Damage to DNA concurrent with lipid peroxidation in rat liver slices

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Lipid peroxidation and DNA damage were evaluated in liver slices incubated for 2 h at 37 °C with 1 mM-t-butyl hydroperoxide (t-BOOH), 1 mM-BrCCl₃ or 50 µM-ferrous iron. t-BOOH induced the greatest amount of damage to DNA and increased the production of thiobarbituric acid-reactive substances (TBARS). Both phenomena depended on the incubation time. Ferrous iron induced both DNA damage and TBARS production, and BrCCl₃ did not induce significant DNA damage and was the weakest TBARS inducer. Butylated hydroxytoluene at 1 mM inhibited both DNA damage and TBARS production. DNA damage and lipid peroxidation in liver slices were correlated, indicating that these events were concurrent.

INTRODUCTION

Oxygen radicals are continuously formed in all living organisms, with deleterious effects that lead to cell injury and death (Tappel, 1973; Chance *et al.*, 1979). Production *in vivo* of oxidative species, mainly products of partial oxygen reduction, occurs under physiological conditions at a controlled rate, but it is dramatically increased in conditions of oxidative stress. Exposure of biological systems to xenobiotics, pollutants, ionizing radiation or u.v. light and certain pathological conditions lead to oxidative stress and, consequently, to increased production of oxyl radicals (Sies, 1985).

Lipid peroxidation seems to be a common consequence of free-radical-mediated reactions that occur *in vivo* in aerobic organisms (Tappel, 1973; Sevanian & Hochstein, 1985). Direct reaction of oxyl radicals with nucleic acids *in vitro* has been extensively reported (Gutteridge & Fu, 1981; Schulte-Frohlinde & Von Sonntag, 1985). Its occurrence *in vivo* may cause DNA modifications that are potentially mutagenic or carcinogenic (Ames *et al.*, 1982). Oxidative damage to DNA in cell culture was recently reported (Meneghini & Hoffmann, 1980; Mello-Filho *et al.*, 1984). Non-radical products of lipid peroxidation also have been reported to induce DNA damage (Summerfield & Tappel, 1984; Frankel *et al.*, 1987). Consequently, under conditions of oxidative stress, DNA damage can occur in parallel with, or can be an effect of, lipid peroxidation.

Study of DNA modifications by oxidative reactions *in vivo* is difficult, given the rapid metabolism of nucleic acid oxidation products, the high activity of repair systems, the delayed expression of such modifications and the fact that nicking in DNA molecules produced during isolation is similar to that produced by oxidative reactions. Tissue slices are a model system in which peroxidative damage can be studied under conditions that are close to physiological (Sano *et al.*, 1986; Fraga *et al.*, 1987, 1988), and DNA damage can be detected with introduction of minimal 'artifactual' damage.

In the present study, DNA damage and lipid peroxidation were observed after exposure of liver slices to compounds known to induce oxidative stress, and there was a close relationship between these two events.

MATERIALS AND METHODS

Animals and tissue slices

Male Sprague-Dawley rats weighing 220–250 g were provided with a standard diet and water *ad libitum*. The rats were decapitated and immediately the livers were dissected, rinsed, then immersed in ice-cold Krebs–Ringer phosphate buffer, pH 7.4 (De Luca, 1972). Liver slices were prepared as previously described (Fraga *et al.*, 1987).

Oxidative reactions

Liver slices were placed in glass serum bottles containing 5 ml of O₂-equilibrated Krebs–Ringer phosphate buffer/10 mM-glucose at 4 °C. BrCCl₃ was prepared as a 10% (v/v) solution in dimethyl sulphoxide (DMSO), and t-BOOH and FeSO₄ were dissolved in Krebs–Ringer phosphate buffer. BHT was prepared as a 4% (w/v) solution in ethanol. These oxidants were added to the serum bottles immediately before incubation. Data for controls incubated in the presence of 5 µl of DMSO or 30 µl of ethanol were not different from data for controls without these additions. t-BOOH, BrCCl₃ and BHT were added at 1 mM final concentration, and FeSO₄ at 50 µM. These concentrations were chosen on the basis of a previous study (Fraga *et al.*, 1988), in order to obtain high level peroxidation while at the same time preventing oxidant-mediated cell death. After incubation at 37 °C with continuous shaking (120 cycles/min), the slices were removed from the incubation medium for DNA isolation and analysis of TBARS. After centrifugation at 1700 g for 10 min at room temperature, the incubation medium was analysed for TBARS.

