THE ROLE OF SUPEROXIDE AND SINGLET OXYGEN IN LIPID PEROXIDATION PROMOTED BY XANTHINE OXIDASE

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Summary - The peroxidative oxidation of extracted rat liver microsomal lipid, assayed as malondialdehyde production, can be promoted by milk xanthine oxidase in the presence of 0.2 mM $FeCl_3$ and 0.1 mM EDTA. The reaction is inhibited by the superoxide dismutase activity of erythrocuprein. The reaction is also inhibited by 1,3-diphenylisobenzofuran, which reacts with singlet oxygen to yield dibenzoylbenzene. During inhibition of the lipid peroxidation reaction by 1,3-diphenylisobenzofuran, o-dibenzoylbenzene was produced. The rate of superoxide production by xanthine oxidase was not affected by 1,3-diphenylisobenzofuran. Lipid peroxidation promoted by ascorbic acid is not inhibited by either erythrocuprein or 1,3-diphenylisobenzofuran. Therefore it is suggested that the peroxidative oxidation of unsaturated lipid promoted by xanthine oxidase involves the formation of singlet oxygen from superoxide, and the singlet oxygen reacts with the lipid to form fatty acid hydroperoxides.

We previously reported that rat liver microsomal NADPH-cytochrome c reductase, in the presence of $FeCl_3$ and EDTA, will catalyze the peroxidation of lipid and the reaction can be inhibited by superoxide dismutase (1). It has very recently been reported that the peroxidation of mitochondrial membranes promoted by glutathione can be inhibited by superoxide dismutase (2). It has also been reported that superoxide dismutase will prevent the peroxidation of erythrocyte membranes induced by dialuric acid (3). This suggests that the superoxide anion (O_2^-) may be involved in lipid peroxidation reactions which occur in a variety of systems.

The most likely role of O_2^- in the lipid peroxidation reaction would be in the initial formation of hydroperoxides either by reacting directly with the unsaturated lipid or by producing some other reactive intermediate. It has been shown that O_2^- in dimethylsulfoxide will evolve singlet O_2 (4). It is therefore likely that the non-enzymatic dismutation of O_2^- in aqueous solvents also produces singlet O_2. The reaction of singlet O_2 with unsaturated fatty acids to form hydroperoxides has been demonstrated directly using both singlet O_2 generated by a radio-frequency gas discharge source and by photoactivation using sensitizers known to generate singlet O_2 (5). It has also been suggested that the formation of fatty acid hydroperoxides catalyzed by soybean lipoxigenase involves a singlet oxygen-like intermediate (6).

In this report, we will present evidence to show that the O_2^-, produced by the aerobic reaction of milk xanthine oxidase (7), will promote the rapid per-

oxidation of extracted rat liver microsomal lipid in reaction mixtures containing $FeCl_3$ and EDTA-Fe (ferric ion chelated with one mole of EDTA). The reaction can be inhibited by either erythrocuprein, the superoxide dismutase isolated from erythrocytes, or by the singlet oxygen trapper, 1,3-diphenylisobenzofuran. In contrast, the lipid peroxidation reaction promoted by ascorbic acid is not inhibited by either erythrocuprein or 1,3-diphenylisobenzofuran.

Methods

Xanthine oxidase was dissolved in 0.05 M Tris-HCl (pH 7.5) and passed through a Sephadex G-50 column immediately prior to use. Erythrocuprein was purified from bovine erythrocytes by the method of McCord and Fridovich (7). The Cu^{++} content of the purified protein was determined by atomic absorption on a Perkin Elmer model 303 atomic absorption spectrophotometer and the amount of erythrocuprein calculated on the assumption of 2 atoms of Cu^{++} per mole (8). The superoxide dismutase activity of the erythrocuprein was destroyed by heating in a boiling water bath for five minutes.

The preparation of rat liver microsomes, the extraction of the microsomal lipid, and the anaerobic preparation of liposomes by sonication have been previously described (1,9). The lipid peroxidation reaction and the assay of malondialdehyde production were performed as previously described (1). The addition of 1,3-diphenylisobenzofuran to the reaction mixture was accomplished by adding the compound to the lipid in chloroform-methanol, removing the solvents under a stream of nitrogen, and then preparing liposomes.

