The oxidation of α-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide

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Peroxynitrite is the product of the reaction between nitric oxide and superoxide. It is an oxidant which can also decompose to form the hydroxyl radical and nitrogen dioxide. In this report we show that a powerful oxidant with reactivity similar to that of the hydroxyl radical is formed from the generation of superoxide from xanthine oxidase and nitric oxide from *S*-nitroso-*n*-acetylpenicillamine (SNAP). Simultaneous generation of these two radicals by either xanthine oxidase/SNAP or the sydnonimine SIN-1 in the presence of low-density lipoprotein (LDL) results in the depletion of α-tocopherol and formation of its oxidised product α-tocopheroquinone. The mechanism of oxidation required both the formation of nitric oxide and superoxide. In contrast to the promotion of LDL oxidation by transition metals the oxidation of LDL by SIN-1 was not sensitive to the addition of exogenous lipid hydroperoxide.

Nitric oxide; Superoxide; Peroxynitrite; Low-density lipoprotein; Atherosclerosis

1. INTRODUCTION

Superoxide (O_2^-) and nitric oxide (NO) are free radicals which are known to be produced in the vasculature under both normal and pathophysiological conditions [1–3]. Nitric oxide is produced by a constitutive NO synthase in endothelial cells and O_2^- by inflammatory cells, or enzymes such as xanthine oxidase [4,5]. It has recently been shown that NO inhibits the respiratory burst enzyme responsible for generating O_2^- in activated neutrophils and this would suggest that it is unlikely that both free radicals are formed simultaneously by these cells [6]. This is important since the product of the reaction of these two species is peroxynitrite which is a powerful oxidant that can decompose to form nitrogen dioxide (NO_2) and the hydroxyl radical ($OH^{\cdot}$) [7]. This reaction, although first described in the gas phase, also occurs under physiological conditions [7–13]. Peroxynitrite is capable of initiating lipid peroxidation, oxidising sulphydryl groups on proteins and promoting the nitration of tyrosine residues [9–11]. It is of interest, therefore to determine whether O_2^- formation from enzymes such as xanthine oxidase results in the formation of potent oxidants in the presence of nitric oxide.

An important human disease to which oxidative stress may contribute is atherosclerosis and a number of lines of evidence suggest that oxidation of low-density lipoprotein (LDL) occurs in the artery wall resulting in the development of an atherosclerotic lesion [14–17]. There may be several mechanisms which could contribute to LDL oxidation including exposure to macrophage-derived 15-lipoxygenase followed by the iron or copper dependent decomposition of lipid peroxides [17,18].

We have recently demonstrated that the simultaneous generation of O_2^- and NO by the compound SIN-1 also results in the formation of an oxidant with 'hydroxyl radical-like' reactivity capable of initiating the oxidation of human LDL [8,14]. Interestingly, SIN-1 increases the extent of lipid peroxidation in the atherosclerotic lesions of cholesterol-fed rabbits [19]. The mechanisms by which O_2^- and NO contribute to oxidative damage to LDL are unclear. However, it has been shown that, in isolation, neither of these free radicals are able to modify LDL to any significant extent [14,20]. If the mechanism of oxidative damage entails the initiation of lipid peroxidation by the formation of peroxynitrite then lipid peroxidation would not require the presence of pre-formed or 'seeding' lipid peroxides. This would be in marked contrast to the transition metal-dependent oxidation of LDL, which has an absolute requirement for lipid peroxides [21,22]. In the present study we show that O_2^- generated from xanthine oxidase

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Abbreviations: LDL, low density lipoprotein; SNAP, *S*-nitroso-*n*-acetylpenicillamine; 13-HPODE, [9*Z*,11*E*,13(*S*)]-13-hydroperoxyoctadecadien-1-oic acid; DTPA, diethylenetriamine penta acetic acid; PBS, phosphate-buffered saline; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

and NO from SNAP form an oxidant with hydroxyl radical-like reactivity which depletes α-tocopherol in LDL. We have shown that the simultaneous formation of O_2^- and NO oxidises α-tocopherol in LDL to α-tocopheroquinone. In addition, unlike transition metal mediated oxidation of LDL, the rate of LDL oxidation by SIN-1 is not accelerated on addition of the exogenous lipid hydroperoxide 13-HPODE.

