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# Oxidised low density lipoproteins induce iron release from activated myoglobin

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Recent reports have detected the presence of iron in human atherosclerotic lesions [Biochem. J. 286 (1992) 901–905]. This study provides evidence for a biochemical mechanism whereby iron is released from myoglobin by low density lipoprotein (LDL) which has become oxidised by the ferryl myoglobin species. The haem destabilisation and iron release are inhibited by monohydroxamate compounds and desferrioxamine through their ability to inhibit the propagation of LDL oxidation. Thus, iron may derive from the myoglobin released from ruptured cells in the oxidising environment of the atherosclerotic lesion.

Oxidised LDL; Monohydroxamate; Desferrioxamine; Iron; Myoglobin; Low density lipoprotein

## 1. INTRODUCTION

The mechanisms underlying the oxidation of low density lipoproteins (LDL) in the artery wall [2] are yet to be elucidated. Evidence has been provided that a range of cell types capable of generating superoxide radicals induce the oxidation of LDL in culture, particularly macrophages/monocytes [3], smooth muscle cells [4] and endothelial cells [5], but only in iron-containing media [6]. Superoxide radical and hydrogen peroxide, its dismutation product, are not reactive per se with polyunsaturated fatty acids, but their reactivity may be amplified by interaction either with available transition metal ions, such as iron or copper, generating the hydroxyl radical [7], or with specific peroxidases, such as myoglobin [8–13] and haemoglobin [14,15], producing the ferryl forms of the haem proteins.

In this study we have investigated the comparative mechanisms by which metmyoglobin and its activated counterpart, ferryl myoglobin, enhance LDL oxidation and how the oxidised LDL influences the reactivity and stability of the haem protein. Analysis of the propensity for the novel monohydroxamate compound, *N*-methyl-*N*-hexanoyl hydroxamate (NMHH) [16] to inhibit the oxidative modification of LDL compared with the trihydroxamatate, desferrioxamine (DFO), have afforded interpretation of their comparative modes of action in terms of the inhibition of the initiating species or chain-breaking scavenging of propagating lipid peroxyl radicals. The results show that, on oxidation mediated by ferryl myoglobin, the resulting oxidised LDL attacks the haem protein, destabilising the haem ring, inducing haem destruction and iron release. NMHH and DFO inhibit the release of iron by suppressing LDL oxidation through the reduction of the initiating species, as well as by acting as inhibitors of the propagation of lipid peroxidation. During metmyoglobin-induced propagation of LDL oxidation under comparative conditions of interaction, the LDL oxidation is less extensive and iron is not made available.

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## 2. MATERIALS AND METHODS

Desferrioxamine mesylate (desferal; DFO) was from CIBA-Geigy; NMHH was synthesized using standard literature methods [17]. Hydrogen peroxide was of Aristar grade, and all other chemicals were of Analar grade and supplied by Merck (Darmstadt, Germany) or Sigma (Poole, UK). Myoglobin (ferric from, horse heart, type III) was prepared as described previously [18] and purified by oxidation with potassium ferricyanide and subsequent separation on a Sephadex G-15 column. Visible spectroscopy was performed on a Beckman DU-65 spectrophotometer fitted with Quant 1 software. Incubations of metmyoglobin (final concentration 20 $\mu$M) were carried out in 10 mM phosphate-buffered saline, pH 7.4, and spectra were run at timed intervals for up to 1.5 h. The concentration of the different myoglobin species were estimated by applying the extinction coefficients for ferryl, met- and oxymyoglobin [13].