Xanthine oxidase (Sigma type I), xanthine, cytochrome c (Sigma type VI), and ascorbic acid were obtained from the Sigma Chemical Company, St. Louis, Missouri. 1,3-Diphenylisobenzofuran (DPIF) and o-dibenzoylbenzene (DBB) were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. All other reagents used were reagent grade.

Results

The ability of xanthine oxidase, catalyzing the oxidation of xanthine, to promote the peroxidation of rat liver microsomal lipid is shown in Table I. The reaction conditions, high ionic strength and the presence of both $FeCl_3$ and EDTA-Fe, are those that were found to be optimal for the production of malondialdehyde in the peroxidation reaction promoted by NADPH-cytochrome c reductase (1). It can be seen that the peroxidation activity required the presence of both xanthine and xanthine oxidase and the amount of activity increases as the concentration of xanthine oxidase increases. Table II shows the reaction conditions required for the peroxidation reaction promoted by either xanthine oxidase or ascorbic acid. Both the xanthine oxidase and the ascorbic acid promoted reactions demonstrate optimal activity in the presence of both $FeCl_3$ and

TABLE I. LIPID PEROXIDATION PROMOTED BY XANTHINE OXIDASE

Reaction System	Malondialdehyde Formed, nMoles/min ml
Complete, 50 μg/ml Xanthine Oxidase	3.45
" , 25 μg/ml "	2.80
" , 10 μg/ml "	1.95
- Xanthine, 50 μg/ml "	0.24
- Xanthine Oxidase	0.15

The complete reaction system contains 0.33 mM xanthine and the amount of xanthine oxidase indicated in peroxidation reaction mixtures containing liposomes (0.5 μMoles of lipid phosphorous/ml), 0.25 M Tris-HCl (pH 6.8 at 37°C), 0.25 M NaCl, 0.1 mM $FeCl_3$, and 0.1 mM EDTA-Fe. The reaction was initiated by the addition of xanthine oxidase and the rate of malondialdehyde production was determined as described under "Methods".

TABLE II. REQUIREMENTS FOR LIPID PEROXIDATION

Reaction System	Malondialdehyde Formed, nMoles/min ml		
		+ Xantine Ox.	+ Ascorbate
Complete	0.15	3.45	6.70
- EDTA-Fe	-	0.30	4.68
- $FeCl_3$	-	0.00	0.00
- NaCl in 0.05 M Tris-HCl	0.88	4.10	7.30

The complete reaction system is the same as the peroxidation reaction mixture described in the legend to Table I. The xanthine oxidase promoted peroxidation reaction mixtures contain 0.33 mM xanthine and 50 μg/ml of xanthine oxidase. The ascorbate promoted peroxidation reaction was initiated by the addition of 0.2 mM ascorbic acid. The rate of malondialdehyde formation was determined as described under "Methods".

EDTA-Fe. Neither reaction system shows any production of malondialdehyde in the absence of EDTA-Fe. The use of high ionic strength is not a necessary condition but preferable since the rate of auto-oxidation is greater at low ionic strength (1).

The involvement of O_2^- in the lipid peroxidation reactions was investigated by adding erythrocuprein to the reaction mixtures. The results of these experiments (Table III) show that the peroxidation activity promoted by xanthine oxidase

TABLE III. THE EFFECT OF ERYTHROCUPREIN ON LIPID PEROXIDATION

Description	Malondialdehyde Formed, nMoles/min ml			
	With Xanthine Oxidase		With Ascorbic Acid	
	Activity	% Control	Activity	% Control
Control	3.55	-	6.45	-
+ Erythrocuprein, 0.37 μM	1.51	43	6.35	98
" , 1.85 μM	0.54	15	6.35	98
+ Boiled Erythrocuprein, 0.37 μM	3.17	89	5.25	81
" " , 1.85 μM	1.81	51	2.44	38
+ $CuCl_2$, 2 μM and $ZnSO_4$, 2 μM	2.05	58	2.64	41

The reaction conditions are the same as those described in the legend to Table II. The xanthine oxidase promoted peroxidation reaction mixtures contain 50 μg/ml of the enzyme.

could be readily inhibited by the presence of erythrocuprein, but no significant amount of inhibition was seen in the reaction promoted by ascorbic acid. When the erythrocuprein was heat inactivated its ability to inhibit lipid peroxidation by xanthine oxidase was only partially reversed. However, the boiled erythrocuprein inhibited the lipid peroxidation promoted by ascorbic acid to a similar degree. This was apparently due to the liberation of Cu^{++} and Zn^{++} during heat inactivation since it was found that the addition of Cu^{++} and Zn^{++}, at concentrations similar to those that could be obtained from the erythrocuprein (8), inhibited both the xanthine oxidase and ascorbic acid promoted peroxidation reactions.

