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Ascorbic Acid Inhibits Lipid Peroxidation but Enhances DNA Damage in Rat Liver Nuclei Incubated with Iron Ions

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In this report we studied DNA damage and lipid peroxidation in rat liver nuclei incubated with iron ions for up to 2 hrs in order to examine whether nuclear DNA damage was dependent on membrane lipid peroxidation. Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) and DNA damage was measured as 8-OH-deoxyguanosine (8-OH-dG). We showed that Fe(II) induced nuclear lipid peroxidation dose-dependently but only the highest concentration (1.0 mM) used induced appreciable 8-OH-dG. Fe(III) up to 1 mM induced minimal lipid peroxidation and negligible amounts of 8-OH-dG. Ascorbic acid enhanced Fe(II)-induced lipid peroxidation at a ratio to Fe(II) of 1:1 but strongly inhibited peroxidation at ratios of 2.5:1 and 5:1. By contrast, ascorbate markedly enhanced DNA damage at all ratios tested and in a concentration-dependent manner. The nuclear DNA damage induced by 1 mM $FeSO_4$/5 mM ascorbic acid was largely inhibited by iron chelators and by dimethylsulphoxide and mannitol, indicating the involvement of OH. Hydrogen peroxide and superoxide anions were also involved, as DNA damage was partially inhibited by catalase and, to a lesser extent, by superoxide dismutase. The chain-breaking antioxidants butylated hydroxytoluene and diphenylamine (an alkoxyl radical scavenger) did not inhibit DNA damage. Hence, this study demonstrated that ascorbic acid enhanced Fe(II)-induced DNA base modification which was not dependent on lipid peroxidation in rat liver nuclei.

Keywords: Nuclei, lipid peroxidation, DNA damage, 8-OH-deoxyguanosine, ascorbic acid, reactive oxygen species

Abbreviations: ROS, reactive oxygen species; OH, hydroxyl radicals; 8-OH-dG, 8-OH-deoxyguanosine; TBARS, thiobarbituric acid-reactive substances; DMSO, dimethylsulphoxide; BHT, butylated hydroxytoluene; LOOH, lipid hydroper-oxides

INTRODUCTION

Aerobes are constantly under attack by reactive oxygen species (ROS) that are produced by various sources which either directly or indirectly damage cellular macromolecules including membrane lipids, proteins and DNA. Oxidative damage to DNA is of great importance because of the growing recognition that such a damage can both initiate and promote carcinogenesis.[1] Hydroxyl radicals (OH) are the most damaging species which react with the DNA base at diffusion-controlled rates by hydroxylation of the pyridine and pyrimidine bases, leading to production of a series of base modification products[2-4]. Among them 8-OH-deoxyguanosine (8-OH-dG), which

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can be released from DNA by enzymic hydrolysis,[4] is a powerful marker of oxidative DNA damage.[2]

A question of interest is the relationship of oxidative damage to membrane lipids and DNA in cellular systems or in intact organelles containing both membrane lipids and DNA. Several in vitro studies have shown that DNA damage is concurrent with or closely related to lipid peroxidation.[5-10] Some of these studies have suggested that DNA damage is a result of membrane lipid *peroxidation*.[8,9] For instance, Hruszkewycz and Bergtold[9] showed that the 8-hydroxyguanine content of isolated mitochondria incubated with iron and either NADPH or ascorbate increased with lipid peroxidation and that α-tocopherol suppressed lipid peroxidation and proportionally attenuated 8-hydroxyguanine production and limited the electrophoretic mobility change of mitochondrial DNA. An in vivo study[11] of $KBrO_3$ toxicity attributed the formation of 8-OH-dG in rat kidney to increased lipid peroxidation because the former appeared after elevation of lipid peroxides. However, it is not clear how membrane lipid peroxidation, which is often a late stage in the oxidative damage mechanism,[12] may cause damage to DNA. Peroxyl and alkoxyl radicals, which can be produced by reactions of iron ions with lipid hydroperoxides,[12,13] are thought to be unlikely to reach nuclear DNA even if they are formed from the nuclear membrane.[14] To help clarify this, we studied lipid peroxidation and DNA damage in rat liver nuclei induced by iron ions. We showed that ascorbic acid inhibited iron-induced nuclear lipid peroxidation but enhanced DNA damage measured as 8-OH-dG, indicating that DNA damage is not necessarily dependent on membrane lipid peroxidation.

