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Lipid Peroxidation Product-Mediated DNA Damage and Mutagenicity

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Abstract : Membrane lipid peroxidation processes yield products that may react with DNA to cause mutations. Lipid hydroperoxides from linoleic acid in the presence of transition metal ions caused strand breaks in plasmid DNA. DNA damage induced by reactive aldehydes known to be produced by decomposition of lipid hydroperoxides, such as 4-hydroxynonenal or malondialdehyde, was repaired by endonucleases and exonuclease III which resulted in the increase of single strand breaks in DNA. Lipid hydroperoxides as well as malondialdehyde and 4-hydroxynonenal also caused mutations in the pUC18 lacZ' gene when measured as a loss of α-complementation. In conclusion, the lipid peroxidation could be an important intermediary event in DNA damage and mutation by oxidative stress.
Key words : aldehydes, DNA damage, lipid peroxidation, mutagenicity

Oxidative modification of cellular constituents including lipids, proteins and DNA has been implicated in the etiology of different pathological conditions, such as diabetes, cataracts, pulmonary emphysema, arthritis, cancer, and in aging (Halliwell, 1987). In biological membranes lipid peroxidation is frequently a consequence of 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 mediated by catalytic transition metal ions may form much more toxic breakdown products such as alkoxy radicals (RO·), peroxyl radicals (ROO·), hydroxyl free radicals (·OH), and reactive aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Slater, 1984; Cerutti, 1985; Ueda *et al.*, 1996). It is possible that in complex biological systems, oxygen free radicals and reactive aldehydes may cause protein and DNA damage indirectly by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation (Fleming *et al.*, 1982).

Oxidative DNA damage is implicated in aging and age-related diseases such as cancer. While the ultimate mechanisms responsible for aging and aging-related diseases are obscure, there is growing evidence that oxygen radicals and lipid peroxidation are involved (Ames, 1983). In particular, lipid peroxidation is proposed to play a key role in membrane-mediated chromosomal damage (Cerutti, 1985). It has been shown that there is concurrent damage to not only lipids but also DNA during lipid peroxidation (Hruszkewycz, 1988; Fraga and Tappel, 1988). Several reports show that lipid hydroperoxides, including hydroperoxy-6,8,11,14-eicosatetraenoic acid and autoxidized methyl linolenate, caused DNA strand breaks, implicating the involvement of hydroxyl free radicals (Inouye, 1984; Ueda *et al.*, 1985; Weitberg and Corvese, 1989). A recent report demonstrates that autoxidized products of methyl linolenate and methyl arachidonate-enriched liposomes induce the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) (Park and Floyd, 1992), which is formed by hydroxyl radical or singlet oxygen attack at the C-8 position of the guanine base (Richter *et al.*, 1988; Park and Kim, 1994). It has also been shown that reactive aldehydes produced by the breakdown of lipid hydroperoxides mediate the formation of various DNA adducts such as pyrimido[1,2-α]purine-10(3*H*)-one (dGM_1) (Marnett *et al.*, 1986), N^6-oxopropenyl-2'-deoxyadenosine (dAM_1) (Chaudhary *et al.*, 1996), and $1,N^2$-ethenodeoxyguanosine (εdG) (Sodum and Chung, 1988) (Fig. 1). Therefore, a study of the relationship between lipid peroxidation and DNA damage is pertinent to understanding aging and carcinogenesis.