Fresh human blood was obtained from human volunteers (with informed consent). LDL was prepared from the fresh plasma in a Kontron 2070 ultracentrifuge fitted with a fixed-angle Kontron rotor, according to the method of Chung et al. [19], as described previously [15]. The concentration of protein was determined by a modified procedure of [20]. LDL (final concentration 0.25 mg/ml protein) was reacted with metmyoglobin (20 $\mu$M) in phosphate-buffered saline at

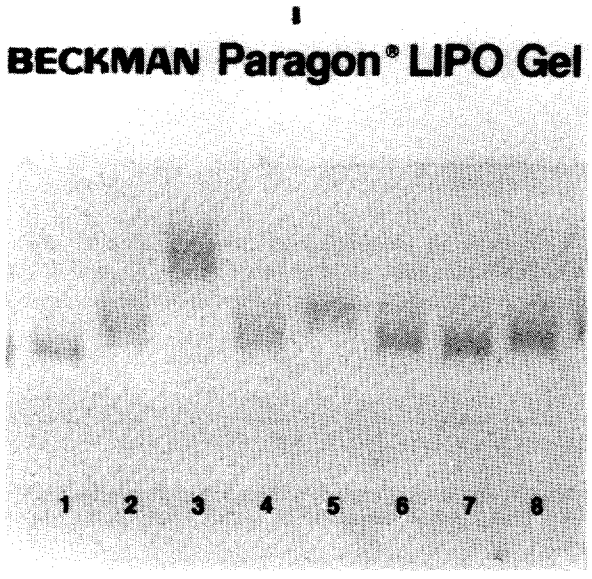


Fig. 1. Altered electrophoretic mobility of LDL (0.25 mg/ml LDL protein) induced by myoglobin (20 $\mu$M ± 25 $\mu$M $H_2O_2$) after 1.5 h incubation, and its inhibition by hydroxamates. Lanes 1,8, native LDL; lanes 2,3, LDL + metmyoglobin or ferryl myoglobin, respectively; lanes 4,6, LDL + metmmyoglobin + DFO (10 $\mu$M) or NMHH (10 $\mu$M); lanes 5,7, LDL + ferryl myoglobin + DFO (10 $\mu$M) or NMHH (10 $\mu$M).

pH 7.4 in the absence, as well as in the presence, of hydrogen peroxide (myoglobin : $H_2O_2$ = 1 : 1.25 mol ratio) to initiate ferryl myoglobin radical formation. Oxidative modification of the LDL was assessed by measuring the increased electrophoretic mobility on agarose gels (Beckman paragon lipo electrophoresis system) stained with Sudan black B stain. The extent of lipid peroxidation was assayed using a modified thiobarbituric acid assay [21] with the absorbance of the chromophore measured at 532 nm, corrected for background absorbance at 580 nm due to possible contributions from haem compounds, and incorporating appropriate controls [22]. Observations of the effects of LDL on the oxidation state of myoglobin as a function of time were measured as difference spectra with prior subtraction of the LDL spectrum.

The concentration of chelatable iron was determined by low temperature (30 K) EPR spectroscopy using a Bruker ESP300 spectrometer fitted with a TE103 rectangular cavity and an Oxford instruments liquid helium flow cryostat ESR900. Spectra were baseline-corrected by subtraction of a cavity spectrum or water/buffer under identical conditions. To allow accurate comparative quantitation, the chelatable iron was always converted to ferrioxamine prior to freezing. To accomplish this, DFO was either present already in the experiment or added 1 min prior to freezing the sample in the EPR tube. To determine the absolute concentration of DFO chelatable iron in the sample, the size of the g = 4.3 EPR signal from ferrioxamine was compared to a standard curve, constructed by adding varying amounts of ferrous sulphate to an aerobic solution of DFO (note: desferrioxamine cannot chelate iron out of undamaged haem proteins, such as myoglobin).

