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Ferrous ion–EDTA-stimulated phospholipid peroxidation

A reaction changing from alkoxyl-radical- to hydroxyl-radical-dependent initiation

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The stimulatory effect of ferrous salts on the peroxidation of phospholipids can be enhanced by EDTA when the concentration of Fe^{2+} in the reaction is greater than that of EDTA. Hydroxyl-radical scavengers do not inhibit peroxidation until the concentrations of Fe^{2+} and EDTA in the reaction are equal. Lipid peroxidation is then substantially initiated by hydroxyl radicals derived from a Fenton-type reaction requiring hydrogen peroxide. Superoxide radicals appear to play some role in the formation of initiating species.

Iron salts can stimulate lipid peroxidation by decomposing lipid peroxides (LOOH) to form alkoxyl ($LO^{\bullet}$) and peroxyl ($LO_2^{\bullet}$) radicals (eqns. 1 and 2), or by directly reacting with molecular O_2 to produce hydroxyl radicals ($OH^{\bullet}$) or a species with similar reactive properties (Scheme 1). Alkoxyl, peroxyl and hydroxyl radicals can initiate lipid peroxidation by abstracting hydrogen atoms from unsaturated fatty acids (LH) (eqns. 3–5), a reaction followed by O_2 uptake (eqn. 6).

$$Fe^{2+} \rightarrow LOOH \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (1)$$

$$Fe^{3+} + LOOH \rightarrow LO_2^{\bullet} + H^{+} + Fe^{2+} \quad (2)$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet} \quad (3)$$

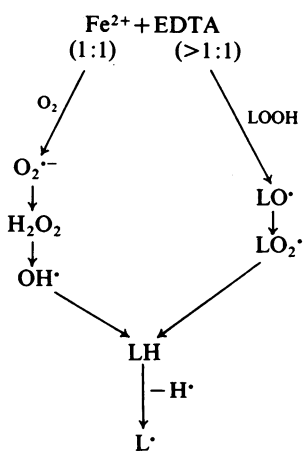
$$LO_2^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (4)$$

$$OH^{\bullet} + LH \rightarrow H_2O + L^{\bullet} \quad (5)$$

$$L^{\bullet} + O_2 \rightarrow LO_2^{\bullet} \quad (6)$$

All lipid preparations examined contain traces of lipid peroxides (Gutteridge & Kerry, 1982), and therefore transition-metal ions, particularly those of iron and copper, may react with these samples in the way described above to produce initiating species. Desferrioxamine, a specific Fe^{3+} chelator, inhibits such reactions when they are iron-dependent (Wills, 1969; Gutteridge *et al.*, 1979). However, several other metal-ion chelators, including EDTA, have been shown to both enhance and inhibit iron-dependent lipid peroxidation (Gutteridge *et al.*, 1979). The ratio of iron salt to chelator in the reaction mixture determines whether peroxidation is enhanced or inhibited.

Studies described in the present paper extend these observations and show that at least two different initiation mechanisms occur when EDTA and a ferrous salt are added to phospholipid liposomes. When equimolar concentrations of ferrous salt and EDTA are present, the reaction is substantially dependent on $OH^{\bullet}$ initiation. However, when the concentration of Fe^{2+} exceeds that



Scheme 1. *Possible pathways by which phospholipid peroxidation is initiated in the presence of ferrous salts and EDTA*

Abbreviations: $LO^{\bullet}$, alkoxyl radical; $LO_2^{\bullet}$, peroxyl radical; $OH^{\bullet}$, hydroxyl radical; $O_2^{\bullet -}$, superoxide radical; LH, polyunsaturated fatty acid; LOOH, lipid hydroperoxide.

of EDTA, peroxidation is stimulated by a reaction independent of OH·.

Materials and methods

Materials

Catalase (bovine liver; thymol-free), superoxide dismutase (bovine erythrocyte), albumin (human; fatty acid-free) diethylenetriaminepenta-acetic acid, bathophenanthrolinesulphonate and Lubrol PX were from Sigma Chemical Co. All other chemicals were of the highest grades available from BDH Chemicals. Desferrioxamine was from Ciba–Geigy. Bovine brain phospholipids were prepared as previously described (Gutteridge, 1977*a*). All reagents were prepared in Chelex-resin-treated distilled water.

