Lipid peroxidation initiated by superoxide-dependent hydroxyl radicals using complexed iron and hydrogen peroxide

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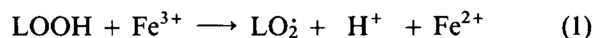
Received 26 April 1984

Iron salts stimulate lipid peroxidation by decomposing lipid peroxides to produce alkoxyl and peroxyl radicals which initiate further oxidation. In aqueous solution ferrous salts produce $OH^{\cdot}$ radicals, a reactive species able to abstract hydrogen atoms from unsaturated fatty acids, and so can initiate lipid peroxidation. When iron salts are added to lipids, containing variable amounts of lipid peroxide, the former reaction is favoured and $OH^{\cdot}$ radicals contribute little to the observed rate of peroxidation. When iron is complexed with EDTA, however, lipid peroxide decomposition is prevented, but the complex reacts with hydrogen peroxide to form $OH^{\cdot}$ radicals which are seen to initiate lipid peroxidation. Superoxide radicals appear to play an important part in reducing the iron complex.

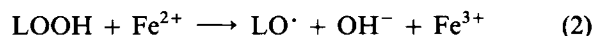
Superoxide-dependent hydroxyl radical *Hydroxyl radical-initiated peroxidation* *Lipid peroxidation* *Superoxide dismutase* *Fenton reaction* *Deoxyribose degradation*

1. INTRODUCTION

Lipid peroxidation has 3 discernible phases, namely, induction, propagation and termination (review [1]). Metal ions and their complexes, particularly iron and copper, stimulate lipid peroxidation by decomposing stable lipid peroxides (LOOH) which are products of the fast propagation phase: (eq.1,2).

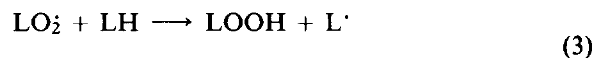


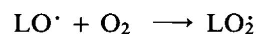
$$LOOH + Fe^{3+} \longrightarrow LO_2^{\cdot} + H^+ + Fe^{2+} \quad (1)$$



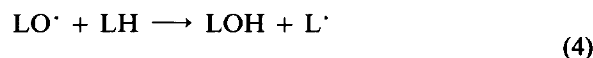
$$LOOH + Fe^{2+} \longrightarrow LO^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

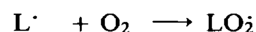
Newly formed peroxyl ($LO_2^{\cdot}$) and alkoxyl ($LO^{\cdot}$) radicals are then capable of initiating further peroxidative sequences: (eq.3,4).





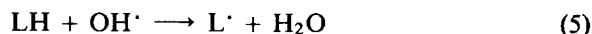
$$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$
$$LO^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \quad (3)$$





$$LO^{\cdot} + LH \longrightarrow LOH + L^{\cdot}$$
$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \quad (4)$$

Hydroxyl radicals ($OH^{\cdot}$) have sufficient energy to abstract hydrogen atoms from unsaturated lipids (LH) and so have the potential to directly initiate lipid peroxidation (eq.5).



$$LH + OH^{\cdot} \longrightarrow L^{\cdot} + H_2O \quad (5)$$

However, although hydroxyl radicals are formed during the auto-oxidation of ferrous salts in aqueous solution, they do not appear to contribute to lipid peroxidation stimulated by addition of iron salts to unsaturated lipids [2,3] in that scavengers of these radicals do not slow the observed rates of peroxidation. Under the conditions used organic oxygen radical reactions (eq.1,2) probably predominate since all unsaturated lipids examined contain variable amounts of lipid peroxides [4] and so most initiation is by $LO^{\cdot}$ and $LO_2^{\cdot}$ and not by $OH^{\cdot}$. However, by carefully controlling the amount of iron salt and metal complexing agent, EDTA, in the mixture it has been possible to demonstrate the role of $OH^{\cdot}$ initiation. EDTA will inhibit iron salt-stimulated lipid peroxide decomposition (eq.1,2) but will promote the decomposition of hydrogen

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peroxide leading to hydroxyl radical formation. These hydroxyl radicals then initiate lipid peroxidation (eq.5).