In this study, lipid peroxidation-mediated DNA dam-

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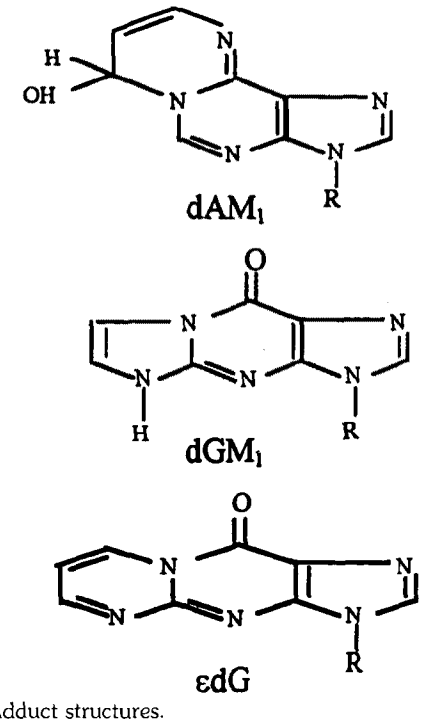

Fig. 1. Adduct structures.

age was investigated with purified lipid peroxidation products. DNA damage and mutation induced by lipid peroxidation products were examined by the induction of single strand breaks, susceptibilty of damaged DNA to repair enzymes and an *in vivo* mutagenicity assay. The results indicate that peroxidation of lipids may be an intermediary event in free radical-induced damage of DNA which presumably resulted in the mutation.

Materials and Methods

Materials

Chemicals and enzymes were obtained from the following sources: linoleic acid, diethylaminoethyl (DEAE)-cellulose, soybean lipoxygenase, 1,1,3,3-tetraethoxypropane (TEP), and isopropyl β-D-thiogalactopyranoside (IPTG) from Sigma Chemical Co. (St. Louis, USA); 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) from Promega (Madison, USA); 4-hydroxynonenal from Biomol (Plymouth Meeting, USA); and exonuclease III from Boehringer Mannheim (Mannheim, Germany).

Preparation of MDA

TEP (1 mmol) was dissolved in 90 ml of double-distilled water and hydrochloric acid (1 ml, 1 N) was added, and then the volume was brought to 100 ml with distilled water. A glass stopper was held firmly in place with parafilm to prevent loss of MDA while heating. After heating the flask in a water bath at 50°C for 60 min, it was cooled to room temperature and the appropriate stock solution was made. The exact concentration of the stock solution was confirmed by measuring its absorbance at 245 nm (ε=13,700 M^{-1} cm^{-1}) (Csallany *et al.*, 1984).

Preparation of lipid hydroperoxide

Freshly prepared ammonium salts of linoleic acid (13.5 μmol) were dispersed in 30 ml of 50 mM Tris-HCl buffer (pH 9.0) in a 250-ml Erlenmeyer flask equilibrated at 30°C. Oxygenation reactions were initiated by the addition of 0.65 ml of a freshly perpared solution containing 1 mg soybean lipoxygenase/ml in 50 mM Tris-HCl buffer, pH 9. After 2 min of incubation at 30°C a second addition of 0.65 ml of soybean lipoxygenase was made and the incubation continued at 30°C for an additional 8 min. Depletion of buffer oxygen in reactions containing excess lipoxygenase and unsaturated fatty acid was avoided by vigorous shaking of the incubation mixture. This procedure substituted for the need for oxygen-saturated buffer or continuous aeration of the reaction mixture during catalysis. Reactions were terminated by the addition of 7.5 ml of ethanol and cooled to 4°C on ice. Reaction mixtures were acidified (pH 3.0) and applied in 5-ml portions to 6-ml C_{18} reversed-phase sample preparation columns (J.T. Baker) which had been previously prewashed sequentially with 25 ml of HPLC-grade ethanol and 25 ml water. The columns were washed sequentially with 25 ml of 20% ethanol in water and 50 ml water to remove proteinaceous material and 10 ml hexane to remove trapped water. Fatty acid hydroperoxides were eluted with 10 ml of methyl formate. Methyl formate fractions were collected and stored at -70°C until used for further experiments (Graff *et al.*, 1990).

Preparation of crude endonucleases

Endonuclease-containing crude extracts from *E.coli* K-12, from which DNA had been removed by DEAE-cellulose chromatography, were prepared according to Riazzudin (1980). Before use, the extracts were diluted with BE buffer (20 mM Tris-HCl, pH 7.5/100 mM NaCl/15 mM EDTA) to a final protein concentration of 1.2 mg/ml.