Oxidation of lipid

The reaction mixture, with a total volume of 0.90ml, contained the following reagents at the concentrations stated: phospholipid, 1mg/reaction mixture; EDTA, 0.112mM or 0.055mM; inhibitors, as indicated in Tables 1–4; ferrous salt, 0.111mM. The pH of the final reaction mixture was 7.7. The reaction mixture was incubated at 37°C for measurement of thiobarbituric acid-reactivity (after 30min) as a non-specific but sensitive indication of the formation of lipid peroxide and fluorescence (after 2h) to detect the formation of secondary carbonyl complexes. For the former, thiobarbituric acid-reactivity was developed by adding 0.5ml of thiobarbituric acid reagent [1% (w/v) in 0.05M-NaOH] and 0.5ml of 25% (v/v) HCl (approx. 2.9M) to the reaction mixture; the tubes were heated for 10min at 100°C and then left to cool, and the resulting chromogen was extracted into 1.5ml of butan-1-ol and its absorbance read at 532nm. Fluorescence was read at 430nm, with excitation at 360nm, after the addition of 0.5ml of 1% (w/v) Lubrol to the incubated reaction mixtures. Units of fluorescence are expressed relative to a standard of tetraphenylbutadiene as previously described (Gutteridge, 1977*a*). The results shown are means for three separate assays, in which results differed by not more than 5%.

Results

The stimulatory effect of ferrous salts on phospholipid peroxidation can be enhanced by EDTA when the concentration of Fe^{2+} is greater than that of EDTA, as previously observed (Gutteridge *et al.*, 1979). Peroxidation, stimulated by ferrous salts alone, is not substantially inhibited by the OH· scavengers formate, ethanol, butan-1-ol, thiourea, glucose, mannitol and Tris (Table 1), in agreement with other studies (Gutteridge, 1982; Searle & Willson, 1983). Catalase and superoxide dismutase at concentrations of 0.03 and 0.006mg/ml similarly showed no significant inhibitory activity (Table 1). The phenolic antioxidant propyl gallate, however, was extremely

Table 1. *Effect of OH·-scavengers on Fe^{2+}-stimulated phospholipid peroxidation*

For experimental details see the text. All concentrations shown are final reaction concentrations. Fluorescence emission was measured at 430nm after excitation at 360nm. Percentage inhibition or stimulation was calculated after subtraction of the blank value. Abbreviations: SOD, superoxide dismutase; RFI, relative fluorescence intensity.

	Thiobarbituric acid-reactivity after 30min incubation		Fluorescence at 430nm after 2.0 incubation	
	A_{532}	Inhibition (I) or stimulation (S) (%)	RFI units	Inhibition (I) or stimulation (S) (%)
Blank: lipid only (subtracted)	(0.012)		(15)	
Control: lipid + Fe^{2+} (0.111mM)	0.258		10	
+ Formate (11.1mM)	0.304	18% S	12	20% S
+ Ethanol (11.1mM)	0.280	9% S	10	0%
+ Butan-1-ol (11.1mM)	0.258	0%	11	10% S
+ Urea (1.11mM)	0.244	6% I	11	10% S
+ Thiourea (1.11mM)	0.240	7% I	10	0%
+ Glucose (11.1mM)	0.310	20% S	15	50% S
+ Mannitol (11.1mM)	0.285	11% S	13	30% S
+ Tris (11.1mM)	0.278	8% S	10	0%
+ Propyl gallate (0.22mM)	0.064	75% I	0	100% I
+ SOD (0.03mg/ml)	0.228	12% I	9	10% I
+ Catalase (0.03mg/ml)	0.236	9% I	15	50% S
+ Albumin (0.03mg/ml)	0.225	15% I	11	10% S

Table 2. *Effect of iron chelators on Fe^{2+}- and Fe^{3+}-stimulated phospholipid peroxidation*

For experimental details see the text. Final reaction concentrations are shown. Fluorescence emission was measured at 430nm after excitation at 360nm. Percentage inhibition or stimulation was calculated after subtraction of the blank value. Abbreviations: DETAPAC, diethylenetriaminepenta-acetic acid; BAS, bathophenanthrolinesulphonate; DEFOM, desferrioxamine; RFI, relative fluorescence intensity units.