MATERIALS AND METHODS

Preparation and Incubation of Nuclei

All chemicals used were of reagent or higher grade. Desferrioxamine mesylate, mannitol, thiobarbituric acid (TBA) were from Sigma Chemical Co. (St. Louis, MO). Dimethylsulphoxide (DMSO) was from BDH Laboratory supplies, UK and diphenylamine from Fluka BioChemica, Switzerland.

Rats weighing 180–230 g were sacrificed by decapitation and livers were quickly frozen in liquid nitrogen and stored at −35°. To prepare nuclei the liver was homogenized in 4 volumes of ice-cold 0.15 M NaCl-0.015 M trisodium citrate, pH 7.0[15] and centrifuged (10 × g, 5 min) to remove tissue and cell debris. The supernatant was then centrifuged at 600 × g for 10 min to obtain crude nuclei which were washed in the same buffer and recentrifuged (600 g, 10 min). The washed nuclei were suspended in 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl. The nuclear suspension containing ca. 4.5 mg protein and 1 mg DNA was incubated (37°) with ferrous sulfate and/or ascorbic acid in a final volume of 1.5 ml in a shaking water bath (180 rpm/min) for up to 2 hrs.

Measurement of Oxidative Damage

Lipid peroxidation was measured as TBA-reactive substances (TBARS) by mixing equal volumes of the reaction mixture with the TBA reagent (0.7% in 0.05 N KOH) and 2.5% TCA.[16] BHT was included at 0.2 mM to prevent sporadic lipid peroxidation during assay. After heating at 100°C for 10 min, the mixture was cooled and centrifuged, and the supernatant was read at 532 nm.

For isolation of DNA the remaining reaction mixture was centrifuged (10,000 × g, 10 min) and the nuclear DNA was isolated using phenol/chloroform/isoamyl alcohol.[17] An equal amount of DNA (200 μg) was then digested with nuclease P1 and alkaline phosphatase, and 8-OH-dG levels were analyzed by HPLC with an electrochemical detector (Bioanalytical Systems, model LC-4C) essentially as described by Shigenaga *et al.*[18]

RESULTS

Wher liver nuclei were incubated with $FeSO_4$ at 37° for 2 hrs, lipid peroxidation increased in a concentration-dependent manner whereas 8-OH-dG was only detected at the highest concentration (1.0 mM) of $FeSO_4$ used (Fig. 1). Ferric chloride up to 1 mM produced minimal lipid peroxidation and no appreciable DNA damage (data not shown). The failure to detect 8-OH-dG at lower concentrations of $FeSO_4$ was not due to insufficient sensitivity because the detection limit of our 8-OH-dG assay was the same as that (ca. 20 fmol 8-OH-dG or 1.9 8-OH-dG/10^5 dG) reported by Shigenaga and Ames.[2] Although some amounts of DNA were released into the medium (indicating the lysis of some of the nuclei during incubation), 8-OH-dG was not detected in the supernatant DNA (data not shown). Time course experiments using 1 mM $FeSO_4$ were carried out in an attempt to determine the sequence of occurrence for nuclear lipid peroxidation and DNA damage. The result showed that the two events appeared to be concurrent (Fig. 2).

Ascorbic acid added with $FeSO_4$ to the nuclei produced a biphasic response in lipid peroxidation (Fig. 3). At an equal molar ratio of ascorbate to $FeSO_4$, ascorbate enhanced Fe(II)-induced lipid peroxidation whereas ascorbate strongly inhibited lipid peroxidation at higher ratios to $FeSO_4$. In contrast, DNA damage induced by 1.0 mM $FeSO_4$ ions was enhanced by ascorbate at all ratios tested and in a manner that was dependent on ascorbate concentration. Time-course studies (Fig. 4) showed that 8-OH-dG levels induced by 5 mM ascorbate + 1 mM $FeSO_4$ increased rapidly with time and reached a maximum at 40 min, while

