The Protective Action of Superoxide Dismutase on Metal-ion Catalysed Peroxidation of Phospholipids

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SUMMARY

The peroxidation of ox-brain phospholipid liposomes catalysed by oxidised and reduced iron and copper ions have been studied by the measurement of fluorescent non-polar MDA-amino complexes. The Inhibitory activity of superoxide dismutase when added to the system implicated superoxide radicals as important intermediates in both reduced and oxidised metal-ion catalysed peroxidation of lipids.

INTRODUCTION

The ions of a number of transition metals are effective catalysts for the rapid peroxidation of unsaturated lipids. These metals all occur in two valence states which are interconvertible by a single electron transfer. Their catalytic peroxidative activity has mainly been ascribed to their ability to accelerate the decomposition of hydroperoxides and to a lesser extent chain initiation following oxygen activation (1,2,3). The initiating sequence is difficult to study by normal laboratory techniques most of which have to rely on the measurement of secondary lipid peroxidation products formed as a result of the chain propagation steps.

Following the discovery of an oxygen free-radical dismutating enzyme it has been possible to study with a certain degree of specificity the involvement of univalently reduced oxygen in autoxidative reactions (4).

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The exact role of the superoxide radical as an intermediate in the initiation of lipid peroxidation is however not altogether clear either in the presence or absence of metal ions (5,6,7).

In this paper the effect of metal-ions on the peroxidation of ox-brain phospholipid model membranes and the involvement of superoxide radicals have been studied by the measurement of non-polar MDA-amino fluorescent complexes. The results suggest that the superoxide radical is an important intermediate in both reduced and oxidised metal-ion catalysed lipid peroxidation.

MATERIAL & METHODS

Ammonium ferrous and ferric sulphates, cuprous and cupric chlorides were of 'Analar' grade. Solvents were of spectroscopic grade and obtained from BDH Ltd. Superoxide Dismutase (bovine erythrocytes) Miles Ltd. Caeruloplasmin (human) Sigma Ltd. Ox-brain Phospholipid (Folch extract) was a white powder free from fluorescent material prepared by rapid extraction from brain tissue (8).

Liposomes containing 5 mg/ml ox-brain phospholipid in 0.15 M NaCl adjusted to pH 7.4 with sodium bicarbonate, were prepared after purging with nitrogen by vigorous vortexing for 5 min. They were allowed to stand for 1 hr at 4°C before use. The metal-ions were dissolved in deionised water and the reduced metal-ions purged with oxygen-free nitrogen. The enzymes superoxide dismutase and caeruloplasmin (ferroxidase) were dissolved in 0.15 M NaCl for use. Heat inactivation was by boiling the dilute working reagent for 5 min.

Peroxidation was carried out in a 10 ml glass tube following the addition of 0.5 ml of phospholipid liposomes. For each metal-ion 4 tests and 1 control were set up to duplicate. The 100% reaction control tube was made to a final volume of 1 ml with 0.15 M NaCl after the addition of 0.1 ml of metal-ion catalyst. The tests for both enzymes were paired

TABLE 1

Metal-ion Catalyst	Fluorescence units per mg / PL*	% INHIBITION OF PHOSPHOLIPID PEROXIDATION			
		Superoxide dismutase 10 μg/ml	Superoxide dismutase 10 μg/ml boiled	Ferroxidase 10 μg/ml	Ferroxidase 10 μg/ml boiled
Ferrous ions 0.1 mM	10.0	40	6	15	2
Ferric ions 0.1 mM	3.3	46	8	42	6
Cuprous ions 0.05 mM	4.2	59	11	0	0
Cupric ions 0.05 mM	14.2	63	2	0	0

*PL=Phospholipid

tubes containing 0.1 ml of enzyme and 0.1 ml boiled enzyme, followed by addition of 0.1 ml of the metal catalyst and adjustment of the final volume to 1.0 ml with saline. The tubes were capped and incubated at 37°C for 2 hr in a shaking waterbath.

The degree of peroxidation was measured spectrofluorimetrically (9) using a Perkin-Elmer MPF-3. After incubation 1 ml of 0.15 M NaCl was added to each tube followed by 3.0 ml chloroform : methanol (2:1 v/v). The tubes were mixed vigorously for 2 min to extract the non-polar MDA-amino fluorescent complexes, followed by centrifugation to separate the two phases. The upper phase was removed and discarded and 0.3 ml of methanol added to clear the lower layer which was read in the spectrofluorimeter with the following instrument settings: Emission WL scanned from 420 - 440 nm (slit width 10 nm), excitation WL 360 nm (slit width 10 nm), sensitivity x 3, scan speed 4, filter 390 nm. The instrument was standardised with reference to quinine sulphate 1 μg / ml in 0.1 N H_2SO_4.