2. MATERIALS AND METHODS

2.1. *Preparation and characterisation of human LDL*

Human LDL was prepared from individual donors as described in [23] and throughout the text all measurements have been normalised to the concentration of LDL protein measured using the BCA protein assay kit supplied by Pierce. The α-tocopherol and α-tocopheroquinone content of samples was determined by HPLC as described in [24,25]. Incubations with SIN-1 were performed in Ca^{2+}/Mg^{2+} free PBS in the presence of 100 μM DTPA at 37°C. Xanthine oxidase (0.05 U/ml) and acetaldehyde (1 mM) were incubated with LDL in a similar fashion in the presence or absence of SNAP (1 mM). Reactions were quenched with butylated hydroxy toluene (100 μM) and superoxide dismutase (100 U/ml).

2.2. *Measurement of superoxide production*

Superoxide was measured using a previously described method [26] in which xanthine oxidase (0.05 U/ml) and acetaldehyde (1 mM) were incubated with cytochrome *c* (50 μM) in PBS at 37°C. The change in absorbance at 550 nm was monitored and the rate of superoxide production was determined using an extinction coefficient of 19.3 $mM^{-1} \cdot cm^{-1}$ for reduced cytochrome *c* minus oxidised cytochrome *c*. The rate of superoxide generation, as determined by the SOD-inhibitable reduction of cytochrome *c*, was 2.9 ± 0.15 $\mu M \cdot min^{-1}$ (mean ± S.D., $n = 3$) and similar to that generated by 500 μM SIN-1. This was sustained for a period of 30–40 min.

2.3. *Measurement of thiobarbituric acid reactive substances (TBARS)*

The production of thiobarbituric acid reactive material (TBARS) from the oxidation of deoxyribose was measured as described elsewhere [27]. Chromophore formed during the reaction was extracted into n-butanol and the visible absorbance spectrum taken. The absorbance values at 532 nm of samples with a well defined absorbance maximum at 532 nm were determined. We are aware that deoxyribose oxidation may generate TBARS that show similar characteristics to the adduct formed between MDA and TBA but have expressed all values in terms of MDA equivalents to aid comparison with the literature. In experiments where acetaldehyde and xanthine oxidase were used in combination, a high background absorbance was observed with no maxima at 532 nm. Samples containing this combination were corrected for this background absorbance. Xanthine oxidase (0.05 U/ml), acetaldehyde (1 mM) and SNAP (2 mM) were incubated in PBS at 37°C, in the presence of DTPA (100 μM), deoxyribose (10 mM) and a range of mannitol concentrations. After incubation for two hours the solutions were tested for TBARS.

3. RESULTS AND DISCUSSION

The combination of xanthine oxidase, acetaldehyde and SNAP was used to generate NO and O_2^- simultaneously and at equal rates (approximately 3 $\mu M \cdot min^{-1}$). The deoxyribose assay was used to assess whether this mixture was able to generate hydroxyl radicals. Table I shows the results of experiments where deoxyribose was incubated with xanthine oxidase, acetaldehyde and SNAP and this indicates that this combination generates an oxidant that is able to degrade deoxyribose to TBARS. Neither xanthine oxidase nor acetaldehyde alone were able to degrade deoxyribose (result not shown). However, in control experiments where SNAP alone was incubated with deoxyribose, a product was formed with a clear absorbance maximum at 532 nm suggesting that SNAP is capable of degrading deoxyribose to TBARS. The mechanisms for this is, as yet, unclear. Nitric oxide alone does not react with deoxyribose but a direct interaction between this sugar and the thiyl radical generated from the homolytic cleavage of SNAP or generation of superoxide from the reaction of the thiol with oxygen cannot be discounted.

