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Inhibition of Heme-promoted Enzymatic Lipid Peroxidation by Desferrioxamine and EDTA

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ABSTRACT

Oxyhemoglobin, methemoglobin and hematin were found to catalyze xanthine-oxidase-induced peroxidation of phospholipid liposomes, while oxy- and met-myoglobin were inactive in this respect. The peroxidation was inhibited by desferrioxamine and by EDTA. Peroxidation catalyzed by 0.4 μM oxyhemoglobin was decreased by 50% by approximately 2 μM desferrioxamine or 20 μM EDTA and completely inhibited by 10 μM desferrioxamine or 100 μM EDTA. Inhibition of hemoglobin-catalyzed peroxidation was not accompanied by any changes in the absorbance spectra of hemoglobin, indicating that the heme iron was not withdrawn by the inhibitor. Inhibition of hematin-catalyzed peroxidation by desferrioxamine may have been due to iron chelation and removal, as judged from changes in absorbance spectra. The peroxidation was apparently not dependent on hydrogen peroxide since catalase did not inhibit peroxidation but on the contrary promoted it in some cases.

INTRODUCTION

Lipid peroxidation is an important component of the progressive tissue destruction observed after hemorrhage in, for example, brain and spinal cord tissue. This peroxidation is believed to be promoted by hemoglobin or hemoglobin degradation products (1,4,15) . Nonenzymatic peroxidation of lipids by heme compounds has been extensively studied (3,10,12,13), whereas heme-catalyzed peroxidation initiated by enzymatically generated oxygen radicals has not received similar attention so far (5,11).

The present study was an attempt to characterize further the heme-catalyzed phospholipid peroxidation initiated by xanthine oxidase. It was found that desferrioxamine and EDTA inhibited the peroxidation. It was also observed that catalase did not inhibit the peroxidation in the experimental system used.

MATERIALS AND METHODS

Phospholipid liposomes. Folch fraction III from bovine brain (Sigma Chemical Co, St Louis, Mo, USA) stored at -20°C as a 10 % solution containing 140 mM phospholipid phosphate in chloroform was used. A 100 µl sample of the chloroform solution was dried under argon, whereafter 1 ml of buffer was added and sonication was performed under argon.

Buffer. A 50 mM phosphate buffer, pH 7.4, containing 1 mM hypoxanthine was used.

Heme compounds. The heme compounds were purchased from Sigma Chemical Co, St. Louis, Mo, USA. Hematin from bovine blood was dissolved in water to a concentration of 500 µM, and a small amount of NaOH was added to bring the hematin into solution. Oxyhemoglobin was prepared from human (Type IV) hemoglobin by addition of dithionite in a slight molar excess, desalting, and bubbling with oxygen. The commercial hemoglobin was used as methemoglobin without further preparation, since no further oxidation was observed after addition of potassium hexacyanoferrate(III). Oxymyoglobin and metmyoglobin were prepared from horse heart (type III) myoglobin, the former by addition of dithionite and bubbling with oxygen, and the latter by adding potassium hexacyanoferrate(III). All protein preparations were made as 500 µM stock solutions and desalted on a Pharmacia PD-10 column before use.

Catalase from beef liver, specific activity 20 000 U/mg, was from Boehringer Mannheim. Desferrioxamine (Desferal^{R}) was manufactured by CIBA.

Lipid peroxidation assay. To 0.5 ml of buffer containing hypoxanthine and test substance was added 10 µl of phospholipid suspension, making the phospholipid phosphate concentration 0.28 mM. The reaction was started by adding 5 µl of xanthine oxidase (1.7 U/ml). The reaction mixture was incubated for 15 min at 37°C, and the reaction was terminated by addition of the thiobarbituric acid reagent. The assay of thiobarbituric acid reactive species (TBARS) was performed as described previously (2), with the addition of 0.02% butylated hydroxytoluene immediately before heating. The results are expressed as absorbance values (532 nm) after subtraction of the background value obtained following incubation of lipid in the presence or absence of xanthine oxidase without addition of the heme compound. The experiments were run in quadruplicate; the coefficients of variation were usually about 1% and never higher than 5%.