2. MATERIALS AND METHODS

Superoxide dismutase (bovine erythrocyte, 3000 units/ml), catalase (bovine liver, 27 000 units/ml, thymol-free), albumin (human fatty acid-free), 2-deoxy-D-ribose, linolenic acid (99% pure) and lipoxidase (soybean) were from Sigma. Units of enzyme activity were as defined in the Sigma catalogue. All other chemicals were of the highest purity available from BDH.

2.1. Preparation of lipids

A fatty acid suspension was prepared by adding 20 μl linolenic acid to 10 ml of 0.15 M NaCl treated with Chelex resin. The solution was vortex mixed for 2 min and the pH adjusted to 7.4 with NaOH. The suspension was further vortex mixed for 2 min and stored at 4°C for 1 h before use. Phospholipid liposomes were prepared containing 5 mg phospholipid per ml of saline (0.15 M, pH 7.4) as in [5]. Linolenic hydroperoxide was prepared as in [6].

2.2. Preparation of iron complexes

Freshly prepared ammonium ferrous sulphate (2 mM) was added to 2 mM EDTA in the ratios 1 : 1.1 and 1 : 1.2 and allowed to stand for 15 min at room temperature before use.

2.3. Peroxidation of lipids

Reactions were carried out in new, clean plastic tubes. Chelex-resin-treated water (0.2 ml), 0.2 ml phosphate–saline buffer (pH 7.4; 0.024 M phosphate, 0.15 M NaCl) and 0.1 ml lipid preparation (either fatty acid or phospholipid) were pipetted

Table 1

Effect of hydroxyl radial scavengers on the peroxidation of linolenic acid stimulated by ferrous salt, and of EDTA on linolenic hydroperoxide decomposition stimulated by ferrous salt

	TBA reactivity/0.5 h	
	$A_{532\text{ nm}}$	% Inhibition (I) or stimulation (S)
(a) 80 μM linolenic hydroperoxide +	0.170	
(b) 80 μM linolenic hydroperoxide + 0.31 mM EDTA	0.079	
(c) 80 μM linolenic hydroperoxide + 0.28 mM Fe^{2+}	0.320	
(d) 80 μM linolenic hydroperoxide + 0.28 mM Fe^{2+} + 0.31 mM EDTA	0.091	
(e) 80 μM linolenic hydroperoxide + 0.28 mM Fe^{3+}	0.342	
(f) 80 μM linolenic hydroperoxide + 0.28 mM Fe^{3+} + 0.31 mM EDTA	0.078	
(1) linolenic acid only	0.058	
(2) linolenic acid + 0.29 mM Fe^{2+}	0.411	
Reaction 2 + 14.3 mM formate	0.375	9 I
Reaction 2 + 14.4 mM ethanol	0.361	12 I
Reaction 2 + 14.3 mM butanol	0.419	2 S
Reaction 2 + 1.43 mM urea	0.425	3 S
Reaction 2 + 1.43 mM thiourea	0.358	13 I
Reaction 2 + 14.3 mM glucose	0.439	7 S
Reaction 2 + 14.3 mM mannitol	0.484	18 S
Reaction 2 + 14.3 mM Tris	0.378	8 I
Reaction 2 + 0.04 mg/ml SOD	0.402	2 I
Reaction 2 + 0.04 mg/ml catalase	0.473	15 S
Reaction 2 + 0.04 mg/ml albumin	0.422	3 S

Readings shown for OH$^{\cdot}$ radical scavengers were calculated after subtraction of control 1. All concentrations shown are final reaction concentrations. Each tube contained phosphate buffer (pH 7.4) (6.9 mM phosphate, 43 mM NaCl). SOD, superoxide dismutase

into each reaction tube. Radical scavengers, or inhibitors, were added to give the final reaction concentrations shown in the tables. Hydrogen peroxide (0.05 ml, 10 mM) was added and the tube contents mixed. Iron–EDTA complex (0.1 ml, 1:1.1) was pipetted into the linolenic acid-containing tubes and 0.1 ml of 1:1.2 iron complex to the phospholipid-containing tubes. The reaction tubes were incubated at 37°C for 30 min.

