The role of superoxide and hydroxyl radicals in phospholipid peroxidation catalysed by iron salts

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Iron(II) salts in aqueous solution, or iron(III) salts in the presence of an $O_2^{\cdot-}$ generating system, can activate dioxygen to produce hydroxyl radicals. These are detected indirectly by their ability to degrade deoxyribose with the formation of thiobarbituric acid-reactive (TBA) products. Iron salts also catalyse the peroxidation of phospholipids resulting in the formation of TBA-reactive products. Hydroxyl radicals were responsible for the degradation of deoxyribose but not for the observed peroxidation of phospholipid. The function of $O_2^{\cdot-}$ in both deoxyribose degradation and phospholipid peroxidation seems to be that of reducing iron(III) into iron(II).

Iron salt dependent hydroxyl radical damage *Iron-catalysed lipid peroxidation* *Superoxide dismutase* *Caeruloplasmin* *TBA-reactivity* *Deoxyribose degradation*

1. INTRODUCTION

Lipid peroxidation is a complex process characterised by three distinct phases: a slow induction period, a rapid autocatalytic phase and a slow termination phase. Free radicals capable of abstracting hydrogen atoms from unsaturated fatty acids, such as the hydroxyl radical, induce lipid peroxidation whereas transition metal ions catalyse propagation reactions. Metal ions of particular interest to biological systems are iron and copper and their various complexes.

Ferrous salts in aerobic aqueous solution readily autoxidise forming active oxygen species (fig. 1) such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$). Of these, only hydroxyl radicals have sufficient reactivity to abstract hydrogen atoms from unsaturated fatty acids. Since ferrous ions are considerably more effective in promoting lipid peroxidation in vitro than are ferric ions, it is widely thought that hydroxyl radicals formed by the reactions shown in fig. 1 play an important part in initiating further lipid peroxidation. Various groups have examined

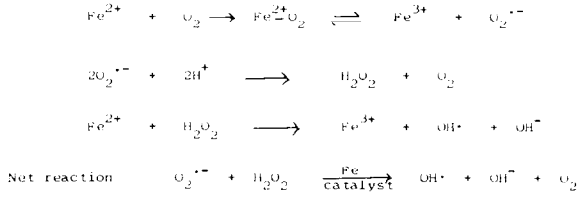

Fig. 1.

the contribution of inorganic oxygen radicals to the process of lipid peroxidation, catalysed by iron salts and their complexes, with differing results [1–4].

Here, hydroxyl radicals were shown to be formed in aqueous solution by the iron-dependent activation of dioxygen. Their damaging effects on both deoxyribose and phospholipids were compared under the same reaction conditions.

2. MATERIALS AND METHODS

Superoxide dismutase (bovine erythrocyte, 3000 units/ml), catalase (bovine liver, 27 000 units/ml),

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xanthine oxidase (grade I, 28.1 units/ml), albumin (human fatty acid free), 2-deoxy-D-ribose, hypoxanthine and caeruloplasmin (type III human) were obtained from the Sigma Chemical Co. Units of enzyme activity were as defined in the Sigma catalogue. Desferrioxamine was from Ciba-Geigy. All other chemicals were of Analar® grade, where available, and obtained from BDH Ltd.

2.1. *Peroxidation of phospholipids*

Bovine brain phospholipid liposomes were prepared as in [5]. This lipid material is a fraction, containing a high percentage of unsaturated fatty acid-containing phospholipids. By careful preparation and storage it has been found to have a low level of pre-formed lipid peroxides.

Liposomes were prepared as multilamellar vesicles, 5 mg phospholipid in 1 ml 0.15 M NaCl (pH 7.4) was vigorously shaken for 2 min in the presence of glass beads. 0.2 ml of liposomal suspension together with 0.2 ml phosphate saline buffer (0.024 M phosphate, 0.15 M NaCl) (pH 7.4) or 0.2 ml 0.15 M NaCl (pH 7.4) as indicated in the different experiments, were added to new, clean glass tubes. 0.1 ml of appropriate radical scavengers or metal chelators was added at this stage followed by, where indicated, 0.1 ml H_2O_2 or 0.2 ml hypoxanthine (saturated aqueous solution). 0.1 ml of either ferric chloride or ammonium ferrous sulphate were added as iron salts. The ferric chloride-hypoxanthine reaction was initiated by the addition of 30 μl of xanthine oxidase (0.7 units/ml). The vol. of each tube was made to 0.8 with Chelex resin-treated distilled water, and the samples incubated at 37°C for 1 h.

2.2 *Degradation of deoxyribose*

Deoxyribose, 0.2 ml, 5 mM, was substituted for phospholipid liposomes in the above reactions and then treated in exactly the same way. All reagents were prepared with Chelex resin-treated distilled water. Solutions of reduced iron salts were purged with nitrogen. All solutions of iron salts were used within 4 min of preparation.