Absorbance spectra were recorded for 50 µM hematin and for 6 µM hemoglobin in an ammonium carbonate solution after at least 10 min preincubation with 500 µM desferrioxamine or EDTA.

Table 1

Effects of EDTA, desferrioxamine and catalase on heme-catalyzed phospholipid peroxidation. The results are expressed as absorbance values (532 nm) after subtraction of the background value obtained following incubation of lipid in the presence or absence of xanthine oxidase without addition of the heme compound.

Additive	Hematin (5 μM)	Oxyhemoglobin (0.4 μM)	Methemoglobin (0.4 μM)
None	0.088	0.046	0.042
Addition before the xanthine oxidase-phospholipid incubation:			
EDTA (20 μM)	-0.014	-0.015	-0.021
Desferrioxamine (20 μM)	-0.015	-0.019	-0.025
Catalase (160 U/ml)	0.089	0.061	0.060
Addition after the xanthine oxidase-phospholipid incubation:			
EDTA	0.085	0.035	0.039
Desferrioxamine	0.084	0.036	0.037
Catalase	0.084	0.043	0.038

RESULTS

Xanthine oxidase induced lipid peroxidation, measured as expression of TBARS, was catalyzed by hematin, oxyhemoglobin and methemoglobin in a dose-dependent fashion as illustrated in Fig. 1. Myoglobin, either in the met- or oxy- form, in concentrations of up to 10 μM did not catalyze the reaction (data not given).

In one series of experiments the heme compound was added after the incubation of phospholipid and xanthine oxidase since it has been reported that hemoglobin interferes with the thiobarbituric acid reaction (9). In another series the phospholipid and heme compound were incubated without xanthine oxidase in order to measure nonenzymatic peroxidation. In these two series only incubation of methemoglobin with phospholipid resulted in appreciable amounts of TBARS (Fig. 1 B).

When desferrioxamine or EDTA was present during the incubation, the expression of TBARS was inhibited (Table 1 and Fig. 3). These compounds did not affect the TBARS assay, since they caused no changes when added after the peroxidation. Desferrrioxamine inhibited oxyhemoglobin-promoted lipid peroxidation already at equimolar concentrations, while EDTA was required in more than tenfold excess. Catalase either had no effect or promoted the peroxidation slightly. The negative values in Table I are probably due to background expression of TBARS due to iron contamination of the buffer. This contaminating iron