RESULTS

Table 1 summarises both the degree of peroxidation catalysed by the metal-ions together with the percentage inhibition brought about by superoxide dismutase. These results are expressed as a mean of 5 duplicate assays.

Both reduced and oxidised iron and copper ions were effective catalysts for the peroxidation of ox-brain phospholipid liposomes. The divalent cations were the most effective catalysts when non-polar peroxidation products were measured spectrofluorimetrically. This data was also confirmed by measurement of polar thiobarbituric acid-reacting (TBA) compounds whose formation were similarly inhibited by superoxide dismutase. However with the TBA test, cupric-ions appeared to be less effective catalysts of lipid peroxidation than ferrous ions reversing the pattern seen with non-polar compounds (8). Ferric and cuprous ions were not so active peroxidation catalysts, the latter have low

solubility at neutral pH and are difficult to prepare in aqueous solution rapidly for addition to the liposomes.

The inhibitory activity of superoxide dismutase at a concentration of 10 μg / ml implicated the superoxide radical as an intermediate in the metal-ion catalysed peroxidation of phospholipid model membranes. Heat inactivation of the superoxide dismutase almost completely abolished its inhibitory activity.

The addition of albumin and a variety of metal containing proteins at the same concentration were not inhibitory. When peroxidation was catalysed by 1.0 mM ascorbic acid (12 fluorescence units /mg phospholipid) it was not inhibited by superoxide dismutase. Ferroxidase (10 μg/ml) was as inhibitory as superoxide dismutase when added to ferric catalysed peroxidation.

DISCUSSION

There is convincing evidence that redox reactions take place between transition metal ions and peroxidic compounds in biological systems with the formation of free-radicals. The autoxidation of a reduced metal ion with the formation of the hydroperoxyl radical ($HO_2^{\bullet}$) and its anion the superoxide radical ($O_2^{\bullet -}$) via intermediates such as the perferryl ion have been implicated in the direct activation of oxygen (3, 10, 11). The catalytic role of oxidised metal-ions is however thought to be one of accelerated hydroperoxide decomposition with the formation of the peroxy free-radical ($RO_2^{\bullet}$).

More recently the discovery of an oxygen-radical dismutating enzyme in all respiring cells (12) has by implication suggested that free-radicals are widely formed during metabolic processes by direct oxygen activation. Free-radicals are an extremely

active chemical species likely to react indiscriminately with macromolecules vital to cell structure and function. The way in which they arise in biological systems and their reaction with cell components are of considerable interest in both physiological and pathological states such as ageing and disease.

The involvement of superoxide radicals directly or as intermediates in the peroxidation of cell lipids has become clouded by a variety of reaction conditions in the presence and absence of metal-ions, chelated forms of iron, different buffers, substrates and methods of detection (7, 13,14). Some of the differences could be explained by changes in the 'susceptibility' of membrane lipids which can be induced by various metal-ions. These changes (also influenced by the lipid composition of the substrate) are possibly in the conformation of the membrane phospholipids (15).

The enzyme ferroxidase (caeruloplasmin) can catalyse the oxidation of ferrous ions to the ferric state. This property may account for most of its apparent antioxidant activity in human serum by fixing the valence state of the metal for binding to the specific protein transferrin (16). When added to ferric catalysed peroxidation it was as inhibitory as superoxide dismutase suggesting that the following overall reaction did occur under these conditions with traces of hydroperoxide in the substrate :-

(a) $Fe^{3} + ROOH \longrightarrow Fe^{2} + RO_2^{\bullet} + H^{+}$

(b) $Fe^{2} + O_2 \longrightarrow Fe^{3} + O_2^{\bullet -}$

Ascorbic acid catalysed peroxidation in the absence of metal ions is thought to involve the monodehydroascorbate radical (17). Superoxide dismutase had no inhibitory effect on liposomal peroxidation catalysed by 1.0 mM ascorbate confirming that the superoxide radical was not involved. It also partly served as a control for the

presence of non-specific antioxidants in the enzyme preparation.

Recent studies show that the superoxide radical is an important intermediate in the formation of singlet oxygen ($^1O_2$) and the hydroxyl radical ($OH^\bullet$) (7, 18, 19) both of which are capable of initiating lipid peroxidation. Singlet oxygen can arise in the absence of superoxide dismutase, catalase, and peroxidases by the spontaneous dismutation of superoxide radicals (20) or by the Haber-Weiss reaction in which superoxide anions react with hydrogen peroxide (7).

From the data obtained under the conditions described it appears that transition metal-ions undergoing reversible oxidation and reduction owe much of their catalytic peroxidative activity to direct oxygen activation via univalently reduced oxygen. In addition to oxygen activation and peroxide decomposition metal-ions may also play an important rôle by inducing conformational changes in membrane lipids.

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