Vol. 117, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 28, 1983 Pages 901-907

SUPEROXIDE-DEPENDENT LIPID PEROXIDATION. PROBLEMS WITH THE USE OF CATALASE AS A SPECIFIC PROBE FOR FENTON-DERIVED HYDROXYL RADICALS

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Received November 14, 1983

Summary. Hydroxyl radicals (OH·) can initiate lipid oxidation by hydrogen abstraction. Transition metals however, particularly iron and copper, stimulate lipid oxidation by reacting with lipid peroxides to form new radical species. The haem-iron protein catalase can react non-specifically with lipid peroxides in this way resulting in loss of their conjugated diene structures. When a superoxide-generating system is used to stimulate lipid autoxidation, catalase can conceivably inhibit the reaction in two ways (A) by decomposing lipid peroxides as they are formed (B) through the removal of hydrogen peroxide preventing OH· radical formation. Results presented here suggest that the latter interpretation, although commonly presented, cannot be automatically assumed.

Free radicals able to abstract hydrogen atoms from unsaturated fatty acids (LH) can initiate lipid oxidation. After initiation a rapid propagation phase, involving oxygen uptake, follows with formation of peroxy radicals ($LO\cdot_2$). These further react with lipid and give rise to stable lipid hydroperoxides (LOOH) [equations 1-3]

$$LH + OH\cdot \longrightarrow L\cdot + H_2O \quad [1]$$

$$L\cdot + O_2 \longrightarrow LO_2\cdot \quad [2]$$

$$LO_2\cdot + LH \longrightarrow LOOH + L\cdot \quad [3]$$

In the presence of transition metal ions, particularly iron and copper, hydroperoxides will decompose to yield more reactive radical species. The hydroxyl radical (OH·) generated by high energy radiation has sufficient reactivity to

0006-291X/83 $1.50

directly abstract hydrogen atoms from unsaturated fatty acids [equation 1] and so initiate lipid oxidation (1,2). However, when OH· radicals are generated in a reaction involving the autoxidation of a ferrous salt in aerobic aqueous solution, they do not enhance the observed rate of phospholipid oxidation (3); iron catalysed reactions predominating. In contrast, however, others have proposed that OH· radical initiation occurs when unsaturated fatty acids are incubated with a superoxide generating system (4,5,6). Here, OH· arise from a superoxide-dependent Fenton reaction [equation 4, 5, 6]

$$O_2\cdot^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \quad [4]$$

$$2O_2\cdot^- + 2H+ \longrightarrow H_2O_2 + O_2 \quad [5]$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + OH\cdot + Fe^{3+} \quad [6]$$

This reaction should respond to inhibition by superoxide dismutase, catalase, a suitable iron chelator and OH· radical scavengers. However, when catalase is added to a lipid system, its haem-iron moiety can itself non-specifically decompose lipid hydroperoxides formed during the reaction. It would thus be possible to confuse inhibition by this destruction of peroxide with inhibition of oxidation by removal of H_2O_2 and hence prevention of OH· formation.

MATERIALS AND METHODS

Materials Superoxide dismutase bovine erythrocyte, catalase bovine liver thymol free, albumin human fatty acid free, xanthine oxidase grade I, lipoxidase soybean, 2-deoxy-D-ribose, linolenic acid 99% pure, and lubrol PX were obtained from Sigma Chemical Co. Haemoglobin 2x crystallised was from Calbiochem. Ltd. All other chemicals were of the highest grades avaiable from BDH Ltd.

Preparation of Lipid Contaminating lipid peroxides were removed from linolenic acid by extraction into hexane from ethanol:water 1:2 v/v. The hexane phase was washed twice with Chelex-resin treated water and the hexane evaporated over nitrogen gas. Lipid micelles were prepared from the purified lipid as a 7mM solution of linolenic acid vigorously vortex mixed with 0.15 M NaCl. The solution was