DNA isolation

Plasmid DNA was prepared from bacteria harboring the plasmid pBluescript or pUC18 (Sambrook *et al.*, 1989). Bacteria were incubated overnight at 37°C with good aeration and shaking. Cells were decanted into a 1.5 ml microcentrifuge tube and collected by centrifugation at 12,000×g for 30 s at 4°C. After removing the supernatant, the pellet was suspended in 100 μl of ice-cold solution I (50 mM glucose/25 mM Tris-Cl, pH 8.0/10

mM EDTA) and 200 μl of freshly prepared solution II (0.2 N NaOH/1% SDS) was added. The contents were mixed by inverting the tube rapidly five times. To this mixture 150 μl of ice-cold solution III (60 ml 5 M potassium acetate/11.5 ml glacial acetic acid/28.5 ml H_2O) were added. After incubation on ice for 3-5 min, the mixture was centrifuged at 12,000×g for 5 min and then the supernatant was transferred to a fresh tube. After the extraction of this solution with an equal volume of phenol/chloroform, DNA was recovered by the addition of 2 volumes of ethanol (-20°C).

Agarose gel electrophoresis

In a typical experiment, 1 μg pBluescript plasmid DNA was incubated with MDA (2.5 mM), HNE (10 mM), or linoleate hydroperoxide (50 μM)/$FeCl_3$ (50 μM) in 5 mM phosphate buffer, pH 7.4. In most cases incubations were at 37°C for 2 h. The reactions were terminated by an addition of cold ethanol and DNA was collected by centrifugation. The treated DNA was incubated with 5 μl of crude endonuclease (1.2 mg/ml) in endonuclease buffer solution (40 mM Tris-HCl, pH 8.0/200 mM NaCl/2 mM EDTA), or exonuclease III (2000 units) in exonuclease buffer solution (40 mM Tris-HCl, pH 8.0/200 mM NaCl/30 mM $CaCl_2$) at 37°C for 2 h. After incubation, 1.5 μl of 10% SDS and 3.3 μl of 6X dye solution were added to each reaction mixture and the samples were subjected to agarose gel electrophoresis. Following electrophoresis, gels were stained with ethidium bromide, irradiated from below with a UV transilluminator box, and photographed. The relative fluorescence intensities of the open circular forms of plasmid were quantitated by measuring the area of a densitometer tracing (Park and Floyd, 1994).

Mutagenicity assay

Exponentially growing *E.coli* JM109 host cells (OD_{600} =0.4) were chilled and pelleted by centrifugation at 12,000×g for 10 min at 4°C. Transformation protocols were performed as described (Sambrook *et al.*, 1989) which are summarized in Fig. 2. The treated pUC18 DNA with same conditions as samples for agarose gel electrophoresis was incubated with 0.2 ml of competent cells at 0°C for 30 min and then the mixture was heat-shocked at 42°C. After 2 min, LB medium was added and incubated at 37°C for 1 h. Transfected cells were plated on LB agar plates containing 0.8 mg of X-gal and 0.8 mg of IPTG and 0.01% ampicillin. The plates were inverted and incubated for 24 h at 37°C before counting colonies.

Replicates

Each result described in this paper is representative of three separate experiments.