Table I

The production of TBARS from deoxyribose using xanthine oxidase, acetaldehyde and SNAP

Condition	[MDA] (μM)
Xanthine oxidase/acetaldehyde/SNAP	2.00 ± 0.15
Xanthine oxidase/acetaldehyde/SNAP + SOD (500 U/ml)	0.87 ± 0.05
Xanthine oxidase/acetaldehyde/SNAP + Catalase (500 U/ml)	1.96 ± 0.10
SNAP (2 mM)	1.02 ± 0.05

All samples contained deoxyribose (10 mM) which was incubated for 2 h in PBS at 37°C and TBARS were detected by reaction with TBA. Where stated samples contained xanthine oxidase (0.05 U/ml), acetaldehyde (1 mM) and SNAP (2 mM). Values are given as the mean ± S.E.M. for 3 experiments.

In order to investigate the mechanism by which the combination of xanthine oxidase/acetaldehyde and SNAP degrade deoxyribose experiments were performed in the presence of the enzymes SOD and catalase and the hydroxyl radical scavenger mannitol (Table I, Fig. 1). Catalase had no effect on the yield of TBARS indicating that deoxyribose degradation did not occur by a route that requires hydrogen peroxide, such as the Fenton reaction. SOD was found to inhibit partially deoxyribose degradation in this system. This result is similar to the results we have reported earlier with SIN-1 [8]. The absence of complete inhibition by the enzyme is either a result of NO competing effectively for O_2^- even in the presence of SOD or the formation of TBARS from SNAP through a mechanism which does not require superoxide [8].

The effects of mannitol, a hydroxyl radical scavenger, on the yield of MDA are shown in Fig. 1. Mannitol clearly inhibits MDA generation in a concentration dependent manner. At the highest mannitol concentration (100 mM), the yield of MDA is only reduced by about 75%. Again this suggests that some TBARS are formed through a process independent of hydroxyl radical generation.

The combination of xanthine oxidase, acetaldehyde and SNAP generates an oxidant, but by a mechanism

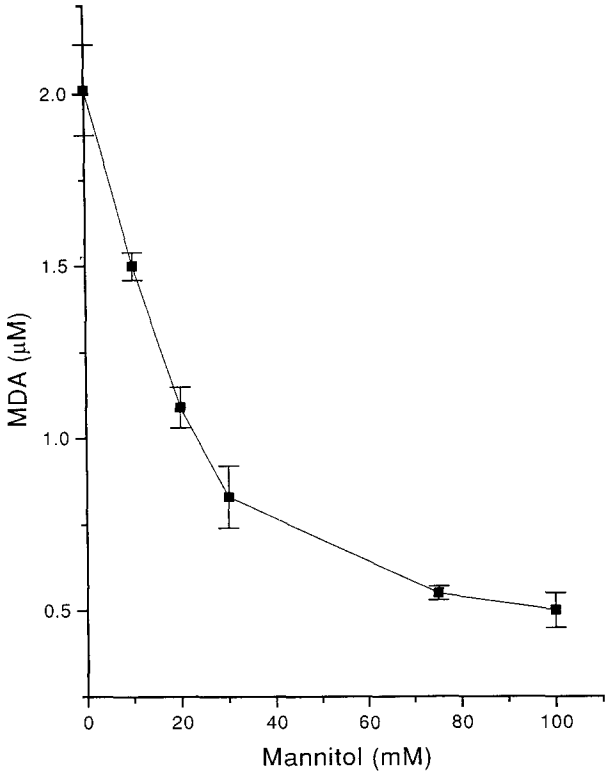

Fig. 1. The effect of mannitol on the xanthine oxidase/acetaldehyde/SNAP dependent degradation of deoxyribose. Deoxyribose was incubated, for 2 h in PBS at 37°C with xanthine oxidase (0.05 U/ml), acetaldehyde (1 mM) and SNAP (2 mM) in the presence of the concentrations of mannitol shown after which TBARS were detected by reaction with TBA. Values are given as the mean ± S.D. for 3 experiments.

different from the Fenton reaction, which exhibits many of the properties of the hydroxyl radical. In this respect this combination of agents acts in a similar way to SIN-1 since they both generate NO and O_2^- and these two free radicals react together to form peroxynitrite which may then decompose to yield the hydroxyl radical and nitrogen dioxide.

