *Arch. Biochem. Biophys.* **312**, 285-291.

Reiss, U. and Tappel, A. L. (1973) Fluorescent product formation and changes in structure of DNA reacted with peroxidizing arachidonic acid. *Lipids* **8**, 199-202.

Slater, T. F. (1984) Free radical mechanism in tissue injury. *Biochem. J.* **222**, 1-15.

Storz, G., Christman, M. F., Sies, H. and Ames, B. N. (1987) Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **84**, 8917-8921.

Tabatabaie, T. and Floyd, R. A. (1994) Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and the protective effect of spin trapping agent. *Arch. Biochem. Biophys.* **314**, 112-119.

Ueda, K., Kobayashi, S., Morita, J. and Komano, T. (1985) Site-specific DNA damage caused by lipid peroxidation products. *Biochim. Biophys. Acta* **824**, 341-348.