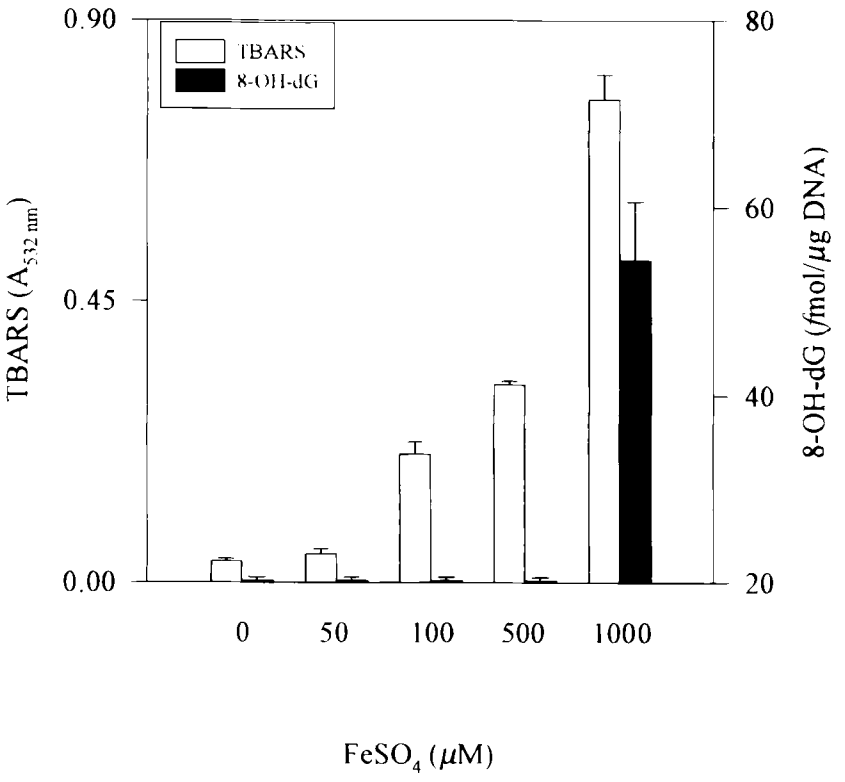

FIGURE 1 Levels of TBARS **(A)** and 8-OH-dG **(B)** in rat liver nuclei incubated with $FeSO_4$ at 37° for 2 hrs. Values are means ± SD, n = 3 – 4.

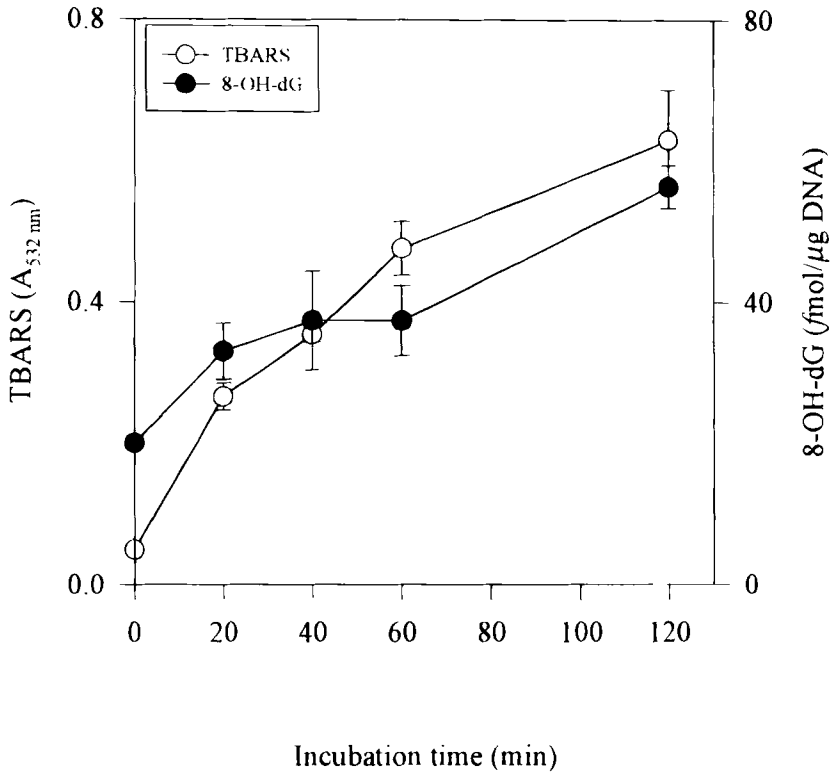

FIGURE 2 Time course of levels of 8-OH-dG and TBARS in rat liver nuclei incubated with 1.0 mM $FeSO_4$ at 37° for 2 hrs. Values are means ± SD, n = 3.

lipid peroxidation was low and only slightly increased during the 2 hr incubation period.