We have previously shown that SIN-1 depletes the α-tocopherol content of human LDL [14]. Since this could be due to a reaction of α-tocopherol with superoxide we tested for this possibility by using xanthine oxidase/acetaldehyde to generate O_2^-. This substrate was chosen rather than xanthine since we found, in agreement with the literature [28], that the urate produced as the product of the reaction was an effective inhibitor of lipid peroxidation under these conditions (result not shown). The results of this experiment are shown in Fig. 2 and demonstrate that whereas treatment with SIN-1 causes a time dependent loss of α-tocopherol, xanthine oxidase had little or no effect. In contrast xanthine oxidase/acetaldehyde in combination with SNAP at concentrations which generate NO and O_2^- at approximately the same rates (3 μM/min) deplete α-tocopherol when incubated with LDL (Table II). Prolonged (16–24

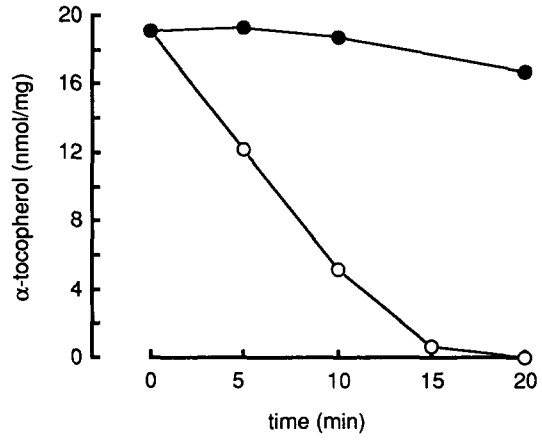

Fig. 2. Depletion of α-tocopherol in LDL by SIN-1 and xanthine oxidase. Human LDL (200 μg/ml) was incubated (37°C) with either 1 mM SIN-1 (○) or xanthine oxidase (0.05 U/ml) and its substrate 1 mM acetaldehyde (●) in the presence of 100 μM DTPA. Samples were taken at the times shown and represent the mean of duplicate samples. The α-tocopherol present in the sample was extracted into heptane before measurement by HPLC.

h) incubations did not result in the modification of the LDL particle to a more electronegative form as occurs with SIN-1 [11]. This is not unexpected since the xanthine oxidase/acetaldehyde system is only capable of generating O_2^- over 40–60 min under these conditions. Analysis of the HPLC chromatogram of the LDL oxidised by SIN-1 revealed a new peak with a characteristic u.v. spectrum (two absorbance maxima at 263 and 270 nm) of the oxidation product of α-tocopheroquinone (Fig. 3) [25]. The simultaneous generation of O_2^- and NO resulted in formation of substantial amounts of α-tocopheroquinone whereas only small amounts were formed

Table II

The α-tocopherol content of human LDL after treatment with SIN-1, xanthine oxidase and SNAP

Sample	α-toc (nmol/mg LDL protein)	α-tocQ (nmol/mg LDL protein)
Control	15.8 ± 0.21	n.d.
Xanthine oxidase	10.96 ± 0.4	0.59 ± 0.21
SNAP	12.6 ± 1.45	0.96 ± 0.17
Xanthine oxidase/SNAP	1.05 ± 0.05	7.69 ± 0.15
Xanthine oxidase/SNAP/SOD	11.8 ± 1.23	1.09 ± 0.24
SIN-1	0.09 ± 0.03	7.81 ± 0.06
SIN-1/SOD	7.79 ± 0.47	3.35 ± 0.10

Samples of human LDL (200 μg/ml) were incubated with 0.05 U/ml xanthine oxidase, 1 mM acetaldehyde and 2 mM SNAP in the combinations shown above for a period of 40 min at 37°C. DTPA was present at a concentration of 100 μM throughout the experiment. Samples were then taken for measurement of their α-tocopherol (α-toc) and α-tocopheroquinone (α-tocQ) content as described previously. The results are reported as the mean ± S.D. of three independent experiments. n.d. = none detected.

