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EVIDENCE FOR MITOCHONDRIAL DNA DAMAGE BY LIPID PEROXIDATION

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When mitochondria of rat liver were incubated in an *in vitro* system containing NADPH and ferrous chloride, marked lipid peroxidation occurred, as evidenced by the evolution of malonic dialdehyde. DNA isolated from these peroxidized mitochondrial preparations had completely different electrophoretic mobility than DNA isolated from mitochondria protected from peroxidation. Scavengers of superoxide anion, hydrogen peroxide and hydroxyl radicals offered no protection against either lipid peroxidation or DNA damage. However, alpha-tocopherol protected against both lipid peroxidation and damage to the mitochondrial genome. These results support the hypothesis that lipid peroxidation can mediate DNA damage.

Oxidative DNA damage is implicated in aging and age-related diseases such as cancer(1). While the ultimate mechanisms are obscure, there is growing evidence that oxygen radicals and lipid peroxidation are involved (2). Oxygen radical species, such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (HO·), are produced by ionizing radiation and during the metabolism of xenobiotics(3,4). Oxygen radicals are also generated *in vivo* as byproducts of normal oxidative metabolism(5,6). Being highly reactive, these free radicals can attack DNA directly(7,8). Alternatively, it has been proposed that in complex biological systems oxygen radicals can cause DNA damage indirectly by initiating lipid peroxidation(1,9), since polyunsaturated side chains of membrane lipids are especially susceptible to free radical initiated oxidation. Peroxidation of lipids in biological membranes is a highly destructive phenomenon which propagates free radicals and leads to the release of a large variety of relatively stable breakdown products, some of which are known to be toxic(10,11). A few of the known products of lipid peroxidation have been shown to cause DNA damage in model systems and prokaryotes(11-18). Although data revealing DNA damage to eukaryotic genes by lipid peroxidation is limited and indirect(2,19,20), lipid peroxidation is thought to play a key role in "membrane-mediated

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chromosomal damage"(21). Thus lipid peroxidation may be a pathogenic mechanism linking high energy radiation, xenobiotic metabolism, and physiologic oxidative stress to DNA damage. This study presents direct evidence that lipid peroxidation affects DNA of eukaryotic cells.

Mitochondria, rather than nuclei, were studied because there are indications that mitochondrial DNA is more sensitive to oxidative stress. The mitochondrial genome is situated near the oxygen radical generating system of the mitochondrial electron transport chain and the easily peroxidizable lipids of the inner membrane. In addition, mitochondrial DNA is not protected by histones(22) or by adequate mechanisms to repair strand breaks (23), as is the case for the nuclear genome. Mitochondrial DNA is a discrete circular molecule of single molecular weight which facilitates detection of small degrees of DNA damage.

Mitochondrial DNA damage by lipid peroxidation has been suggested as a mechanism of inducing the age-related decline in mitochondrial function, since mitochondrial DNA encodes key enzymes of the respiratory chain(9). Also, many carcinogenic agents have been shown to preferentially attack mitochondrial DNA(24-26), and there is growing evidence that mutation of the mitochondrial genome may be important in the mechanism of carcinogenesis(27). Therefore, a study of the relationship between lipid peroxidation and mitochondrial DNA damage is pertinent to our understanding of aging and the carcinogenic potency of the prooxidant state(28).

MATERIALS AND METHODS

Basic Experimental Design: Rat liver mitochondria were incubated in an in vitro system in which lipid peroxidation was either stimulated or prevented. DNA was then extracted from the mitochondrial suspensions and its integrity assessed by agarose gell electrophoresis. Lipid peroxidation was monitored by determining the evolution of malonic dialdehyde (MDA).

Preparation of Liver Mitochondrial Fraction: Mitochondria were isolated from livers of male rats of the Sprague-Dawley strain (Hilltop Laboratories, Inc.). The rats had been maintained on standard Purina Laboratory Chow and weighed between 300 and 450 gm. The animals were sacrificed by decapitation, and their livers were removed and chilled in an ice-cold solution containing 0.25 M sucrose and 5 mM Hepes (pH 7.4). Livers were then homogenized in 9 volumes of this solution using a glass Potter-Elvehjem homogenizer equiped with a Teflon pestle. The homogenate was centrifuged at 600 x g for 12 minutes in a Sorval SS-34 rotor at 2-4°C. The supernatant fraction was then centrifuged at 9,000 x g for 12 minutes. The "fluffy layer" was removed by aspiration and the remaining pellet, which contained sedemented mitochondria, was washed three times with 0.15 M Tris-HCl (pH 7.4) and finally resuspended in the same buffer.