We then examined the ROS involved in nuclear DNA damage induced by 5 mM ascorbate + 1 mM $FeSO_4$. Lipid peroxidation was not determined due to the strong inhibition by ascorbate. The results showed that desferrioxamine, DMSO and mannitol effectively inhibited DNA damage (Table I). EDTA stimulated DNA damage by 30% when added at 2 mM but inhibited the damage by 60% at 10 mM. 8-OH-dG was decreased ca. 40% by catalase and 15% by superoxide dismutase. The effects of catalase and superoxide dismutase were abolished by boiling the enzymes for 5 min (data not shown).

The chain-breaking scavengers BHT and diphenylamine, an alkoxyl radical scavenger,[19] were tested and compared with ethanol since BHT and diphenylamine were dissolved in ethanol (0.6% or 130 mM) (Table I). The result showed that ethanol, BHT and diphenylamine decreased 8-OH-dG levels to approximately the same extent and that there were no concentration effects for BHT and diphenylamine. Thus, inhibition of 8-OH-dG formation by BHT and diphenylamine was attributed to solvent (ethanol) effect.

DISCUSSION

Under the present experimental conditions ferrous ions induced nuclear lipid peroxidation in a concentration-dependent manner but only the highest concentration (1.0 mM) used produced detectable amounts of 8-OH-dG. Ferric ions induced minimal lipid peroxidation and no appreciable amounts of 8-OH-dG. From these observations it is tempting to conclude that DNA

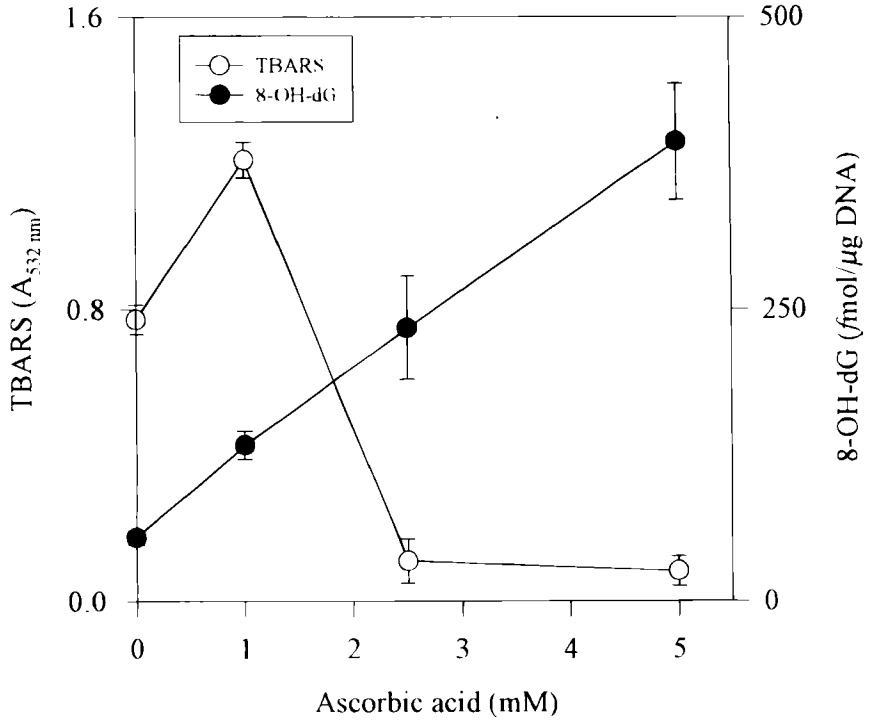

FIGURE 3 Concentration effects of ascorbic acid on levels of 8-OH-dG and TBARS in rat liver nuclei incubated with 1 mM $FeSO_4$ at 37° for 2 hrs. Values are means ± SD, n = 3. Ascorbic acid (5 mM) alone induced ca. 50 fmol 8-OH-dG / μg DNA but no appreciable lipid peroxidation.

damage is closely related to, contributed by, or a result of nuclear lipid peroxidation.