## 3. RESULTS

To investigate the potential for release of iron from the haem protein on interaction with LDL the effects of ferryl myoglobin as an initiating oxidising species, and the propagation effects of metmyoglobin, were studied. After 90 min interaction both metmyoglobin and, to a greater extent, ferryl myoglobin cause an increase in the relative electrophoretic mobility of the LDL, indicating oxidation and change in the surface charge of the LDL protein (Fig. 1). The level of thiobarbituric acid-reactive compounds, as a measure of breakdown products of lipid peroxidation, mediated by metmyoglobin and ferryl myoglobin, reached 6.9 ± 0.5 nmol/mg LDL protein (corresponding to $A_{532}-A_{580}$ 0.69/mg LDL protein) and 40 ± 1.0 nmol/mg LDL protein, respectively, at 1.5 h ($n$ = 8).

Changes in the oxidation state as the haem protein is activated by hydrogen peroxide in the presence and absence of LDL are shown in Fig. 2. The difference spectra were analysed for the presence of ferryl myoglobin applying the Whitburn algorithms. Activation of metmyoglobin in the absence of the LDL substrate generated ferryl myoglobin to the extent of 60% of the total haem, with very little change after 90 min interaction (Fig. 2, panels a), as previously reported [23]. In the presence of LDL the maximal ferryl formation is attained more rapidly and the actual level is enhanced initially, 72% by 6.5 min interaction, sustained up to 14.5 min, but declines progressively as the reaction with LDL continues, becoming progressively reduced to metmyoglobin with only 39% ferryl myoglobin remaining after 90 min interaction.

Interaction between LDL and metmyoglobin in the absence of hydrogen peroxide showed no detectable changes in the optical spectrum of the haem protein. The oxidative modification of the LDL is shown clearly both in terms of lipid peroxidation and altered surface charge, and is evidence for metmyoglobin-mediated propagation of oxidation.

Pretreatment of myoglobin with monohydroxamate and trihydroxamate hydrogen-donating compounds prior to activation with hydrogen peroxide (in the absence of LDL) substantially inhibited the development of the ferryl myoglobin (Fig. 2). In the presence of LDL, pretreatment with NMHH (100 $\mu$M) was more effective at reducing the ferryl myoglobin back to the met state than DFO when at concentrations in excess of the myoglobin but not at lower concentrations (10 $\mu$M). At hydroxamate concentrations of 10 $\mu$M, both DFO and NMHH inhibited the oxidation of LDL, as shown by the suppression of the altered electrophoretic mobility (Fig. 1), the monohydroxamate being more effective than the trihydroxamate. At excess concentrations of both drugs (100 $\mu$M) the change in surface charge is totally inhibited.

Inhibition of peroxidation of the polyunsaturated fatty acyl chains of the LDL by the drugs when present at the time of activation to ferryl myoglobin in the presence of the LDL is shown in Table I. At 10 $\mu$M levels, the monohydroxamate was almost as effective as 100 $\mu$M concentrations, but 10 $\mu$M DFO only inhibited to the extent of 60%. Metmyoglobin-induced propaga-