2.4. Deoxyribose degradation

This was carried out in the same way as for lipid oxidation described above but 0.1 ml of 10 mM deoxyribose was substituted for the lipid preparation. Both iron complexes were used.

2.5. Thiobarbituric acid reactivity

After incubation of the reaction mixtures 0.5 ml thiobarbituric acid (TBA) reagent containing 1% (w/v) TBA in 0.05 M NaOH was added to each tube followed by 0.5 ml appropriate acid reagents [7]: 25% (v/v) HCl for phospholipids, 2.8% (w/v) trichloroacetic acid for deoxyribose and buffer (pH 3.5) (0.05 M phthalate, 8.2 mM HCl) for linolenic acid. The contents of the plastic tubes were transferred to glass tubes which were heated for

Table 2

Hydroxyl radical scavenger protection against damage to lipids and deoxyribose by OH. Radicals generated with complexed iron and hydrogen peroxide

	TBA reactivity/0.5 h					
	Linolenic acid		Phospholipid		Deoxyribose	
	$A_{532\ nm}$	% Inhibition (I) or stimulation (S)	$A_{532\ nm}$	% Inhibition (I) or stimulation (S)	$A_{532\ nm}$	% Inhibition (I) or stimulation (S)
(1) Iron–EDTA complex	0.045		0.030		0.020	
(2) Iron–EDTA + hydrogen peroxide (0.7 mM)	0.112		0.200		0.770	
(2) + formate (13 mM)	0.026	79 I	0.030	85 I	0.122	84 I
(2) + ethanol (13 mM)	0.045	60 I	0.076	62 I	0.038	95 I
(2) + butan-1-ol (13 mM)	0.044	61 I	0.082	59 I	0.008	99 I
(2) + thiourea (1.3 mM)	0.019	83 I	0.054	73 I	0.015	98 I
(2) + urea (1.3 mM)	0.122	9 S	0.180	10 I	0.785	2 S
(2) + glucose (13 mM)	0.064	43 I	0.068	66 I	0.038	95 I
(2) + mannitol (13 mM)	0.054	52 I	0.038	81 I	0.054	93 I
(2) + Tris (13 mM)	0.027	76 I	0.075	62 I	0.031	96 I
(2) + SOD (0.03 mg/ml)	0.024	79 I	0.066	67 I	0.038	95 I
(2) + SOD (0.006 mg/ml)	0.049	56 I	0.080	60 I	0.069	91 I
(2) + SOD (0.03 mg/ml, heat-denatured)	0.107	5 I	0.122	39 I	0.269	65 I
(2) + catalase (0.03 mg/ml)	0.007	94 I	0.002	99 I	0.002	100 I
(2) + catalase (0.006 mg/ml)	0.027	76 I	0.008	96 I	0.008	99 I
(2) + catalase (0.03 mg/ml, heat-denatured)	0.106	5 I	0.186	7 I	0.759	2 I
(2) + albumin (0.03 mg/ml)	0.127	13 S	0.188	6 I	0.782	2 S
(2) + albumin (0.006 mg/ml)	0.124	11 S	0.220	10 S	0.801	4 S
(2) + albumin (0.03 mg/ml, heat denatured)	0.143	28 S	0.194	3 I	0.778	1 S

Values shown were calculated after subtraction of control 1. Concentrations are final reaction concentrations. All reactions contained phosphate–saline buffer (pH 7.4, 6.4 mM phosphate and 40 mM NaCl). The iron–EDTA concentrations in the linolenic acid reactions were 0.13 mM Fe:0.14 mM EDTA and in the phospholipid reactions 0.12 mM Fe:0.15 mM EDTA. Both ratios of iron:EDTA gave essentially the same result in the deoxyribose assay. SOD, superoxide dismutase

15 min at 100°C. The results shown are means of 3 separate experiments which differed by less than ±6%.