Abbreviations used: BHT, butylated hydroxytoluene; t-BOOH, t-butyl hydroperoxide; EBF, ethidium bromide fluorescence; TBA(RS), 2-thiobarbituric acid (-reactive substances); BrCCl₃, bromotrichloromethane; DMSO, dimethyl sulphoxide.

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Table 1. Effect of t-BOOH, BrCCl₃ and ferrous iron on DNA damage and TBARS production by liver slices

Liver slices were incubated for 2 h at 37 °C. Treatments were initiated immediately before incubation. BHT was added at 1 mM. DNA damage was calculated as indicated in the Results section. TBARS evaluated in the incubation medium are mean results $\pm$ S.E.M.; *n* is shown in parentheses. Significance: **P* < 0.01; ^anot significant; ^c*P* < 0.05, all as compared with control.

Treatment	DNA damage (%)		TBARS (nmol/g of liver)	
	– BHT	+ BHT	– BHT	+ BHT
None (control)	0 (9)	0 (3)	23 $\pm$ 1 (9)	25 $\pm$ 1 (3)
+ 1 mM-t-BOOH	35 ^a (8)	13 (3)	118 $\pm$ 4 ^a (7)	41 $\pm$ 3 ^a (3)
+ 1 mM-BrCCl ₃	24 ^b (5)	13 (3)	96 $\pm$ 7 ^a (5)	55 $\pm$ 3 ^a (3)
+ 50 μ M-Fe ²⁺	30 ^c (5)	0 (2)	141 $\pm$ 19 ^a (5)	27 $\pm$ 2 ^b (3)

dependent upon the amount of DNA. Factors other than the amount of DNA also influenced the enhancement of EBF; for equivalent amounts of A_{260} , smaller increases in EBF were observed after DNA renaturation than before denaturation. The enhancement of EBF by renatured DNA isolated from slices incubated in the presence of 1 mM-t-BOOH was smaller than that for DNA from control liver slices incubated without oxidant. When DNA was assayed before denaturation–renaturation, the increase in EBF was not different from that for DNA isolated from control or t-BOOH-incubated slices (Fig. 1). This indicates that oxidative damage induced by t-BOOH did not modify the DNA molecule sufficiently to affect the amount of ethidium bromide that intercalated the DNA before heat denaturation. The modifications in DNA introduced by oxidative damage did not permit DNA to return to its original configuration after denaturation, thus the EBF was diminished. Fig. 2 shows that the damage induced by t-BOOH was dependent on incubation time. When liver slices were incubated with 1 mM-BrCCl₃ or 50 μ M-ferrous iron, the isolated DNA assayed before and after renaturation enhanced EBF qualitatively the same as did DNA from slices incubated with t-BOOH. On the basis of data presented in Fig. 1, DNA damage induced by incubation of liver slices with oxidants was calculated by using the following equation:

$$\text{DNA damage (\%)} = 100 \times \frac{\left(\frac{\text{EBF after denaturation}}{\text{EBF before denaturation}} \right)_{\text{oxidized}}}{\left(\frac{\text{EBF after denaturation}}{\text{EBF before denaturation}} \right)_{\text{control}}}$$

The EBF value used to calculate oxidative DNA damage was expressed per unit of A_{260} . The absorbance spectra of DNA between 200 and 400 nm before denaturation and after renaturation were not significantly different. The denaturation–renaturation process produced only minor alterations in the A_{260} value ($\pm 10\%$), and the A_{260}/A_{280} ratio was always over 1.8. Both results indicated DNA purity and that no additional damage was caused by heating (Maniatis *et al.*, 1982).

Table 1 shows the damage to DNA induced by incubation of liver slices with the oxidants. Oxidative damage was significant in the DNA isolated from slices incubated with 1 mM-t-BOOH and 50 μ M-ferrous iron. Incubation with 1 mM-BrCCl₃ did not induce significant damage. Addition of 1 mM-BHT diminished the damage

induced by iron, t-BOOH and BrCCl₃ to the extents of 100, 61 and 44 % respectively. Addition of BHT did not modify the DNA isolated from control liver slices incubated in absence of inducer.