Incubation Procedures: All reaction mixtures contained mitochondrial suspensions at a concentration of 1.6 mg of protein per ml in media with 150 mM Tris-HCl (pH 7.4) and 1.0 mM KH_2PO_4. Additions of 0.1 mM $FeCl_2$ and 0.4 mM NADPH were made as indicated in Table 1. The final volume of each reaction mixture was 40 ml. Anaerobic controls were prepared by flushing

the reaction mixtures, contained within 125 ml flasks, with 10 liters of oxygen-free nitrogen. Mitochondrial suspensions treated with superoxide dismutase, catalase, mannitol, or alpha-tocopherol were incubated with these agents for five minutes prior to addition of NADPH and $FeCl_2$, as described by I. Tong Mak et al.(29). Superoxide dismutase (specific activity, 5500 units/mg) and catalase (specific activity, 11,000 units/mg) were prepared from bovine liver. Control and experimental test systems were incubated and shaken at 37°C for 20 minutes. Immediately after incubation, all test systems were cooled to 4°C, made 10 mM with respect to EDTA, and aliquots (1.2 ml) were removed for MDA determination. Malonic dialdehyde was assayed according to Ghoshal and Recknagel(30). Mitochondrial protein content was measured by the method of Lowry et al.(31).

Preparation of Mitochondrial DNA: Mitochondrial DNA was isolated by a modification of the method of Palva and Palva(32). After the incubation procedure, mitochondria were concentrated by centrifuging each reaction mixture (15,000 x g x 15 min). The resulting pellets were resuspended to a final volume of 1.5 ml in ice cold buffer containing 50 mM of glucose, 1.5 mM EDTA, and 25 mM Tris-HCl (pH 7.4). To lyse mitochondria and denature any contaminating nuclear DNA, 4.0 ml of alkaline SDS solution was mixed into each suspension. Two ml of 3.0 M potassium acetate was then added and the resultant precipitate removed by centrifugation (10,000 x g x 10 min). The supernatant was then mixed with an equal volume of isopropanol and kept at -20°C for 12 hours, after which the DNA was collected by centrifugation (12,000 x g x 30 min) and dried. Each DNA sample was resuspended in 1.5 ml of a solution containing Tris-HCl (pH 7.8), 5.0 mM EDTA, and 0.5% SDS, and treated with 6 units of Proteinase K for 30 minutes at 37°C. The solutions were then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The aqueous phase was then mixed with three volumes of 100% ethanol. The reprecipitated DNA was collected by centrifugation and drying. The DNA was then resuspended in 50 ul of water. Quantitative estimation of DNA isolation was performed by spectrophotometric analysis. Restriction endonuclease analyses were performed with EcoR I endonuclease obtained from Bethesda Research Laboratories. DNA electrophoresis analysis was performed with 1.0% agarose slab gels, followed by staining with ethidium bromide.

RESULTS

Table 1 shows that the test system provided conditions for assessing the relationship between mitochondrial lipid peroxidation and mitochondrial DNA damage. Aerobic incubation of mitochondria with the addition of NADPH and $FeCl_2$ led to marked lipid peroxidation as evidenced by the evolution of MDA. Peroxidation was markedly diminished in the absence of either NADPH or ferrous chloride. The degree of peroxidation was not affected by the addition of superoxide dismutase, catalase, or mannitol. Addition of alpha-tocopherol protected mitochondria from peroxidation. Lipid peroxidation was also prevented by anaerobic conditions.

Agarose gel electrophoresis of the DNA samples extracted from the controls (Fig. 1A, lane 3) showed the typical banding pattern of undigested mitochondrial DNA. The exact nature of the DNA was determined by electrophoresis of the EcoR I restriction endonuclease digest (Fig. 1A, lane 2), which shows a banding pattern characteristic of mitochondrial DNA from Sprague Dawley rats(33). DNA isolated from mitochondria which had under-

Table 1. Malonic dialdehyde production in mitochondrial suspensions

Additions	Malonic dialdehyde formed (nmoles/mg protein)	
none	0.44 ± 0.27[a]	(6)[b]
NADPH	4.64 ± 4.74	(4)
$FeCl_2$	4.02 ± 0.62	(3)
$FeCl_2$ + NADPH	16.37 ± 3.69	(6)
$FeCl_2$ + NADPH + Superoxide dismutase (50ug/ml)	16.67 ± 3.16	(3)
$FeCl_2$ + NADPH + Catalase (50ug/ml)	17.84 ± 4.19	(3)
$FeCl_2$ + NADPH + Mannitol (10mM)	15.13 ± 2.38	(3)
$FeCl_2$ + NADPH + Alpha-tocopherol (10mM)	0.90 ± 0.55	(3)
$FeCl_2$ + NADPH (anaerobic)	0.53 ± 0.20	(4)

[a] The data are expressed as mean ± SD.
[b] The number of experiments is indicated in parentheses. At least four determinations were made in each experiment.