Results

We investigated lipid peroxidation product-mediated DNA damage by an increase in the susceptibility of damaged DNA for repair enzymes such as endonuclease and exonuclease III, which acts as an AP endonuclease. Plasmid DNA (pBluescript or pUC18) was treated with MDA or HNE at 37°C for 2 h; the damaged DNA was recovered by ethanol precipitation and redissolved in water. The treated plasmid DNA was incubated with crude endonuclease (1.2 mg/ml) or exonuclease III (2000 units). An increase of strand breaks in DNA which resulted in the repair activity of endonuclease and exonuclease III was measured by agarose gel electrophoresis. While exonuclease III is known to recognize sites of base loss (AP-sites) specifically (Rogers and Weiss, 1980), the repair enzyme-containing crude extracts from *E. coli* contain glycosylases and endonucleases recognizing a variety of base modifications (Epe

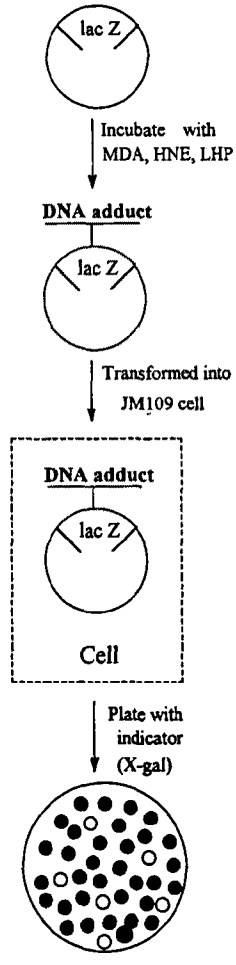

Fig. 2. *In vivo* assay for the mutagenicity of lipid peroxidation products.

et al., 1988). It has been shown that repair enzymes can be used as sensitive probes to analyze DNA damage. The assay uses supercoiled DNA as a target, in which both strand breaks and the incision of repair enzymes (endonuclease-sensitive sites) can be easily detected and quantitated (Epe *et al.*, 1989). When MDA-modified DNA was incubated with repair enzymes, single strand breaks reflected by the conversion of covalently closed circular double-stranded supercoiled DNA (ccc) to open (relaxed) circular double-stranded DNA (oc) were significantly increased (Fig. 3). In contrast, untreated DNA induced no significant increase in strand breaks with treatment of repair enzymes. However, there was only a small increase of single strand breaks in the HNE-treated DNA after incubation with endonuclease, as shown in Fig. 4. This result may indicate that MDA causes more extensive damage to DNA compared to HNE. As shown in Fig. 5, the extensive strand breaks of DNA were observed with linoleate hydroperoxide/Fe^{3+} even without treatment of repair enzymes, which may be caused by the direct attack of hydroxyl radicals generated from the breakdown of lipid hydroperoxide.

To examine the mutagenicity of lipid peroxidation products, pUC18 plasmid DNA was treated with MDA, HNE, or linoleate hydroperoxide/Fe^{3+}, and then damaged DNA was recovered. *E. coli* (JM109 competent cells) spheroplasts were transformed with treated DNA. *E. coli* expressing fully active β-galactosidase produce dark blue colonies on the indicator substrate X-gal, whereas mutation within the lacZ' α segment of pUC18 DNA resulted in the expression of much less active β-

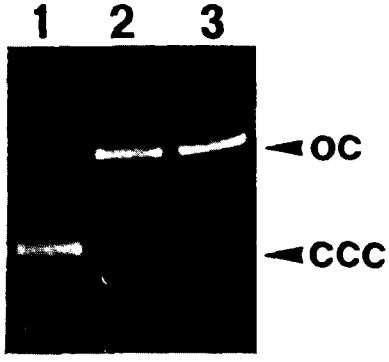

Fig. 3. Agarose gel electrophoresis of plasmid DNA treated with MDA. Reactions were carried out for 2 h at 37°C and then damaged DNA was further incubated with repair enzymes for 2 h at 37°C. Lanes correspond to reaction conditions as follows: 1, no repair enzymes; 2, + endonucleases; 3, + exonuclease III. ccc, covalently closed circular DNA; oc, open circular DNA.