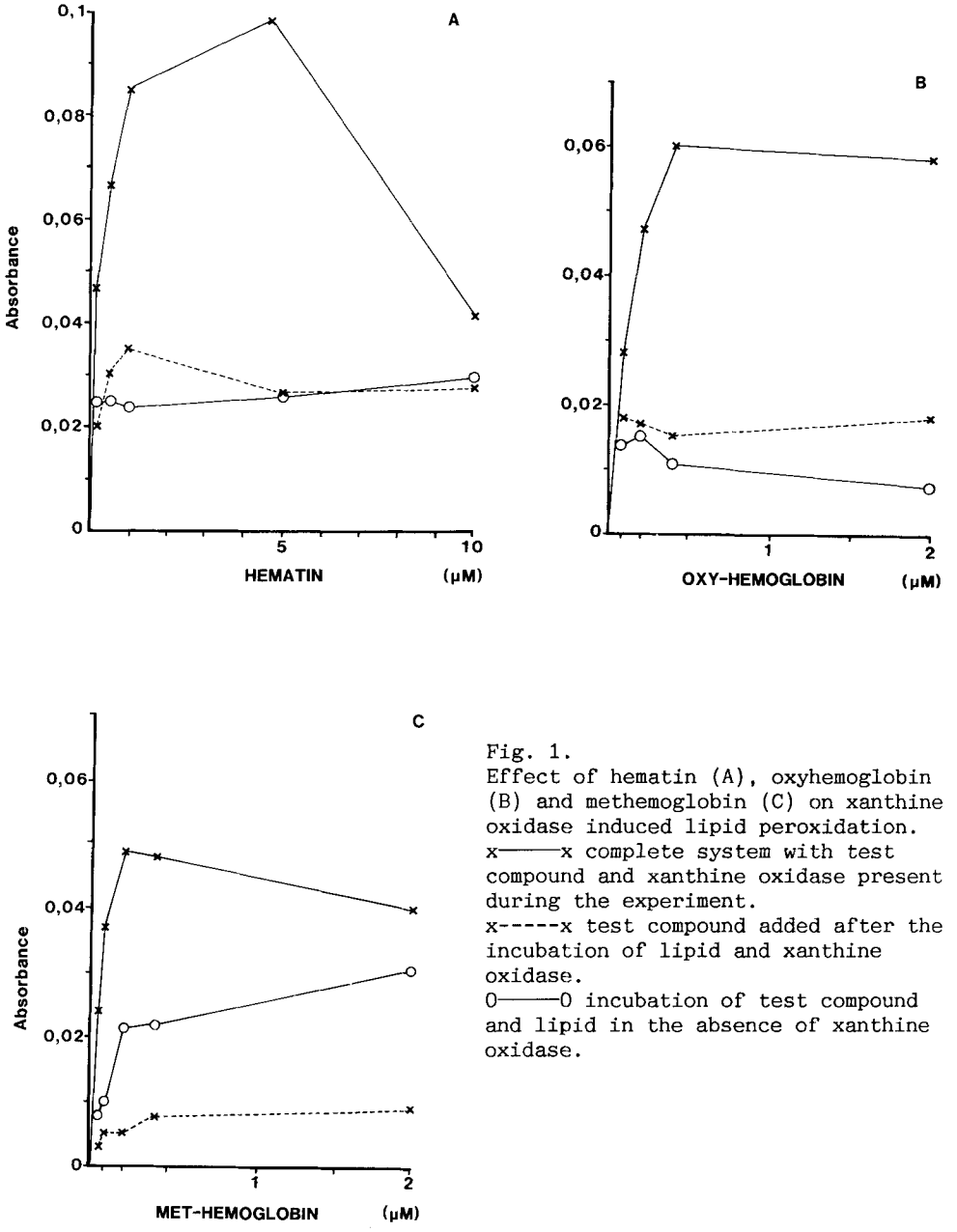

Fig. 1.
Effect of hematin (A), oxyhemoglobin (B) and methemoglobin (C) on xanthine oxidase induced lipid peroxidation.
x———x complete system with test compound and xanthine oxidase present during the experiment.
x-----x test compound added after the incubation of lipid and xanthine oxidase.
0———0 incubation of test compound and lipid in the absence of xanthine oxidase.