	Thiobarbituric acid-reactivity after 30min incubation				Fluorescence at 430nm after 2.0h incubation			
	Fe^{2+} (0.111mM)		Fe^{3+} (0.111mM)		Fe^{2+} (0.111mM)		Fe^{3+} (0.111mM)	
	A_{532}	Inhibition (I) or stimulation (S)	A_{532}	Inhibition (I) or stimulation (S)	RFI	Inhibition (I) or stimulation (S)	RFI	Inhibition (I) stimulation (S)
Blank: lipid only (subtracted)	(0.012)		(0.012)		(15)		(15)	
Control: lipid + iron salt	0.258		0.202		10		7	
+ EDTA (0.055mM)	0.426	65% S	0.245	21% S	37	270% S	14	100% S
+ DETAPAC (0.055mM)	0.415	60% S	0.228	13% S	34	240% S	9	29% S
+ BAS (0.055mM)	0.358	39% S	0.321	59% S	–*	–	–*	–
+ 2,2′-Dipyridyl (0.055mM)	0.450	74% S	0.443	119% S	40	300% S	14	100% S
+ Ferrozine (0.055mM)	0.408	58% S	0.231	14% S	52	420% S	7	0
+ DEFOM (0.055mM)	0.220	15% I	0.158	22% I	3	70% I	2	71% I

* Bathophenanthroline interfered with fluorescence measurements.

effective as an inhibitor at low concentrations (Table 1).

The iron-ion chelators EDTA, diethylenetriaminepenta-acetic acid, bathophenanthrolinesulphonate, dipyridyl and ferrozine stimulated lipid peroxidation when both Fe^{2+} and Fe^{3+} ions were present (Table 2). Desferrioxamine, however, under the same conditions was inhibitory. EDTA most effectively enhanced peroxidation, under the conditions described, when the ratio of Fe^{2+} to EDTA in the reaction was 1:0.5 (Fig. 1 and Table 3). This reaction too, was not inhibited by the $OH^{\bullet}$ scavengers; indeed, most were slightly stimulatory (Table 3). However inhibition by superoxide dismutase at concentrations of 0.03 and 0.006mg/ml suggests some participation of the superoxide radical ($O_2^{\bullet -}$) in the EDTA-enhanced peroxidation. At the same protein concentration catalase and albumin had no significant inhibitory effect. When the molar ratio of ferrous salt to EDTA in the reaction was unity, the reaction became sensitive to inhibition by the $OH^{\bullet}$ scavengers and by superoxide dismutase and catalase (Table 4). Urea and albumin were added as controls for non-specific scavenging effects.

The effect of formate on EDTA-enhanced lipid peroxidation is shown in Fig. 1. At high ratios of Fe^{2+} to EDTA, formate increased the rate of peroxidation; these reactions were not $OH^{\bullet}$-dependent. However, formate substantially inhibited peroxidation at equimolar concentrations of Fe^{2+} and EDTA (Fig. 1).