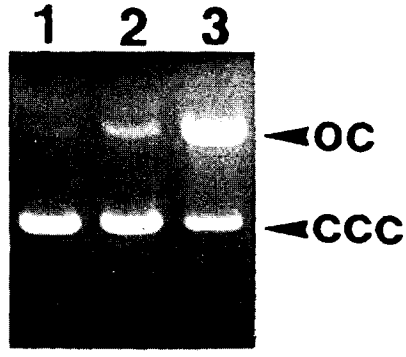

Fig. 5. Cleavage of supercoiled pBluescript plasmid DNA in the presence of linoleate hydroperoxide/Fe^{3+}. Lanes correspond to reaction conditions as follows: 1, DNA; 2, DNA + Fe^{3+}; 3, DNA+linoleate hydroperoxide/Fe^{3+}

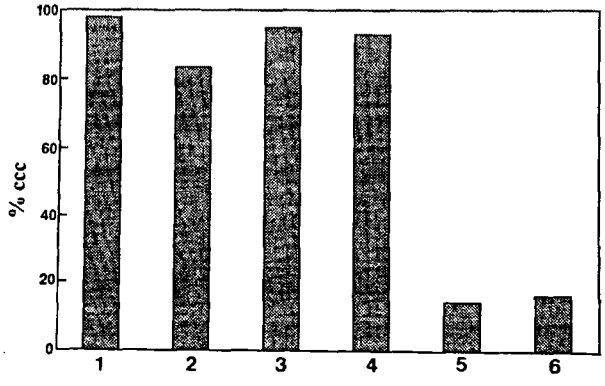

Fig. 4. Repair of DNA damage induced by HNE and MDA. The damaged DNA was treated with repair enzymes and was analyzed by agarose gel electrophoresis and the amount of uncleaved ccc DNA was measured. 1, HNE-treated DNA; 2, HNE-treated DNA + endonucleases; 3, HNE-treated DNA + exonuclease III; 4, MDA-treated DNA; 5, MDA-treated DNA + endonucleases; 6, MDA-treated DNA + exonuclease III. The amount of ccc DNA of untreated DNA was used as 100%.

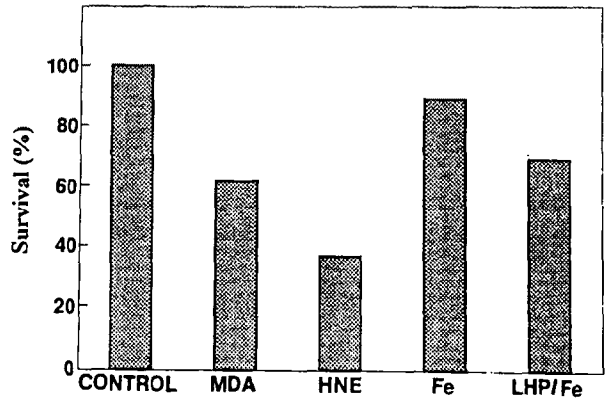

Fig. 6. Transforming ability of lipid peroxidation product-treated plasmid DNA in *E. coli* JM109. Damaged DNA as described in the Material and Methods was transfected into competent bacteria. Transformants were selected on media containing ampicillin and the colonies were scored after overnight incubation at 37°C. The relative survival was determined by the ratio between the number of colonies obtained with treated and untreated DNA.

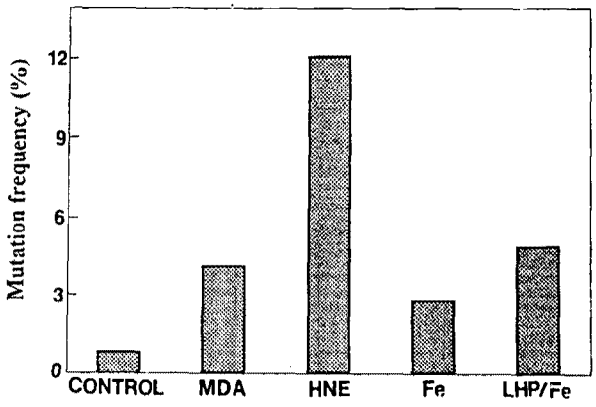

Fig. 7. Mutagenicity of lipid peroxidation products-treated plasmid DNA in *E. coli* JM109. Transfection was carried out as described in Fig. 6. Mutagenic frequency was expressed as the ratio between the mutant colonies and total colonies.