Lipid peroxidation

Estimation of the amount of TBARS released into the incubation medium by liver slices is an index of lipid peroxidation and, consequently, of oxidative reactions occurring in the slices (Sano *et al.*, 1986; Fraga *et al.*, 1987, 1988). The TBARS released into the incubation medium from liver slices was increased by 1 mM-t-BOOH, 1 mM-BrCCl₃ or 50 μ M-ferrous iron (Table 1). The values agree with those previously reported (Fraga *et al.*, 1988). Co-incubation of liver slices with 1 mM-BHT and ferrous iron, t-BOOH or BrCCl₃ decreased TBARS release to the extents of 100, 85 and 60 % respectively, over the release from slices incubated without BHT (Table 1). The release of TBARS into the incubation medium was not accompanied by accumulation of TBARS in the slices. TBARS in the slices not only rapidly equilibrate with the incubation medium but are actively metabolized by hepatic aldehyde dehydrogenase (Sano *et al.*, 1986). TBARS in homogenates prepared from the slices incubated with t-BOOH and BrCCl₃ were increased slightly (14 %), but significantly (*P* < 0.05), over the control value (88 $\pm$ 3 nmol/g of liver). TBARS in slices incubated with ferrous iron were not increased. By analysing the amount of TBARS in the chromatin fraction, it is possible to estimate the amount of TBARS bound to the DNA and/or nucleoproteins. The amount of TBARS in the chromatin fraction isolated from the liver slices incubated with BrCCl₃ was increased 26 % (*P* < 0.01) over the control values (3.4 $\pm$ 0.1 nmol/g of liver). Ferrous iron and t-BOOH did not increase the amount of TBARS in the chromatin fraction.

DISCUSSION

Lipid peroxidation is a key process occurring in aerobic tissues exposed to oxidative conditions (Tappel, 1973). Oxidative damage to DNA has been postulated to have biological importance in mammals (Ames, 1983). Consequently, the study of DNA damage under oxidative stress and its relationship to lipid peroxidation is relevant.

Results presented here show that DNA modifications occurred concurrently with lipid peroxidation. Each value obtained for DNA damage and TBARS released into the incubation medium under all the conditions used

were linearly correlated ($r = -0.60$, $P < 0.01$). Considering that the time course for DNA damage and TBARS production were highly correlated and that BHT diminished both events with comparable antioxidant potency, it is possible to suggest a cause-effect relationship. The effect of BHT, a lipid-soluble chain-breaking antioxidant, suggests that the oxidative species generated in hydrophobic domains, i.e., membranes, are responsible for DNA damage. The small changes in the TBARS content in the slices and in the chromatin fraction and their lack of correlation with DNA damage indicate that TBA reactive products of lipid peroxidation, i.e., malonaldehyde and other aldehydes, did not directly damage DNA.

The oxidants used cover a broad range of the commonly used lipid peroxidation inducers, i.e., halogenated hydrocarbons, peroxides and metals. All have been reported to share free-radical mechanisms for induction of oxidative damage. Oxidative damage to DNA is believed to depend on metal-catalysed peroxide decomposition. The metal, probably ferrous iron, would be complexed to a macromolecule in the nucleus. By reaction with this metal, peroxides yield a hydroxyl radical, which is claimed to be the ultimate species that damages DNA (Meneghini & Hoffmann, 1980; Mello-Filho & Meneghini, 1984). Hydroxyl radical may produce specific modifications in DNA bases (Floyd *et al.*, 1986). The oxidants used induced lipid peroxidation, thus increasing the steady-state level of intracellular peroxides and other products of lipid peroxidation that can damage DNA. Ferrous iron and t-BOOH can also damage DNA without mediation of lipid peroxidation. BrCCl₃ needs to be activated, mainly by the endoplasmic reticulum, and it induces peroxide production through lipid peroxidation. A significant effect of this compound at sites distant from the endoplasmic reticulum seems unlikely. This is reflected by the insignificant damage observed in DNA isolated from the slices incubated with BrCCl₃. This result with BrCCl₃, and the fact that t-BOOH and iron induced DNA damage, also suggests an important role for the nuclear membrane in promoting oxidative conditions that can damage DNA. Direct damage induced by iron and t-BOOH without mediation of lipid peroxidation cannot be totally disregarded, but it seems unlikely given the prevention of damage by BHT.

Since study of DNA damage *in vivo* is difficult, the use of systems that closely approximate physiological conditions may provide valid approximations of processes occurring *in vivo*. Detection of DNA damage concurrent with the occurrence of oxidative reactions in tissue slices is important, since this system is closer to conditions *in vivo* than are more biochemical systems (homogenates, subcellular fractions, etc.). Tissue slices maintain a significant amount of organization, which is an advantage over cultured or isolated cell systems wherein the cells are removed from their natural environment. However, since tissues slices represent a complete and heterogenous system, the results obtained reflect integration of the various processes occurring within the tissue.

In summary, incubation of physiologically active liver

slices with inducers of oxidative stress resulted in DNA damage that appeared to be a consequence of concurrently occurring lipid peroxidation.

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