Table 1

Ferrous salt-dependent damage to phospholipids and deoxyribose

	Phospholipids				Deoxyribose	
	TBA-reactivity		Fluorescence		TBA-reactivity	
	A_{532}	% Inhib.	RFI units Ex. 360 Em. 430	% Inhib.	A_{532}	% Inhib.
Blank (lipid or deoxyribose)	0.03		14		0	
Control (Fe^{2+} 0.12 mM)	0.30		43		0.26	
(less PO_4)	0.31		21		0.13	
+ catalase 0.06 mg/ml	0.34	0	47	0	0.07	73
+ SOD 0.06 mg/ml	0.21	30	36	24	0.27	0
+ Caeruloplasmin 0.12 mg/ml	0.12	60	27	55	0.08	69
+ Albumin 0.12 mg/ml	0.25	16	40	10	0.25	4
+ Mannitol 12.5 mM	0.31	0	39	14	0.04	85
+ Thiourea 1.2 mM	0.29	3	37	21	0.12	54
+ Urea 1.2 mM	0.32	0	41	7	0.27	0
+ Desferrioxamine 0.25 mM	0.05	83	16	93	0.04	85
+ Propyl gallate 0.12 mM	0.06	80	13	100	0.04	85

All samples contained 6 mM phosphate. Results are expressed as a mean of 2 separate assays and calculated after subtraction of the blank. SOD, superoxide dismutase; Inhib., Inhibition

2.3. *TBA-reactivity*

TBA-reactivity for free radical damage to lipids and carbohydrates was determined as in [6]. Following incubation, phospholipid TBA-reactivity was developed by heating for 15 min at 100°C after adding 0.5 ml 1% TBA in 0.05 M NaOH and 0.5 ml 25% (v/v) HCl, and deoxyribose TBA-reactivity by the use of 2.8% (w/v) trichloroacetic acid instead of HCl. Resulting chromogens were measured at A_{532} nm against appropriate blanks.

2.4. *Phospholipid autofluorescence*

Following incubation of liposomes, 1 ml of 0.15 M NaCl was added to each tube, followed by 1 ml of methanol and 2 ml of chloroform. Tubes were then vigorously vortex-mixed for 1 min to extract fluorescent material. After centrifugation, to separate the phases, the upper aqueous-methanol phase was removed and discarded. The lower chloroform phase was 'cleared' by the addition of 0.4 M methanol and the fluorescence measured against a reference block standard containing 10^{-7} M tetraphenylbutadiene set to 100 units at excitation 350 nm and emission 440 nm, sensitivity × 1. Relative fluorescence intensity (RFI) in the samples was measured at excitation 360 nm and emission 430 nm, sensitivity × 3 [7].

3. RESULTS

Ferrous salts in aerobic solutions cause deoxyribose degradation in a reaction inhibited by catalase and hydroxyl radical scavengers but not by superoxide dismutase (table 1). Peroxidation of phospholipids, although stimulated by ferrous ions, was not similarly inhibited by catalase or hydroxyl radical scavengers (table 1). Superoxide dismutase offered some protection as in [8] whereas catalase slightly stimulated peroxidation.

Ferric salts alone induced a little peroxidation

Table 2

Ferric salt-dependent damage to phospholipids and deoxyribose in the presence of a superoxide generating system

	Phospholipids				Deoxyribose	
	TBA-reactivity		Fluorescence		TBA-reactivity	
	A_{532}	% Inhib.	RFI units Ex. 360 Em. 430	% Inhib.	A_{532}	% Inhib.
Blank (lipid or deoxyribose)	0.03		12		0	
Fe^{3+} 0.06 mM	0.07		17		0	
Hypoxanthine/XOD only	0.09		17		0.20	
Control Fe^{3+}/O_2^- (less PO_4)	0.35		35		0.10	
Control Fe^{3+}/O_2^-	0.40		49		0.72	
+ Catalase 0.06 mg/ml	0.38	5	55	0	0.06	92
+ SOD 0.06 mg/ml	0.06	85	13	97	0.05	93
+ Caeruloplasmin 0.12 mg/ml	0.17	58	21	76	0.06	92
+ Albumin 0.12 mg/ml	0.44	0	51	0	0.78	0
+ Mannitol 12.5 mM	0.46	0	52	0	0.06	92
+ Thiourea 1.2 mM	0.39	2	49	0	0.33	54
+ Urea 1.2 mM	0.39	2	49	0	0.81	0
+ Desferrioxamine 0.25 mM	0.02	95	14	95	0.06	92
+ Propyl gallate 0.12 mM	0.01	98	12	100	0.04	95

All samples contained 6 mM phosphate. Results are expressed as means of 2 separate assays and calculated after subtraction of the blank value. XOD, xanthine oxidase; SOD, superoxide dismutase

but no deoxyribose degradation. The simultaneous presence of a ferric salt and an $O_2^{\cdot-}$ generating system caused both processes to occur (table 2). Deoxyribose degradation was inhibited by catalase and hydroxyl radical scavengers, whereas phospholipid peroxidation was not (table 2), but both processes were inhibited by superoxide dismutase.