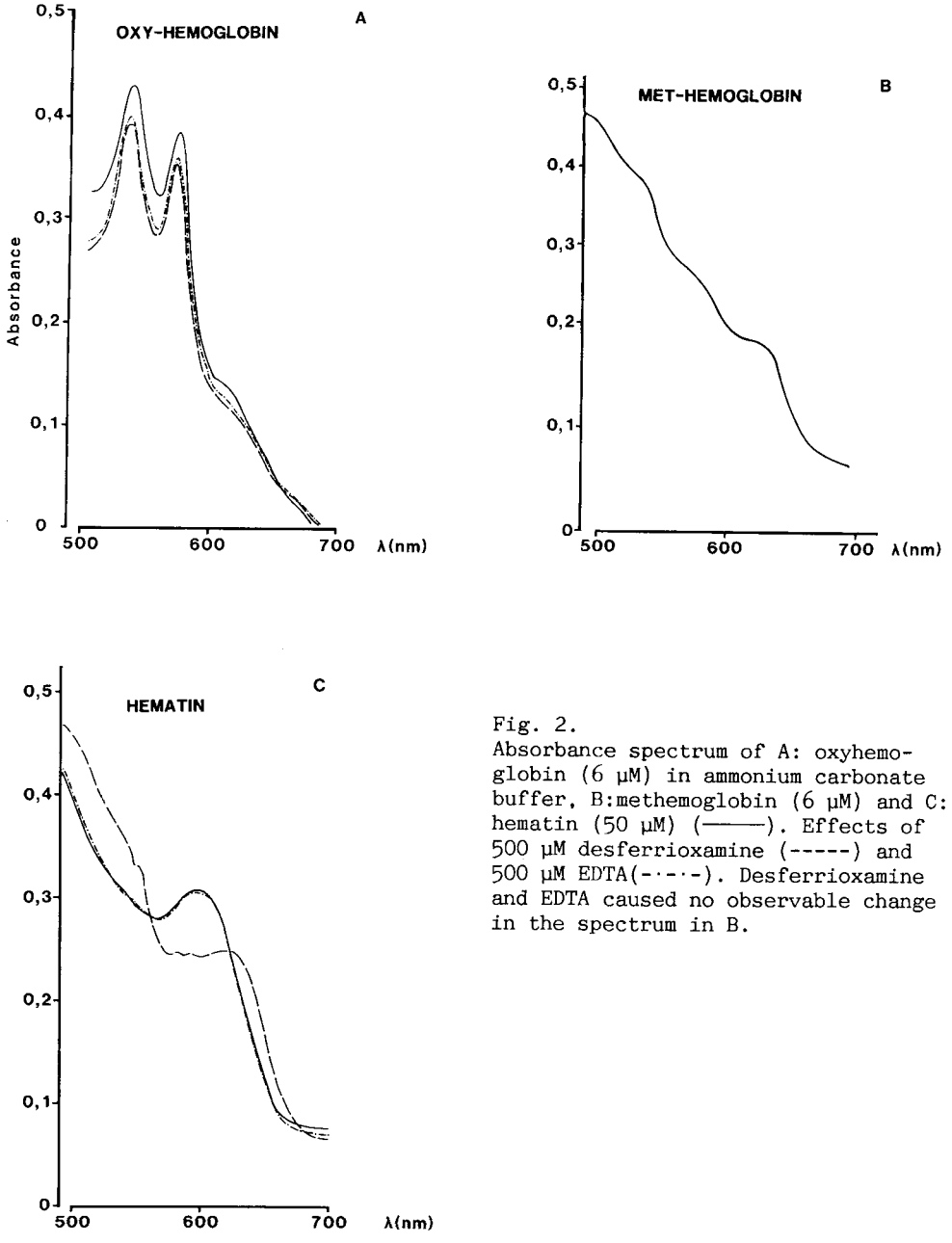

Fig. 2.
Absorbance spectrum of A: oxyhemoglobin (6 μM) in ammonium carbonate buffer, B:methemoglobin (6 μM) and C: hematin (50 μM) (———). Effects of 500 μM desferrioxamine (-----) and 500 μM EDTA(-·-·-). Desferrioxamine and EDTA caused no observable change in the spectrum in B.

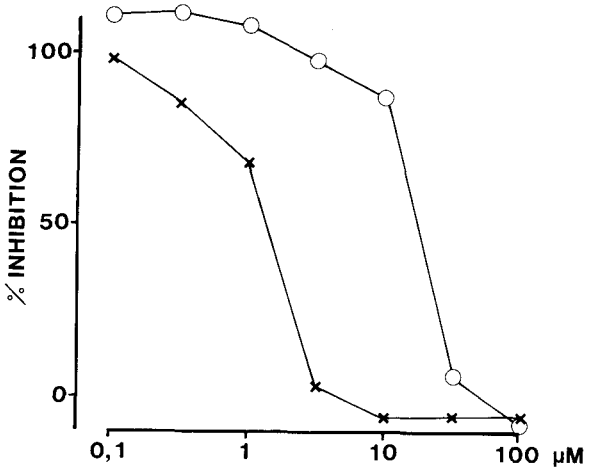

Fig. 3.
Effects of EDTA (o) and desferrioxamine (x) on xanthine oxidase promoted peroxidation of phospholipid liposomes in the presence of oxyhemoglobin (0.4 µM).

was chelated by desferrioxamine and EDTA, leading to values lower than background values. Similar (negative) values were obtained when xanthine oxidase and phospholipid were incubated with EDTA or desferrioxamine in the absence of heme (data not shown).