However, the results obtained from nuclei incubated with $FeSO_4$ (1 mM) and ascorbate (5 mM) indicated that nuclear DNA damage was independent of lipid peroxidation. The nuclear lipid peroxidation may be induced by reaction of $FeSO_4$ with preformed lipid hydroperoxides (LOOH) (Reactions 1 and 2)[12,13] which are likely to be present in the nuclei. Traces of LOOH are often formed during preparation of biological membrane systems[20] and low-density lipoproteins.[13,21]

$$LOOH + Fe\ (II) \rightarrow LO^{\bullet} + OH^{-} + Fe(III) \quad (1)$$

$$LOOH + Fe(III) \rightarrow LOO^{\bullet} + H^{+} + Fe(II) \quad (2)$$

At low concentration ratios of ascorbate to Fe(II), ascorbate may enhance lipid peroxidation by reduction of Fe(III) to Fe(II). At higher concentration ratios, the antioxidant property of ascorbate becomes dominant, leading to inhibition of lipid peroxidation. This is consistent with the fact that ascorbate is both an antioxidant and a prooxidant in vitro.[22]

In contrast to nuclear lipid peroxidation, ascorbate enhanced DNA damage in a concentration-dependent manner. It is possible that, as its concentration increases, ascorbate becomes available to react with Fe(II) ions bound upon or very close to DNA molecules. Using isolated rat liver nuclei incubated with iron ions, Shires[7] clearly demonstrated the binding of ^{59}Fe to DNA. Intracellular iron ions may also be released in response to oxidative stress from their storage sites with subsequent binding to DNA.[22,23] Thus, ·OH can be formed in DNA molecules and can attack DNA in a site-specific manner that is

