ROLE OF SUPEROXIDE RADICALS IN THE LIPID PEROXIDATION OF INTRACELLULAR MEMBRANES

D. D. TYLER

Division of Biochemistry, Royal Veterinary College, University of London, London, N.W.1, England

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1. Introduction

Mainly due to the work of Fridovich [1], mammalian cells are known to contain several enzymes, including xanthine oxidase, that generate superoxide radicals (O_2^-); and also to contain the enzyme superoxide dismutase (SOD), which destroys O_2^- by catalysing reaction (1).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

Superoxide radicals are involved in mechanisms of lipid peroxidation which may be concerned in the process of aging and turnover of intracellular components [2–4]. Fong et al. [4] have recently suggested that the radical-like agent responsible for the peroxidation and lysis of purified lysosomes, incubated with xanthine, xanthine oxidase and chelated iron, is the hydroxyl radical (.OH) formed from O_2^- according to reaction (2); (see [5]).

$$O_2^- + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + .OH \qquad (2)$$

In the present communication, it is shown that the rapid O_2^--dependent lipid peroxidation of liver subcellular fractions occurs by a mechanism not involving .OH radicals. It is concluded, in agreement with a recent paper [6], that .OH radical formation in liver cells is unlikely to be of physiological significance.

2. Materials and methods

Beef liver catalase (thymol-free), ADP, xanthine (sodium salt) and xanthine oxidase (Grade 1) were obtained from the Sigma Chemical Company and other reagents from British Drug Houses Ltd. AR grade reagents were used whenever possible. Digitonin was recrystallized from ethanol before use.

Subcellular fractions were isolated from rat liver in 0.25 M sucrose and 5 mM Tris–Cl buffer, pH 7.4, according to the method of Duve et al. [7]. When boiled subcellular fractions were prepared for incubation, they were first disrupted by freezing and thawing. Inner membrane-matrix particles were prepared from the heavy mitochondrial fraction, using sucrose–Tris solution containing 0.5 mg/ml bovine plasma albumin, by the method of Schnaitman and Pedersen [8]. Superoxide dismutase was prepared by the method of McCord and Fridovich [9]. Denatured enzymes were prepared by boiling dilute, unbuffered enzyme solution for 10 minutes and cooling the solution to 1°C. The lipid peroxidation assays used 2 ml samples of stock suspensions of each subcellular fraction, containing 0.27 mg of protein/ml in 0.12 M KCl and 20 mM Tris–Cl, pH 7.4. When boiled fractions were used, before sampling, the stock suspension in KCl–Tris buffer and 10 μM EDTA was stirred in a flask placed in a boiling water bath for 10 min, cooled and homogenized to disperse the denatured protein and re-aerate the suspension. In the complete system, samples of the stock suspension also contained: ADP, 1.5 mM; $FeCl_3$, 0.1 mM; xanthine, 0.4 mM; and xanthine oxidase, 0.015 Sigma units (20–30 μg of protein). The samples were incubated for 30 min at 36°C before the measurement of lipid peroxide formation by a thiobarbituric acid (TBA) method [10]. Oxygen uptake and catalase activity were assayed with a Clark oxygen electrode [11]. Protein was estimated by the biuret method [12].

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3. Results and discussion

When samples of heavy mitochondrial fraction were incubated with xanthine and xanthine oxidase, a significant lipid peroxidation was observed only when ferric iron was added to the reaction mixture (table 1).

The maximal effect of added iron was obtained in the presence of added ADP, which presumably served to chelate the iron and maintain it in solution. In the presence of ADP, added iron had a half-maximal effect at a concentration of about 13 μM. When xanthine or xanthine oxidase was omitted, or when denatured xanthine oxidase was used, the peroxidation observed was similar to that obtained with added ADP-Fe^{3+} alone, indicating that rapid peroxidation was dependent on xanthine oxidase activity. In the presence of 0.15 mM $MnCl_2$, a potent inhibitor of lipid peroxidation [13], the TBA colour of all samples was similar to that obtained with no further addition made to the reaction mixture. Thus the increase in TBA colour, obtained in response to the various additions shown in table 1, can be attributed specifically to a lipid peroxidation reaction. This conclusion is supported by measurements showing that an increase in the TBA colour was paralleled by an increase in oxygen uptake (table 1). The rapid lipid peroxidation observed in the complete system was abolished by SOD, but unaffected by denatured SOD (fig.1) and was therefore entirely dependent on superoxide ions generated by xanthine oxidase activity. SOD also inhibited the extra oxygen uptake associated with rapid lipid peroxidation (table 1) and partly inhibited lipid peroxidation induced by ADP-Fe^{3+} alone (fig.1).