adjusted to pH 7.4 with 1 M NaOH gassed with nitrogen, vortex mixed for 2 min and stored at 4°C for use.
Oxidation of lipid 0.2 ml of lipid micelles were added to 0.2 ml of phosphate-saline buffer pH 7.4 (0.024 M Phosphate, 0.15 M NaCl). 50 µl EDTA 1 mM and 0.1 ml of protein solution were added and the reaction started by the addition of 0.1 ml xanthine oxidase 0.7 units/ml and 20 µl acetaldehyde (100 mM). After the addition of 20 µl Lubrol 1% w/v the volume was made to 1.0 ml with Chelex-resin treated distilled water. Samples were incubated at 25°C for 1 hr.
Deoxyribose degradation This was carried out as described above for the lipid, but 25 mM deoxyribose substituted for the linolenic acid suspension.
Measurement of oxidation After incubation, samples were read at A 234 nm against appropriate blanks for diene conjugation. Thiobarbituric acid (TBA) reactivity was measured as A 532 nm after heating the samples with 0.5 ml TBA 1% w/v in 0.05 M NaOH and 0.5 ml acid buffer pH 3.5 (50 mM phthalate, 8.2 mM HCl). 50µl of ferric chloride 1 mM was added to each tube to complex with the EDTA present (7). Samples were heated at 100°C for 15 min to develop the colour. Samples containing deoxyribose were similarly treated, but using 2.8% trichloroacetic acid w/v instead of the acid buffer and without addition of ferric chloride.
Preparation of linolenic hydroperoxides 10 µl of linolenic acid (9.2 mg) was dissolved in 0.1 ml ethanol and added to 150 ml of Tris buffer 0.1 M, pH 8.5. 25 µl of lipoxidase was added to the solution kept well oxygenated by continuous stirring at 25°C for 20 min. After incubation, the pH was adjusted to 4.0 with 1 M HCl and the solution extracted 3 times with diethyl ether (antioxidant free grade). The ether phases were evaporated over nitrogen and the residue dissolved in methanol. The concentration of hydroperoxide was calculated using a molar extinction coefficient at 234 nm of 25,000 (6).

RESULTS

Linolenic acid incubated with a superoxide generating system oxidised, after a short lag phase, at a linear rate (Figure 1). This is shown as an increase in absorbance at A 234 nm due to formation of conjugated dienes. A similar increase in TBA-reactive substances also occurred as a function of time (data now shown). Lipid autoxidation was dependent on the presence in the reaction of both xanthine oxidase and acetaldehyde (Figure 1). The reaction was inhibited by superoxide dismutase at a final reaction concentration of 0.02 mg/ml. Albumin had little effect on the rate of oxidation whereas catalase and haemoglobin substantially inhibited it (Figure 1). Although catalase at

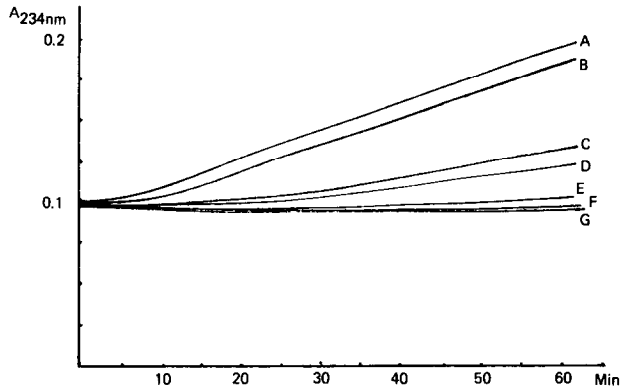

Figure 1.

A = superoxide-dependent linolenic acid oxidation control (continuous trace). B = control + albumin 0.02 mg/ml. C = control + haemoglobin 0. 02 mg/ml. D= control + catalase 0.02 mg/ml. E = control + SOD 0.02 mg/ml. F = control less acetaldehyde. G = control less xanthine oxidase.

final reaction concentrations ranging from 0.02 to 0.1 mg/ml appeared to decrease oxidation, measured in this way, it appeared to stimulate oxidation as measured by TBA-reactivity (Figure 2). Heat denatured catalase also decreased the observed diene conjugation (Figure 2). Changes resulting from the addition of proteins to the superoxide-dependent linolenic acid autoxidation were confirmed by adding the same proteins to a preparation of synthesised linolenic hydroperoxide (Table 1).

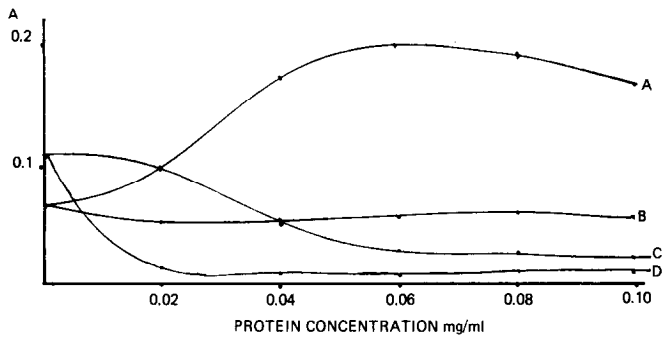

Figure 2.