galactosidase and yields light blue or white colonies. The loss in viability (survival of colony-forming ability) of pUC18 plasmid DNA upon incubation with lipid peroxidation products was in a range of 40-60% compared to untreated DNA (Fig. 6). The frequency of mutation induced by lipid peroxidation products was 4.5-12%, as shown in Fig. 7. The results indicate that both linoleate hydroperoxide/Fe^{3+} and reactive aldehydes form mutagenic lesions in DNA. Although MDA-treated DNA formed a significantly higher level of lesions susceptible to repair enzymes, a much lower mutation frequency was observed compared to HNE-treated DNA. This implies that HNE creates highly mutagenic lesions in DNA. Fe^{3+} itself induced mutagenic lesions in DNA, which is consistent with previous findings with iron-treated ϕX174 *am3* (amber 3 mutation) DNA (Loeb *et al.*, 1988). Addition of linoleate hydroperoxide moderately increased mutagenic frequency (Fig. 7).

Discussion

It has been proposed that lipid peroxidation is a continual process in living aerobic cells, is maintained at a low level, and 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 and thus cause widespread lipid peroxidation (Lee and Park, 1995).

The DNA-damaging action of peroxidized lipids may contribute to disorders in genetic information transfer; thus, peroxidized lipids act as etiological agents in biological aging and tumorigenesis (Ueda *et al.*, 1985). Consequently, the study of DNA damage under oxidative stress and its relationship to lipid peroxidation is relevant. 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). A major problem in elucidating the mechanisms behind the proposed genotoxicity of the lipid peroxidation process is the discrimination between DNA damage caused by the direct action of free radicals and their role in the initiation of lipid peroxidation and the damage caused by the reaction of DNA with the final products of the lipid peroxidation process (Vaca *et al.*, 1988). Several reports demonstrate that hydroperoxides can cause DNA strand breaks (Inouye, 1984; Ueda *et al.*, 1985 Hruszkewycz, 1988). One report shows that lipid peroxide interaction with guanine nucleotide causes double-strand DNA breaks specifically (Ueda *et al.*, 1985), but the mechanism is not known and the DNA adducts have not been identified. Others have demonstrated that peroxidizing arachidonic acid causes structural changes in isolated DNA (Reiss and Tappel, 1973).

Aldehyde compounds such as MDA and HNE, together with similar compounds generated in lower amount such as 4-hydroxynonenal, are associated with lipid peroxidation (Esterbauer *et al.*, 1991). MDA undergoes reactions with DNA and a decrease in template activity of liver DNA from rats fed with MDA was observed (Klamerth and Levinsky, 1969). It has been also shown that MDA is mutagenic in certain strains of *Salmonella typhimurium* (Basu and Marnett, 1983) and murine L 5178 Y lymphoma cells (Yau, 1979). HNE has a conjugated aldehyde structure, which enables it to react readily with DNA, and is 4000 times more efficient than MDA in inducing an SOS response in *S. typhimurium* (Benamira and Marnett, 1992). Consequently HNE, together with MDA and other aldehydes, have been proposed to be partly responsible for the mutagenic properties of lipid peroxidation (Esterbauer *et al.*, 1990).

The reaction of secondary free radicals and reactive aldehydes, which are produced from the breakdown of unstable lipid hydroperoxide, with DNA is likely to be important in the overall action of these toxic species. We exposed purified plasmid DNA to lipid peroxidation products *in vitro* and measured alterations of biological activity. Present results clearly indicate that lipid peroxidation products are highly mutagenic. Therefore, lipid peroxidation may represent an important intermediary step in the process of oxygen radical-induced genetic damage.

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