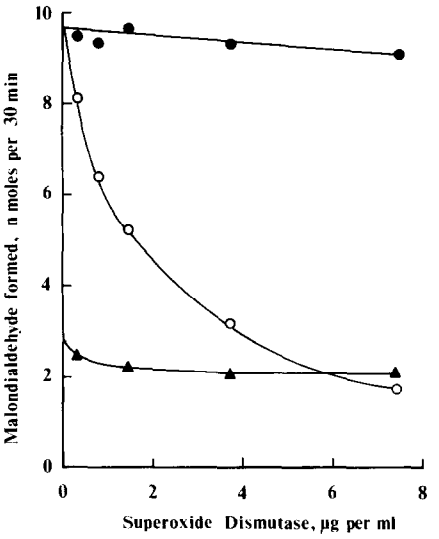

Fig.1. Effect of SOD on the lipid peroxidation of heavy mitochondrial fraction. Reaction mixtures contained the complete reaction mixture specified in the Materials and methods section. ○, SOD; ●, denatured SOD; ▲, with SOD but omitting xanthine oxidase.

Lipid peroxidation of the heavy mitochondrial fraction is similar to that induced by Fe^{2+} [10], since both phenomena result in extensive mitochondrial swelling and the release of matrix enzymes. When

Table 1
Lipid peroxidation of heavy mitochondrial fraction during xanthine oxidase activity

Additions to stock suspension	Malondialdehyde formed (nmol/30 min)	Oxygen uptake (nmol/30 min)
No further addition	1.0	
Complete system	10.1	320 (190*)
Complete omitting Fe^{3+}	1.5	
Complete omitting ADP	4.6	
Complete omitting ADP, Fe^{3+}	1.0	170
Complete omitting Xanthine	2.8	
Complete omitting xanthine oxidase	2.6	
Complete omitting xanthine and oxidase	2.6	30

* with 25 μg/ml SOD present

xanthine, xanthine oxidase and Fe^{3+} were replaced by Fe^{2+}, lipid peroxidation was unaffected by SOD. These observations suggest that rapid lipid peroxidation (table 1) is due to the formation of Fe^{2+} according to reaction (3).

$$ADP\text{-}Fe^{3+} + O_2^- \longrightarrow ADP\text{-}Fe^{2+} + O_2 \quad (3)$$

The occurrence of reaction (3) is supported by the finding that ADP-Fe^{3+} inhibits cytochrome c reduction by O_2^-, in agreement with previous results [4].

When the experiment of table 1 was repeated using inner membrane-matrix particles instead of heavy mitochondrial fraction, the rate of lipid peroxidation induced by ADP-Fe^{3+} alone was either unchanged or stimulated only slightly by the further addition of xanthine and xanthine oxidase. In these experiments lipid peroxidation during xanthine oxidase activity was stimulated considerably by catalase, whereas denatured catalase had no effect (fig.2). Lipid peroxidation was also stimulated to a lesser extent by benzoate or ethanol, which are known to be .OH radical trapping agents [14]. The stronger effect of benzoate compared to ethanol (fig.2) is consistent with the observation that the .OH radical reacts faster with benzoate than with ethanol [14]. The effect of these compounds was largely or totally abolished by SOD, but not by denatured SOD, as in the previous experiments. In the presence of added catalase, the rate of lipid peroxidation observed was similar to that found with samples of heavy mitochondrial fraction. The amount of added catalase required to induce a near-maximal effect was equivalent to the amount of catalase removed by digitonin treatment during the preparation of the inner membrane-matrix particles (fig.2). Thus in these experiments, the ability of xanthine oxidase activity to induce rapid lipid peroxidation depended on catalase activity. The greater stimulation of peroxidation caused by catalase, compared to benzoate and ethanol, is explained by the fact that removal of H_2O_2 by catalase blocks O_2^- consumption by both reactions (2) and (4) below [1,5], whereas the trapping of .OH radicals by benzoate or ethanol blocks only the consumption of O_2^- by reaction (4):