Superoxide-dependent linolenic acid oxidation. A = Catalase by TBA-reactivity. B = Heat denatured catalase by TBA-reactivity. C = Catalase by diene conjugation. D = Heat-denatured catalase by diene conjugation.

TABLE 1.

Effect of Proteins on Linolenic Hydroperoxide

Proteins added to linolenic hydroperoxide 31.6uM	TBA-reactvity A532nm	Diene conjugation A234nm
Control (no proteins added)	0.070	0.791
" + catalase 0.02 mg/ml	0.117	0.624
" + catalase 0.10 mg/ml	0.357	0.185
" + haemoglobin 0.02 mg/ml	0.061	0.382
" + haemoglobin 0.10 mg/ml	0.041	0.311
" + albumin 0.02 mg/ml	0.074	0.730
" + albumin 0.10 mg/ml	0.078	0.580

Concentrations shown are final reaction concentrations.

Superoxide-dependent deoxyribose degradation was inhibited by catalase but stimulated by heat-denatured catalase (Figure 3). Albumin and haemoglobin were included as controls, and had little effect on deoxyribose degradation.

DISCUSSION

Hydroxyl radicals (OH·) are formed in superoxide generating systems, when traces of iron salts are present,

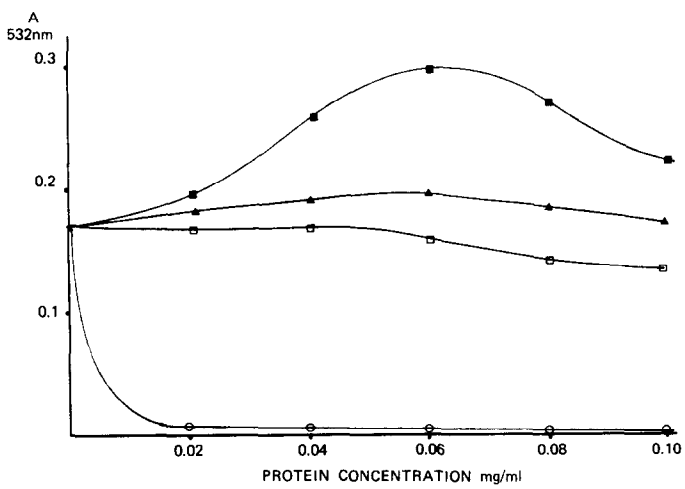

Figure 3.

Superoxide-dependent deoxyribose degradation.
■ Catalase heat-denatured (5 min. 100°C)
▲ Albumin
□ Haemoglobin
○ Catalase

by a Fenton-type reaction [equations 4,5,6] (8,9). The OH· radical can abstract hydrogen atoms from unsaturated lipids [equation 1.] and so initiate lipid oxidation. Based on inhibition studies with specific and non-specific scavengers of the superoxide radical, hydroxyl radical, hydrogen peroxide and iron salts, several recent reports have suggested that lipid oxidation can be initiated by OH· radicals formed in a superoxide generating system (4,5,6), whereas others have found it to contribute little to the observed oxidation process (3,10).

Catalase when added to simple systems provides a specific probe for the Fenton reaction as shown here by its ability to completely inhibit deoxyribose degradation. Heat-denatured catalase, however, appeared to stimulate deoxyribose degradation; and this may be attributed to the release of iron from the protein, since total reactivity was abolished by the addition of 0.2 mM desferroxamine (data not shown). Haemoglobin and albumin, included as controls, had little effect on this system. When catalase is added to lipid hydroperoxides, the haem-iron moiety of the protein can non-specifically decompose the peroxides, seen here as a loss of diene conjugation or an increase in TBA-reactivity. Decomposition of lipid peroxides by haem-iron was confirmed by the addition of haemoglobin to the reaction. The haemoglobin was shown not to be contaminated with catalase since it did not inhibit deoxyribose degradation.

These results show that superoxide-dependent lipid oxidation cannot necessarily be ascribed to the OH· radical merely because it is inhibited by the protein catalase. The haem-iron protein catalase, like haemoglobin, can readily decompose lipid peroxides resulting in loss of their diene

conjugation. It therefore becomes difficult to delineate inhibition of peroxide formation from loss of peroxide by decomposition.

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