The ability of desferrioxamine and EDTA to complex and remove the heme iron was studied by light absorbance spectroscopy. Since the absorbance spectra of hematin and hemoglobin differ from those of desferrioxamine-iron or EDTA-iron complex, a change in the the iron chelation would be expected to result in an altered spectra.

The results presented in Fig. 2 indicate that desferrioxamine was able to chelate hematin iron but not oxy- or methemoglobin iron and that EDTA had no effect on any of these substances.

DISCUSSION

Oxyhemoglobin was found to promote xanthine-oxidase-induced lipid peroxidation essentially as described by Miura and Ogiso (11). Peroxidation was also promoted by methemoglobin and hematin.

Desferrioxamine inhibited the lipid peroxidation promoted by all the heme compounds studied. In the case of hematin, changes in the absorbance spectrum indicated chelation and removal of the heme iron. However, similar changes in the spectra of oxy- and methemoglobin were not found. Nor was this expected in the case of oxyhemoglobin, where, at least formally, the iron is in the ferrous state and thus has a low affinity for desferrioxamine (8). Furthermore, earlier

studies have shown that hemoglobin iron is not withdrawn by desferrioxamine (8).

It is possible that the lipid preparation used in the present investigation contained traces of peroxides. Gutteridge (6) found that preformed lipid peroxides reacted with hemoglobin to release iron which could be complexed to *e.g.* desferrioxamine. The released iron - not the heme-iron - was suggested to be the catalyst of lipid peroxidation in hemoglobin-containing systems, since it was unlikely that heme-iron could act as a catalyst for the Fenton reaction. However, lipid peroxidation does not require hydroxyl radicals (14), which are the products of the Fenton reaction, and we found no effect of the Fenton reaction-inhibitor catalase in the study. It is thus possible that heme-iron, like ADP-iron (14), promotes hydroxyl radical-independent lipid peroxidation.

The ability of desferrioxamine to block hemoglobin-promoted lipid peroxidation therefore suggests an interaction with hemoglobin, but further studies are required to elucidate the nature of this possible interaction. A similar case of complex formation was described by Winterbourn (16), who found that an EDTA-lactoferrin complex, in contrast to lactoferrin alone, catalyzed the production of hydroxyl radicals from superoxide and hydrogen peroxide. In the present study EDTA was found to be less effective than desferrioxamine as an inhibitor of oxyhemoglobin promoted peroxidation, but at high concentration it inhibited all the heme compounds studied.

Nonenzymatic lipid peroxidation promoted by hemoglobin has been studied by Winterbourn and coworkers. Szebeni *et al.* (12) found that hemoglobin entrapped in an emulsion of unsaturated phospholipids was rapidly oxidized under the formation of lipid peroxides, which suggested that the peroxidation was initiated by oxidation of oxyhemoglobin, leading to H_2O_2 production, or by a reaction between oxyhemoglobin and peroxide contaminants of the lipid preparation. Catalase inhibited both lipid peroxidation and hemoglobin oxidation. Carrell *et al.* (3) discussed the concept that oxyhemoglobin may be analogous to a ferric superoxide and a potential producer of superoxide ions for oxidative stress reactions according to the formula:

$$(Hb)Fe^{2+} + O_2 \langle\!=\!\rangle (Hb)Fe^{3+}\cdot\cdot O_2^- \longrightarrow (Hb)Fe^{3+} + O_2^- .$$

Grisham (5) found that arachidonic acid was peroxidized by xanthine-oxidase-generated radicals in the presence of myoglobin and hemoglobin. However, like the nonenzymatic peroxidation discussed above, and in contrast to the present findings, this peroxidation was sensitive to catalase inhibition and was enhanced by superoxide dismutase. The mechanism of myoglobin catalysis of arachidonic acid therefore seems to be quite different from the peroxidation observed in the present study, and is more similar to the phospholipid peroxidation induced by H_2O_2-activated metmyoglobin and methemoglobin described by Kanner and Harel (7).