A mixture of ferrous salts and hydrogen peroxide is a known source of hydroxyl radicals and resulted in substantial degradation of deoxyribose (table 3). This reaction was inhibited by catalase and OH$^{\cdot}$ radical scavengers. Phospholipid peroxidation, however, was hardly affected by the greatly increased generation of OH$^{\cdot}$ radicals (table 3).

4. DISCUSSION

The addition of ferrous salts to an aqueous solution produces hydroxyl radicals as shown by deoxyribose degradation [9]. This reaction is inhibited by catalase and OH$^{\cdot}$ radical scavengers [10,11]. Similarly, ferric salts together with $O_2^{\cdot-}$ produce OH$^{\cdot}$ radicals in a reaction which is inhibited by superoxide dismutase [11]. Here, the superoxide radicals reduce ferric ions to the ferrous state. Ferrous salts, or ferric salts with $O_2^{\cdot-}$, promote lipid peroxidation in a reaction which is not OH$^{\cdot}$ radical-dependent. In this $O_2^{\cdot-}$ generating system ferric ions are also being reduced to the ferrous form since the reaction is inhibited by superoxide dismutase.

Radiation studies have shown that hydroxyl radicals can induce lipid peroxidation [12]. However, this is not the species responsible for the observed iron salt-dependent reaction. The species promoting lipid peroxidation has not yet been identified, the perferryl complex (Fe^{2+}–O_2) has been proposed [4,13] but this species is an unlikely candidate since it is poorly reactive, a more likely

Table 3

Ferrous salt-dependent damage to phospholipids and deoxyribose in the presence of hydrogen peroxide

	Phospholipids				Deoxyribose	
	TBA-reactivity		Fluorescence		TBA-reactivity	
	A_{532}	% Inhib.	RFI units Ex. 360 Em. 430	% Inhib.	A_{532}	% Inhib.
Blank (lipid or deoxyribose)	0.03		14		0	
Fe^{2+} 0.12 mM only	0.31		35		0.13	
H_2O_2 0.12 mM only	0.03		15		0	
Control Fe^{2+}/H_2O_2 (plus 6 mM PO_4)	0.14		27		0.48	
Control Fe^{2+}/H_2O_2	0.26		41		0.91	
+ Catalase 0.06 mg/ml	0.32	0	41	0	0.05	95
+ SOD 0.06 mg/ml	0.20	23	32	33	0.67	26
+ Caeruloplasmin 0.12 mg/ml	0.17	35	27	52	0.56	38
+ Albumin 0.12 mg/ml	0.31	0	30	37	0.70	23
+ Mannitol 12.5 mM	0.34	0	45	0	0.09	90
+ Thiourea 1.2 mM	0.34	0	42	0	0.08	91
+ Urea 1.2 mM	0.24	8	39	7	0.92	0
+ Desferrioxamine 0.25 mM	0.07	73	18	85	0.09	90
+ Propyl gallate 0.12 mM	0.05	81	19	81	0.08	91

Samples were phosphate free. Results are expressed as means of 2 separate assays. SOD, superoxide dismutase

reactant being a ferryl species (FeO^{2+}) [14].

Caeruloplasmin has been found to be a powerful inhibitor of lipid peroxidation and some other oxygen radical reactions [15,16]. The mechanisms by which caeruloplasmin inhibits oxygen radical reactions is not entirely clear, but may well involve its ferroxidase activity [15]. It has been suggested that caeruloplasmin can scavenge $OH^{\cdot}$ radicals [17]. However, such a role would not explain its protection against lipid peroxidation seen here.

Ferrous ions will react with lipid hydroperoxides (ROOH) to form alkoxy radicals ($RO^{\cdot}$), whereas ferric ions will react to form peroxy radicals ($ROO^{\cdot}$). The former are more reactive than peroxy radicals and might explain the greater stimulatory effects seen with ferrous ions. However, cupric salts equimolar to ferrous salts are more stimulatory towards lipid peroxidation [18]. Since cupric ions would be expected to form peroxy radicals it is unlikely that ferrous salt stimulation can simply be attributed to alkoxy radical formation.

The hydroxyl radical plays no part in the observed peroxidation of phospholipids catalysed by iron salts even though it is formed in the reaction mixture. The iron–oxygen complex responsible for promoting lipid peroxidation still remains to be identified.

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