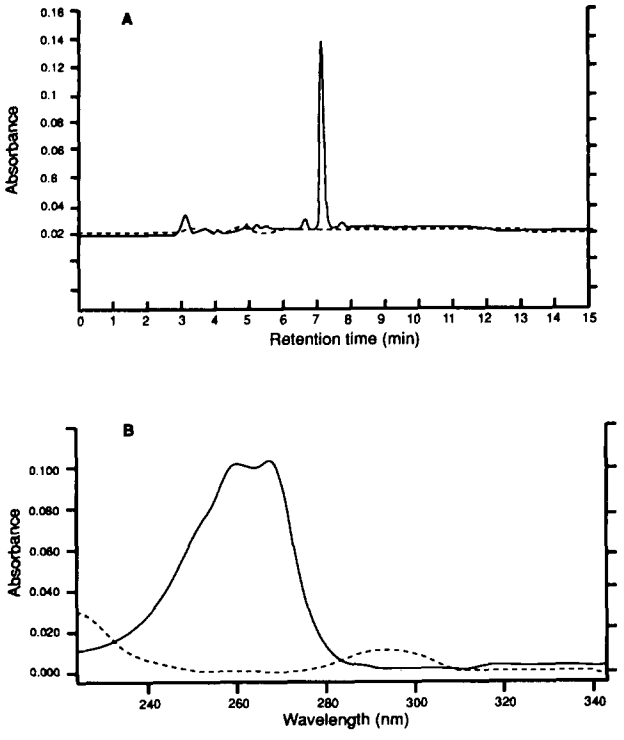

Fig. 3. Formation of α-tocopheroquinone during the SIN-1-dependent oxidation of LDL. Human LDL (200 μg/ml) was incubated with 1 mM SIN-1 for 60 min before extraction with heptane and being subjected to analysis by HPLC. Panel A: The UV absorbance traces (λ = 270 nm) for control LDL (- - -) and SIN-1 treated LDL (———) are shown. Panel B: The UV spectra of the major peak in SIN-1 treated LDL (retention time = 7.2 min) is shown compared to α-tocopherol, retention time = 6.6 min).

by xanthine oxidase or SNAP when either was incubated with LDL alone (Table II). This oxidation of α-tocopherol was substantially inhibited by SOD (Table II), which is consistent with the hypothesis that the oxidant is either peroxynitrite or its decomposition products. It has long been known that NO_2 oxidises α-tocopherol to α-tocopheroquinone and it has also been suggested that the direct reaction of nitric oxide and α-tocopherol results in the formation of tocopheroquinone [25,30]. However, under the conditions described here, and by others, little loss of α-tocopherol by reaction with NO was detected, suggesting that either the reaction of NO with oxygen to form NO_2 is inefficient compared to its formation via the decomposition of peroxynitrite, or the oxidising agent is peroxynitrite or the hydroxyl radical (Table II and [14,20]). It is possible, however, that in the presence of higher concentrations of NO than those used here, the reaction with oxygen to form the oxidant NO_2 occurs and generates peroxyl radicals or reacts directly with α-tocopherol [29,30].

It has been shown that bicarbonate reacts with peroxynitrite, probably yielding a bicarbonate radical [10]. In the case of the peroxynitrite-dependent killing of bacteria, the reaction with bicarbonate decreased its toxicity [10]. Since bicarbonate is probably present in the artery wall, where oxidation of LDL is thought to occur during the early stages of atherosclerosis, we tested its effects on the rate of loss of α-tocopherol in the presence of SIN-1. The results are reported in Table III and indicate that the rate of α-tocopherol depletion is accelerated in the presence of bicarbonate.

The oxidation of LDL can be mediated by the addition of transition metals such as iron or copper [16,18,21,22]. We, and others, have shown that the most likely mechanism for this reaction is the promotion of lipid peroxidation through the transition metal-mediated decomposition of lipid peroxides endogenous to the LDL particle to form peroxyl and alkoxyl radicals [21,22]. In contrast, it is thought that the reaction of peroxynitrite with unsaturated fatty acids involves direct abstraction of a hydrogen atom from the fatty acid side chain to form an alkyl radical [11]. To test for this possibility we have measured the rate of oxidative modification of LDL as prepared and supplemented with the lipid hydroperoxide 13-HPODE. The results are shown in Fig. 4 and indicate that the rate of LDL oxidation, as measured by the change in electrophoretic mobility relative to native LDL, is not significantly affected by the presence of 13-HPODE. Under the same conditions the rate of modification of LDL supplemented with lipid peroxide was greatly accelerated when promoted with copper [22].