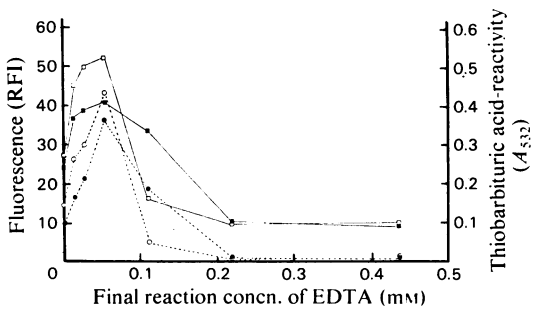


Fig. 1. *Effect of formate on ferrous-salt-stimulated lipid peroxidation at different EDTA concentrations*

For experimental details see the text. ■, Thiobarbituric acid-reactivity after incubation with Fe^{2+}/EDTA; □, thiobarbituric acid-reactivity after incubation with Fe^{2+}/EDTA + formate; ●, fluorescence RFI units (relative fluorescence intensity) after incubation with Fe^{2+}/EDTA; ○, fluorescence RFI units (relative fluorescence intensity) after incubation with Fe^{2+}-EDTA + formate.

Discussion

Ferrous salts in aerobic aqueous solution autoxidize with the formation of $OH^{\bullet}$ radicals, or a species with similar reactive properties (eqns. 7–9).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} - O_2^{\bullet -} \rightleftharpoons Fe^{3+} + O_2^{\bullet -} \quad (7)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad (9)$$

Table 3. *Effect of OH·-scavengers on Fe^{2+}/EDTA (1:0.5)-stimulated phospholipid peroxidation*
For experimental details see the text. All concentrations shown are final reaction concentrations. Fluorescence emission was measured at 430nm after excitation at 360nm. Percentage inhibition or stimulation was calculated after subtraction of the blank value. Abbreviations: SOD, superoxide dismutase; RFI, relative fluorescence intensity.

	Thiobarbituric acid-reactivity after 30min incubation		Fluorescence at 430nm after 2.0 incubation	
	A_{532}	Inhibition (I) or stimulation (S)	RFI units	Inhibition (I) or stimulation (S)
Blank: lipid + EDTA (0.055mM) (subtracted)	(0.016)		(15)	
Control: lipid + Fe^{2+} (0.111mM) + EDTA (0.055mM)	0.426		37	
+ Formate (11.1mM)	0.541	27% S	43	16% S
+ Ethanol (11.1mM)	0.455	7% S	34	8% I
+ Butan-1-ol (11.1mM)	0.422	1% I	28	24% I
+ Urea (1.11mM)	0.471	11% S	38	3% S
+ Thiourea (1.11mM)	0.455	7% S	28	24% I
+ Glucose (11.1mM)	0.516	21% S	39	5% S
+ Mannitol (11.1mM)	0.463	9% S	42	14% S
+ Tris (11.1mM)	0.438	3% S	40	8% S
+ Propyl gallate (0.22mM)	0.127	70% I	0	100% I
+ SOD (0.03mg/ml)	0.311	27% I	21	43% I
+ SOD (0.006mg/ml)	0.303	29% I	20	46% I
+ Catalase (0.03mg/ml)	0.406	5% I	46	24% S
+ Albumin (0.03mg/ml)	0.401	6% I	35	5% I

Addition of EDTA greatly accelerates the autoxidation of ferrous ions, whereas an Fe^{2+}–diethylenetriaminepenta-acetic acid complex only slowly autoxidizes. In contrast, bathophenanthrolinesulphonate, dipyridyl and ferrozine form relatively stable ferrous complexes with characteristic visible-region-absorption spectra. Desferrioxamine, however, which only binds Fe^{2+} ions, stimulates the rapid oxidation of Fe^{2+} ions by displacing the equilibrium between Fe^{2+} and Fe^{3+} ions in solution. When Fe^{3+} is complexed to desferrioxamine it cannot be readily reduced and so does not take part in radical reactions (Gutteridge *et al.*, 1979). Changes in iron-ion solubility, and the ability of the complexed iron to undergo redox-cycling, probably explain the enhancing effect of EDTA and other iron-ion chelators on lipid peroxidation when the reaction is not dependent on OH·-radical initiation. In addition, the enhancing effect of added OH· scavengers such as formate and glucose may suggest the formation of a reducing species from these scavengers.