The role of singlet O_2 in the peroxidation reaction was investigated by the use of DPIF, which reacts very readily with singlet O_2 to form DBB (10). The results obtained using liposomes containing the singlet O_2 trapper are shown in Table IV. The reaction promoted by xanthine oxidase was strongly inhibited by the presence of DPIF, but no significant inhibition was observed in the ascorbic acid promoted reaction. These reactions were performed in a dark room under a safelight to prevent the generation of singlet O_2 by any type of photosensitization and to rule out the possible effect of photoexcited DPIF, even though similar results were obtained under ordinary light. The formation of DBB in the lipid peroxidation mixture containing xanthine, xanthine oxidase, and DPIF was verified by thin layer chromatography of chloroform extract of the reaction mixture (Fig. 1). The possible effect of DPIF on the rate of O_2^- generation by xanthine oxidase

TABLE IV. THE EFFECT OF 1,3-DIPHENYLISOBENZOFURAN ON LIPID PEROXIDATION

Description	Malondialdehyde Formed, nMoles/min ml			
	With Xanthine Oxidase		With Ascorbic Acid	
	Activity	% Control	Activity	% Control
Control	2.48	-	6.40	-
+ 1,3-Diphenylisobenzofuran:				
0.2 mole/mole lipid P	0.58	23	6.20	97
1.0 mole/mole lipid P	0.15	10	6.15	96

The 1,3-diphenylisobenzofuran was added to the liposomes as described under "Methods" The reactions were all performed in a dark room where the only source of illumination was a safelight with a Wratten series 1A filter. The reaction conditions are the same as those described in the legend to Table II. The xanthine oxidase promoted peroxidation reaction mixtures contain 25 μg/ml of the enzyme.

was checked by assaying cytochrome c reduction in both the absence and presence of erythrocuprein. The results in Table V demonstrate that lipid containing DPIF had no apparent effect on the rate of cytochrome c reduction by xanthine oxidase or the inhibition of this activity by erythrocuprein.

Discussion

The results presented in this communication demonstrate that milk xanthine oxidase, while catalyzing the oxidation of xanthine, can promote the peroxidation of lipid included in the reaction mixture. The inhibition of the lipid peroxidation by both erythrocuprein and DPIF strongly suggests that the reaction mechanism involves the generation of singlet O_2 from the O_2^- produced by xanthine oxidase and the reaction of the singlet O_2 with the unsaturated fatty acids in the lipid to form fatty acid hydroperoxides. The inability of either erythrocuprein or DPIF to inhibit the ascorbic acid mediated peroxidation reaction eliminates the possibility that their inhibition of the xanthine oxidase promoted peroxidation was the result of any general antioxidant properties. The DPIF also had no effect on the rate of O_2^- generation by xanthine oxidase. It should be pointed out however, that erythrocuprein is also reported to be an effective scavenger of singlet O_2 (11). But, since the xanthine oxidase promoted peroxidation activity is inhibited by DPIF in the dark, it is unlikely that the singlet O_2 was derived from any source other than O_2^-.