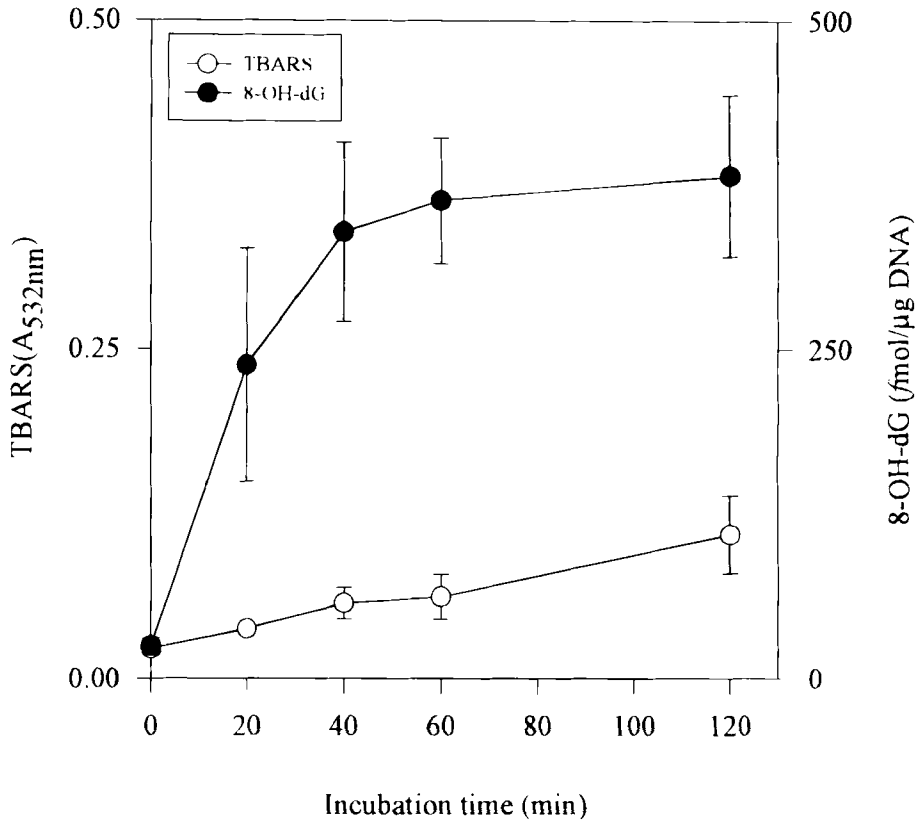

FIGURE 4 Time course of levels of 8-OH-dG and TBARS in rat liver nuclei incubated with 1.0 mM $FeSO_4$ + 5 mM ascorbate at 37° for 2 hrs. Values are means ± SD, n = 2 – 3.

TABLE I Effect of scavengers of reactive oxygen species on the levels of 8-OH-dG in rat liver nuclei incubated (37°, 2 hrs) with 1 mM $FeSO_4$/5 mM ascorbic acid

Addition	8-OH-dG (fmol/μug DNA)	Change (%)
$FeSO_4$/ascorbate	373 ± 56	—
+ EDTA, 2 mM	477 ± 16	+28
+ EDTA, 10 mM	154 ± 5	–59
+ Desferrioxamine, 1 mM	101 ± 8	–73
+ Dimethylsulphoxide, 20 mM	335 ± 30	–10
+ Dimethylsulphoxide, 50 mM	132 ± 8	–65
+ Mannitol, 20 mM	335 ± 11	–10
+ Mannitol, 50 mM	152 ± 23	–59
+ Superoxide dismutase, 40 μg/ml	315 ± 10	–15
+ Catalase, 40 μg/ml	220 ± 11	–41
+ Ethanol, 0.6 % (130 mM)	193 ± 15	–48
+ Butylated hydroxytoluene, 0.1 mM in 0.6% ethanol	194 ± 19	–48
1 mM in 0.6% ethanol	219 ± 30	–41
+ Diphenylamine, 0.1 mM in 0.6% ethanol 1 mM in 0.6% ethanol	209 ± 10	–43
1 mM in 0.6% ethanol	175 ± 15	–53

Data are means ± SD of 3–5 assays.

difficult to protect against.[3,4,22] The involvement of ·OH in the ascorbate-enhanced nuclear DNA damage by $FeSO_4$ was demonstrated by the inhibition of DNA damage by ·OH scavengers and by desferrioxamine which binds iron ions into chelates unable to generate ·OH. Although superoxide anions and H_2O_2 are considered unlikely to attack DNA directly,[4] they may do so through generation of ·OH by Fenton reaction. Additional reaction mechanism may also be involved in the formation of 8-OH-dG since none of the scavengers used completely inhibited DNA damage. In this context, Kasai and Nishimura[24] have shown that ascorbic acid itself is able to hydroxylate deoxyguanosine at the C-8 position in the presence of oxygen in 0.1 M phosphate buffer and that this 'direct hydroxylation' may not involve ·OH because ethanol fails to inhibit it. The conclusion reached by Kasai and Nishimura[24] is somewhat difficult to interpret since the 'direct hydroxylation by ascorbic acid' may have involved traces of iron contamination which are commonly present in biological reagents especially in the phosphate buffer;[22] and, as discussed above, site-specific damage to DNA is not always accessible to ·OH scavengers (ethanol was the only ·OH scavenger used by them). Even if this 'direct hydroxylation' did occur in our experimental system, its contribution to the overall level of 8-OH-dG was relatively small since ascorbic acid (5 mM) alone produced only ca. 50 fmol 8-OH-dG/μg DNA (Fig. 3 legend) while 5 mM ascorbic acid + 1 mM $FeSO_4$ produced ca. 370 fmol 8-OH-dG/μg DNA (Table I).

In summary, the present study demonstrated that ascorbate inhibited nuclear lipid peroxidation but enhanced DNA damage measured as 8-OH-dG. Our results do not exclude the possibility that membrane lipid peroxidation may precede, be concurrent, or contribute to DNA damage in other systems; rather, they demonstrate that lipid peroxidation and DNA damage are not necessarily closely related or dependent on each other.

Acknowledgements

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