When EDTA and Fe^{2+} ions are present in equimolar concentrations, lipid peroxidation appears to be substantially initiated by OH·. To achieve equimolar concentrations of Fe^{2+} ions and EDTA it may be necessary to add slightly more EDTA, since trace amounts of intrinsic iron will be present in spite of Chelex resin treatment of reagents. When the concentration of EDTA exceeds that of the Fe^{2+} ions, lipid peroxidation is substantially inhibited (Wills, 1965; Gutteridge *et al.*, 1979; Tien *et al.*, 1982). Superoxide dismutase has been observed to partially inhibit Fe^{2+}-stimulated lipid peroxidation (Gutteridge, 1977*b*), an activity that is not always destroyed by heat (Halliwell & Gutteridge, 1981). However, increased inhibition by superoxide dismutase when peroxidation is initiated by OH· suggests a role for $O_2^{\cdot-}$ in the formation of these radicals, possibly in the reduction of the Fe^{3+}–EDTA complex.

Inhibition by catalase of OH·-initiated peroxidation suggests that OH· radicals are formed by a Fenton-type reaction in which the Fe^{2+}–EDTA complex decomposes H_2O_2 (eqn. 9). Stimulation of thiobarbituric acid-reactivity and fluorescence formation by catalase when added to lipids have previously been described in detail and relate to the haem-iron moiety of the protein (Gutteridge *et al.*, 1983). EDTA can influence the reactivity of lipid peroxides in the thiobarbituric acid reaction as well as of unsaturated lipids in the incubation mixture (Gutteridge & Quinlan, 1983). Analyses were therefore carried out by both fluorescence and the thiobarbituric acid reaction, and results were shown to be in general agreement. Buffers were not used in these studies, to avoid metal-ion

Table 4. *Effect of OH'-scavengers on phospholipid peroxidation stimulated by equal concentrations of ferrous salt and EDTA*
For experimental details see the text. All concentrations shown are final reaction concentrations. Fluorescence emission was measured at 430nm after excitation at 360nm. Percentage inhibition or stimulation was calculated after subtraction of the blank value. Abbreviations: SOD, superoxide dismutase; RFI, relative fluorescence intensity.

	Thiobarbituric acid-reactivity after 30min incubation		Fluorescence at 430nm after 2.0h incubation	
	A_{532}	Inhibition (I) or stimulation (S)	RFI units	Inhibition (I) or stimulation (S)
Blank: lipid+EDTA (0.112mM) (subtracted)	(0.025)		(15)	
Control: lipid+Fe^{2+} (0.111mM) +EDTA (0.112mM)	0.365		17	
+Formate (11.1mM)	0.195	47% I	6	65% I
+Ethanol (11.1mM)	0.164	55% I	1	94% I
+Butan-1-ol (11.1mM)	0.171	53% I	1	94% I
+Urea (1.11mM)	0.372	2% S	14	18% I
+Thiourea (1.11mM)	0.219	40% I	4	76% I
+Glucose (11.1mM)	0.229	37% I	8	53% I
+Mannitol (11.1mM)	0.178	51% I	4	76% I
+Tris (11.1mM)	0.253	31% I	6	65% I
+Propyl gallate (0.22mM)	0.083	77% I	0	100% I
+SOD (0.03mg/ml)	0.164	55% I	5	71% I
+SOD (0.006mg/ml)	0.188	49% I	6	65% I
+SOD (heat-denatured) (0.03mg/ml)	0.185	49% I	14	18% I
+Catalase (0.03mg/ml)	0.188	49% I	8	53% I
+Catalase (0.006mg/ml)	0.246	33% I	9	47% I
+Catalase (heat-denatured) (0.03mg/ml)	0.348	5% I	38	123% S
+Albumin (0.03mg/ml)	0.348	5% I	15	12% I
+Albumin (0.006mg/ml)	0.375	3% S	16	6% I
+Albumin (heat-denatured) (0.03mg/ml)	0.379	4% S	15	12% I

complexing and OH'-scavenging effects associated with all buffers likely to be useful in the physiological pH range.

The results described here suggest that EDTA can enhance ferrous-salt-stimulated phospholipid peroxidation by at least two different mechanisms, one of which involves hydroxyl radicals (Scheme 1).

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