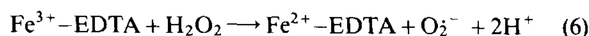
3. RESULTS

The rate of lipid peroxidation stimulated by a ferrous salt was not significantly influenced by the addition of hydroxyl radical scavengers (table 1). EDTA, however, substantially inhibited both ferric and ferrous salt-dependent decomposition of preformed lipid hydroperoxides (table 1), as measured by their resulting TBA reactivity, showing that EDTA did not act as an inhibitor by accelerating the oxidation of ferrous ions.

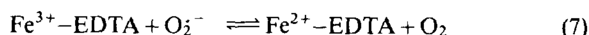
With a defined concentration of EDTA in the reaction, iron salts no longer stimulated lipid peroxidation, but the resulting iron–EDTA complex was able to decompose added hydrogen peroxide and increase the observed rate of lipid peroxidation (table 2). Under the same reaction conditions, deoxyribose was substantially degraded to release TBA-reactive material. Damage to lipids and deoxyribose by iron–EDTA and hydrogen peroxide was markedly inhibited by the addition of a range of hydroxyl radical scavengers, superoxide dismutase and catalase (table 2) but not by urea, albumin and heat-denatured catalase which were included as controls for non-specific effects. Heat-denatured superoxide dismutase, however, retained some activity in the deoxyribose and phospholipid systems as previously observed [8].

4. DISCUSSION

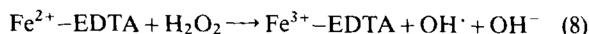
Several studies have shown that hydroxyl radicals, produced by high-energy radiation, initiate lipid peroxidation [9–11], and others that superoxide generating systems produce initiating radicals [12,13]. However, the demonstration of hydroxyl radical initiation when $OH^{\cdot}$ radicals are generated by iron salts is complicated by the predominance of oganic oxygen radical reactions (eq.1–4) [2]. Here, iron salts have been prevented from participating in these peroxide decomposition reactions by complexing with EDTA. The iron–EDTA complex can, however, readily decompose hydrogen peroxide in a Fenton-type sequence releasing $OH^{\cdot}$ radicals which initiate lipid peroxidation. When a ferrous salt is mixed with EDTA the complex rapidly auto-oxidises to form the ferric complex [8]. However, addition of hydrogen peroxide to a ferric–EDTA complex still resulted in the formation of hydroxyl radicals, as shown by the inhibitory properties of the hydroxyl radical scavengers tested. Since superoxide dismutase also strongly inhibits this reaction, it suggests that the ferrous–EDTA complex, essential for reaction with hydrogen peroxide to form $OH^{\cdot}$ radicals (eq.8) must have substantially arisen from the reduction of ferric–EDTA by superoxide radicals (eq.7) formed by the reaction of ferric–EDTA with hydrogen peroxide (eq.6).



$$Fe^{3+}\text{–EDTA} + H_2O_2 \longrightarrow Fe^{2+}\text{–EDTA} + O_2^{\cdot-} + 2H^+ \quad (6)$$



$$Fe^{3+}\text{–EDTA} + O_2^{\cdot-} \rightleftharpoons Fe^{2+}\text{–EDTA} + O_2 \quad (7)$$



$$Fe^{2+}\text{–EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{–EDTA} + OH^{\cdot} + OH^- \quad (8)$$

Catalyse can interfere with lipid oxidation studies by non-specifically decomposing lipid peroxides or stimulating TBA reactivity [11]. However, in this study it strongly inhibited lipid peroxidation and deoxyribose degradation, although its specificity was not required to substantiate involvement of hydrogen peroxide.

Albumin, included as a control for non-specific protein effects, stimulated peroxidation and deoxyribose degradation. This may result from loosely bound copper associated with commercial preparations (unpublished). Heat-denatured superoxide dismutase did not totally lose its enzymic activity, as observed in [8], and this probably reflects recombination of released copper and protein to restore some dismutase activity.

The results show that when reactions 1 and 2 are prevented the role of hydroxyl radicals in initiating lipid peroxidation can be clearly seen.

ACKNOWLEDGEMENT

J.M.C.G. is grateful to Dr Barry Halliwell for helpful discussions.

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