Differences between the xanthine oxidase and ascorbic acid promoted peroxi-

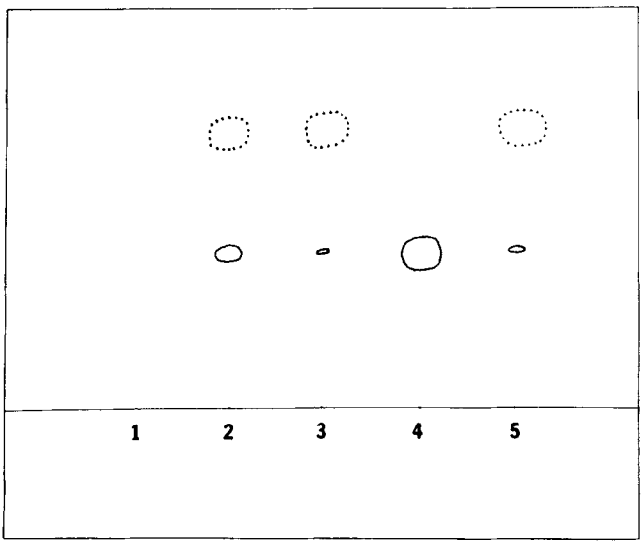

FIGURE 1. A TRACING OF THE THIN LAYER CHROMATOGRAM DEMONSTRATING THE FORMATION OF O-DIBENZOYLBENZENE IN XANTHINE OXIDASE PROMOTED LIPID PEROXIDATION MIXTURES CONTAINING 1,3-DIPHENYLISOBENZOFURAN. #1, Complete incubation mixture minus 1,3-diphenylisobenzofuran; #2, Complete incubation mixture; #3, Complete incubation mixture minus xanthine oxidase; #4, 250 nMoles of o-dibenzoylbenzene; #5, 250 nMoles of 1,3-diphenylisobenzofuran. Only the spots observed by their fluorescence (dotted circles) or after being sprayed with 0.5% 2,4-dinitrophenylhydrazine in 2 N HCl (solid circles) are shown. The complete reaction mixture contained 1.0 mole of 1,3-diphenylisobenzofuran per mole of lipid phosphorous and xanthine oxidase as described in the legend to Table IV. For chromatographic analysis, 2.0 ml of each reaction mixture was removed after 7 minutes incubation and extracted with 0.5 ml of $CHCl_3$. One-tenth ml of each extract was applied to the Silica Gel G thin layer plate and developed by heptane-dioxane (3:1). All operations were performed in a dark room as described in the legend to Table III.

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dation reactions can also be demonstrated by the effect of $FeCl_3$ and EDTA-Fe. Both reaction systems require $FeCl_3$ but only the xanthine oxidase promoted reaction requires EDTA-Fe. However, the rate of peroxidation promoted by ascorbic acid is greatest in the presence of both $FeCl_3$ and EDTA-Fe. Ascorbic acid and ferric ion will also catalyze aromatic hydroxylation and this activity is also increased by the addition of EDTA, but in contrast to the peroxidation reaction, the hydroxylation activity is not inhibited by EDTA concentrations in excess of the ferric ion concentration (12). This suggests that in the peroxidation reaction, $FeCl_3$ is required for the radical-generating degradation of hydroperoxides and subsequent production of malondialdehyde.

There appear to be a variety of means by which O_2^- is generated in biological systems (13, 14) and the ubiquitous presence of superoxide dismutase in organisms

TABLE V. THE EFFECT OF 1,3-DIPHENYLISOBENZOFURAN ON SUPEROXIDE PRODUCTION BY XANTHINE OXIDASE AS MEASURED BY THE RATE OF CYTOCHROME C REDUCTION

Description	nMoles Cytochrome c reduced/min ml	
	- Erythrocuprein	+ Erythrocuprein
Control	5.80	2.05
+ Lipid	5.95	1.90
+ Lipid with Diphenylisobenzofuran, 1.0 mole/mole lipid P	5.90	1.95

The cytochrome c reductase activity was measured at 37°C in reaction mixtures containing 0.05 M Tris-HCl (pH 6.8), 0.33 mM xanthine, 75 μM cytochrome c, and 25 μg/ml of xanthine oxidase. The erythrocuprein was present at a concentration of 0.125 nmole/ml where indicated. The reduction of cytochrome c was measured at 550 nm and the rate of reduction calculated by using ϵ (reduced-oxidized) = 2.11×10^4 cm^2/mmole.

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living in the presence of O_2 (15) certainly suggests that O_2^- is detrimental to biological systems. It is very likely that the generation of singlet O_2 from O_2^- may be one of the primary reasons for the toxicity of O_2^- in biological systems. It has been reported that the lungs of animals exposed to ozone show signs of peroxidative damage (16). While ozone is a very reactive species, it is known that singlet O_2 is generated from a number of ozone oxidation reactions (17) and therefore singlet O_2 may also be in part responsible for the effects of ozone. Singlet O_2 is also produced by photosensitization in biological systems. It has been shown that protoporphyrin IX generates singlet O_2 when photoactivated (18) and people who suffer from certain types of porphyria in which high levels of free protoporphyrin exist in their external tissues are very sensitive to light (19). It has also been shown that singlet O_2 produced by photosensitization may be involved in the carcinogenic effect of polycyclichydrocarbons (20).

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