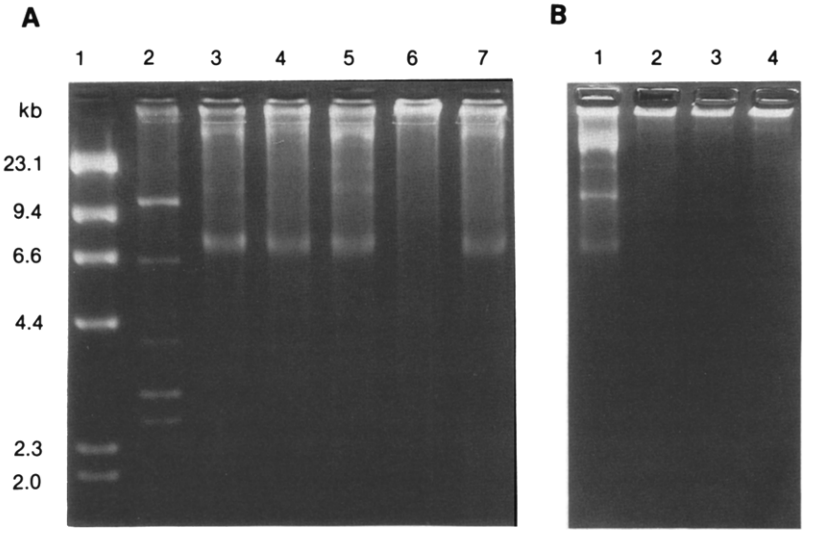

Figure 1. Panel A: Electrophoretic analysis of DNA extracted from incubated mitochondria. (Lane 1) molecular size markers produced by digesting Lambda phage DNA with Hind III endonuclease; (lane 2) EcoR I digest of DNA extracted from untreated mitochondrial preparation; (lane 3) undigested DNA from untreated mitochondrial preparation; (lane 4) undigested DNA incubated aerobically with NADPH alone; (lane 5) undigested DNA incubated aerobically with $FeCl_2$ alone; (lane 6) undigested DNA incubated aerobically with $FeCl_2$ and NADPH; (lane7) undigested DNA incubated anaerobically with $FeCl_2$ and NADPH.
Panel B: Electrophoresis patterns of DNA extracted from mitochondrial suspensions preincubated with selected antioxidants before aerobic incubation with NADPH and $FeCl_2$. (Lane 1) alpha-tocopherol; (lane 2) catalase; (lane 3) superoxide dismutase; (lane 4) mannitol.
The results are typical of those obtained in three to six separate experiments.

gone no or little lipid peroxidation showed similar prominent DNA bands (Fig. 1A, lanes 3,4,5, and 7; Fig. 1B, lane 1). However, whenever there was marked evolution of MDA, there was obliteration of the banding pattern of intact mitochondrial DNA (Fig. 1A, lane 6; Fig. 1B, lanes 2,3, and 4). Densitometric analysis of agarose gels showed that a major portion of the nucleic acid from peroxidized mitochondria was unable to migrate into the agarose gel, remaining at the wall of the well, suggesting the occurrence of DNA crosslinking.

DISCUSSION

These experiments show a strong association between mitochondrial lipid peroxidation and damage to mitochondrial DNA. The observation that there was simultaneous protection against lipid peroxidation (Table 1) and DNA damage (Fig. 1B, lane 1) by alpha-tocopherol, an antioxidant that inactivates lipid radicals, supports the view that the peroxidative decomposition of membrane lipids plays an important role in causing the genetic damage. Superoxide dismutase, catalase, and mannitol, scavengers of $O_2^{\cdot-}$, H_2O_2, and $HO\cdot$ offered little protection against either lipid peroxidation (Table 1) or DNA damage (Fig. 1B, lanes 2, 3, and 4). Therefore, a direct role of active oxygen species in the causation of genotoxicity could not be demonstrated with this system. Since it is possible that the mitochondrial locus where DNA damage occurred was not accessible to these scavengers, the same experiment was performed with dimethyl sulfoxide, a hydroxyl radical scavenger which can easily penetrate membranes. The results with dimethyl sulfoxide were indistinguishable from those obtained with mannitol, showing no attenuation of either lipid peroxidation or DNA damage.

These experiments support the hypothesis that peroxidation of membrane lipids is an important intermediary event in free radical induced DNA damage in living systems. Although these findings do not offer a precise mechanism by which lipid peroxidation affects DNA, one obvious possibility is an attack on DNA by a lipid radical, such as an alkoxyl radical ($LO\cdot$). The effect of such a free radical reaction could be similar to the one postulated for radiation in which there is an attack on DNA by hydroxyl radicals(8). Schaich and Borg have reported lipid radical interactions with DNA(34). Another possible mechanism is an attack on DNA by end products of lipid peroxidation. For example, malonic dialdehyde has been shown to cause DNA crosslinking in model systems(16). However, in view of the large array of potentially genotoxic compounds produced by lipid peroxidation, it is not possible at this time to assert which of these products can penetrate the elaborate cellular antioxidant defenses and play an important role in pathophysiologic mechanisms of genetic transformation. Further

study of DNA change in mitochondria undergoing oxidative stress may provide this information.

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