In summary, we have shown that the production of O_2^- and NO, in solution, by two distinct pathways, xanthine oxidase/SNAP and SIN-1 results in the formation of an oxidant with hydroxyl radical-like reactivity. This is consistent with the hypothesis put forward by Beckman and co-workers that the product of NO and O_2^-, peroxynitrite, is a pro-oxidant of physiological relevance [7–13]. In agreement with the proposal that per-

Table III

The effects of bicarbonate on the loss of α-tocopherol during the oxidation of LDL by SIN-1

Sample	α-Tocopherol (nmol/mg)
Control	11 ± 1.65
SIN-1 (1 mM)	
(2.5 min)	6.1 ± 0.57
(5 min)	3.95 ± 0.70
SIN-1 (1 mM) and 25 mM HCO_3	
(2.5 min)	2.5 ± 0.7
(5 min)	n.d.

Human LDL (200 μg/ml) was incubated (37°C) with SIN-1 under the conditions shown and samples taken for measurement of the α-tocopherol content. Results are reported as the mean ± S.D. for three independent experiments. n.d. = none detected.

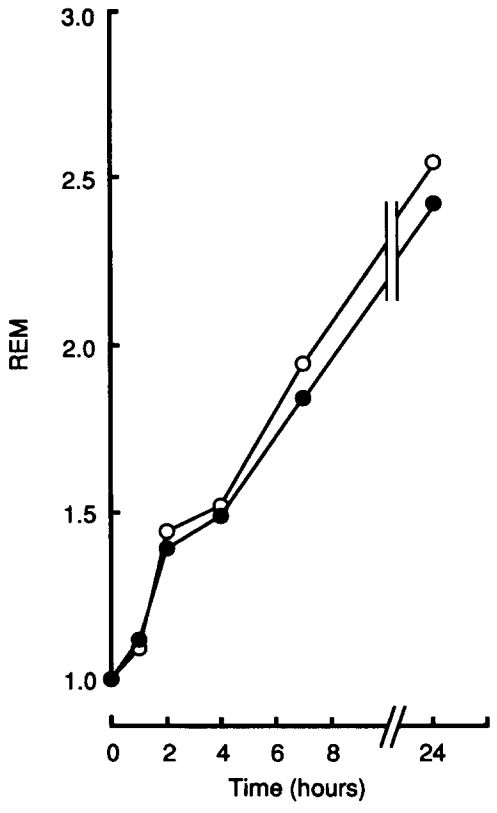

Fig. 4. The SIN-1-dependent oxidation of LDL in the presence of 13-HPODE. Human LDL (200 μg/ml) as prepared (○) or supplemented with 20 μM 13-HPODE (●) was incubated with SIN-1 (1 mM) and samples taken for measurement of electrophoretic mobility relative to native LDL (R.E.M.). Each point represents the mean of duplicate experiments.

oxynitrite may initiate lipid peroxidation directly we have found no evidence for a lipid peroxide-dependent component in the pro-oxidant reaction of NO and O_2^- in promoting LDL oxidation. One of the earliest oxidation products formed during the reaction of NO and O_2^- in combination with LDL is α-tocopheroquinone. This could occur only by direct reaction of oxidants with the antioxidant or through secondary reactions with lipid-derived peroxyl radicals. Interestingly, although α-tocopherol was also depleted rapidly during the oxidation by transition metals we were unable to detect α-tocopheroquinone (result not shown).

It has been shown that the formation of O_2^- by the NADPH oxidase of neutrophils is inhibited by NO [6]. Our results with xanthine oxidase suggest that the formation of O_2^- by this enzyme is not impaired by NO and it could, therefore, contribute to the formation of peroxynitrite if activated in the presence of NO. Our study also supports the hypothesis that the reaction between NO and O_2^- is likely to be strongly pro-oxidant rather than antioxidant as suggested by some investigators [31]. However, the relevance of these reactions in vivo remains a matter of conjecture.

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