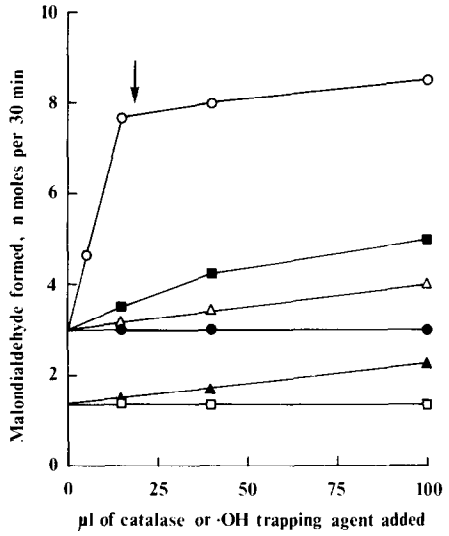

Fig.2. Lipid peroxidation of the inner membrane-matrix fraction. Reaction mixtures contained the complete reaction mixture and additional components as follows: ○, catalase; ■, K benzoate; △, ethanol, ●, boiled catalase; ▲, catalase with 15 µg/ml SOD: □,K benzoate or ethanol with 15 µg/ml SOD. The stock solutions of catalase or .OH trapping agent used contained: catalase, 0.2 mg/ml; benzoate, 0.4 M; ethanol, 16 M. The vertical arrow indicates the amount of added catalase equal in activity to the catalase removed during the preparation of the inner membrane-matrix particles.

$$O_2^- + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + .OH \quad (2)$$
$$.OH + O_2^- \longrightarrow OH^- + O_2 \quad (4)$$

Catalase, benzoate and ethanol can therefore stimulate lipid peroxidation by diverting O_2^- away from reactions (2) and (4) and into reaction (3).

These conclusions are supported by the results of similar experiments using subcellular fractions before and after depletion of their catalase activity by boiling. Catalase and benzoate had little or no effect on the lipid peroxidation of non-boiled samples, but stimulated the peroxidation of boiled samples (table 2). Lipid peroxidation of boiled samples was also stimulated by ethanol, or by mannitol, another .OH trapping agent [14], and was inhibited by SOD.

The results of all these experiments indicate that even when catalase activity is low or absent, the formation of .OH radicals fails to initiate significant lipid peroxidation despite the fact that, under these conditions, .OH radical formation appears to occur more readily than the reduction of ferric iron. Most mammalian cells appear to contain sufficient catalase and SOD to control O_2^--dependent lipid peroxidation

Table 2
Effect of boiling on the lipid peroxidation of subcellular fractions

Fraction	Additions to complete system	Malondialdehyde formed (nmol/30 min)			
		Non-boiled		Boiled	
		– SOD	+	– SOD	+
H	None	10.3	1.9	4.8	2.0
	Catalase	11.0	2.9	10.5	3.0
	Benzoate	10.4	2.0	7.9	2.1
L	None	8.3	1.8	2.7	1.6
	Catalase	8.3	2.1	6.6	1.9
	Benzoate	8.5	1.9	4.7	1.6
P	None	17.3	2.9	9.8	2.2
	Catalase	17.3	3.1	14.7	4.8
	Benzoate	17.0	2.8	11.7	2.3

H, heavy mitochondrial fraction; L, light mitochondrial fraction; P. microsome fraction. Additions made as indicated, were catalase 10 μg/ml; SOD, 10 μg/ml; K benzoate, 19 mM.

mediated either through iron-dependent or .OH-dependent reactions and no metabolic defect is found even in catalase deficient individuals [15]. If intracellular O_2^--dependent lipid peroxidation does contribute to the turnover of membrane poly-unsaturated fatty acids or initiate membrane lesions under pathological conditions, it seems probable that the process involves iron catalysis [16] rather than .OH radical formation.

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