Our results are in good accordance with those of Miura and Ogiso (11), who found that oxyhemoglobin promoted xanthine-oxidase-induced peroxidation of erythrocyte ghosts, and that this peroxidation was inhibited by superoxide dismutase but not by catalase. Superoxide dismutase was included in our preliminary experiments and inhibited peroxidation completely (results not shown), but was considered to be of little interest since it would prevent the reaction between superoxide and heme compound. It is generally thought that in the absence of an appropriate complex of iron (or of certain other transition metals), superoxide is not able to bring about peroxidation, and the results of the present study are consistent with the notion that the lipid peroxidation is not caused by superoxide separated from the heme environment, since no lipid peroxidation was seen in the absence of heme compounds despite the presence of xanthine-oxidase-generated superoxide. It seems more likely that the peroxidation is initiated by a complex between heme and active oxygen. The inability of catalase to inhibit the reaction also supports the view that the heme compounds may be similar to ADP-iron complexes, which are also catalase-insensitive initiators of phospholipid peroxidation (14).

REFERENCES

1. Anderson, D.K. & Means, E.D.: Lipid peroxidation in spinal cord: $FeCl_2$ induction and protection with antioxidants. Neurochem Pathol 1:249-264, 1983.

2. Carlin, G.: Peroxidation of linolenic acid promoted by human polymorphonuclear leucocytes and xanthine oxidase. J Free Rad Biol Med 1:255-261, 1986.

3. Carrell, R.W., Winterbourn, C.C. & French, J.K.: Haemoglobin - a frustrated oxidase? Implications for red cell metabolism. Hemoglobin 1:815-827, 1977.

4. Demopolous, H.B., Flamm, E.S., Pietronigro, D.D. & Seligman, M.Y.: The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol Scand Suppl 492:91-119, 1980.

5. Grisham, M.B.: Myoglobin-catalyzed hydrogen peroxide dependent arachidonic acid peroxidation. J Free Rad Biol Med 1:227-232, 1985.

6. Gutteridge, J.M.C: Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. FEBS Lett 201:291-295

7. Kanner, J. & Harel, S.: Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. Arch Biochem Biophys 237:314-321, 1985.

8. Keberle, H.: The biochemistry of desferrioxamine and its relation to iron metabolism. Ann NY Acad Sci 119:758-768, 1964.

9. Kellogg, E.W. & Fridovich, I.: Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J Biol Chem 250:8812-8817, 1975.

10. Kühn, H., Schewe, T. & Rapoport, S.M.: Catalytic activities of haemoglobin related to lipid peroxidation. Biomed Biochim Acta 42:S139-S143, 1983.

11. Miura, T. & Ogiso, T.: Lipid peroxidation of erythrocyte membrane induced by xanthine oxidase system: Modification of superoxide dismutase effect by hemoglobin. Chem Pharm Bull 30:3662-3668, 1982.

12. Szebeni, J., Winterbourn, C.C. & Carrell, R.W.: Oxidative interactions between haemoglobin and membrane lipid. Biochem J. 220:685-692, 1984.

13. Tappel, A.L.: Unsaturated lipide oxidation catalyzed by hematin compounds. J Biol Chem 217:721-733, 1955.

14. Tien, M., Svingen, B.A., & Aust, S.D.: An investigation into the role of hydroxyl radical in xanthine oxidase-dependent lipid peroxidation. Arch Biochem Biophys 216:142-151, 1982.

15. Willmore, L.J. & Rubin, J.J.: Formation of malonaldehyde and focal brain edema by subpial injection of $FeCl_2$ into rat isocortex. Brain Res. 246:113-119, 1982.

16. Winterbourn, C.C.: Lactoferrin-catalyzed hydroxyl radical production. Additional requirement for a chelating agent. Biochem. J. 210:15-19, 1983.

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