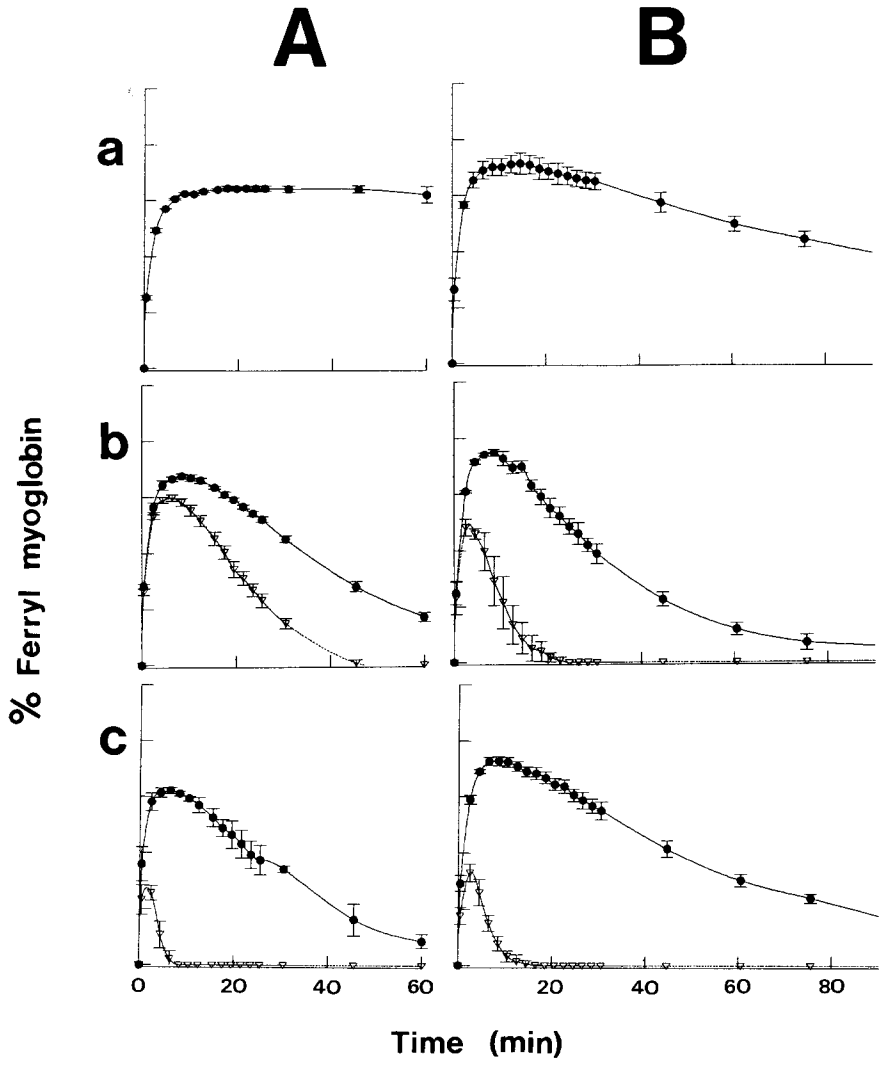


Fig. 2. Changes in the oxidation state as metmyoglobin is activated to ferryl myoglobin in the absence (A) and presence (B) of LDL and the effects of hydrogen-donating compounds. Panels a, no drugs; panels b, + DFO 10 $\mu$M (●), 100 $\mu$M (▽); panels c, + NMHH 10 $\mu$M (●), 100 $\mu$M (▽). Reaction concentrations: [metmyoglobin] 20 $\mu$M, [hydrogen peroxide] 25 $\mu$M, [LDL] 0.25 mg/ml, [hydroxamates] 10 $\mu$M, 100 $\mu$M.

tion of oxidation was again inhibited extensively by 100 $\mu$M monohydroxamate, but, in this case, 100 $\mu$M and 10 $\mu$M DFO were only effective to 55% and 30% inhibition, respectively. 10 $\mu$M NMHH is twice as effective as DFO as a chain-breaking antioxidant of LDL oxidation.

The stability of the haem ring of the myoglobin on interaction with LDL was assessed by studying the increase in non-haem iron by EPR spectroscopy and by calculating the decrease in the amount of haem remaining by visible spectroscopy applying the Whitburn algorithms, and from the intensity of the absorption maximum in the Soret region applying the extinction coefficient. The visible spectroscopic results reveal a decline in the total haem content of 25% after interaction of ferryl myoglobin with LDL with time of exposure up to 90 min, whereas the haem ring remains intact when metmyoglobin was applied under the same conditions and when metmyoglobin is activated by hydrogen peroxide in the absence of LDL (data not shown). Confirmation of the iron release from ferryl myoglobin consequent to its interaction with LDL is also provided from experiments applying low temperature EPR spectroscopy (Fig. 3) from the g-values at 6 and 4.3, respectively [24]. After the 90 min interaction between ferryl myoglobin and LDL, 25% of the original total haem was detected as non-haem iron; thus after this duration of interaction, the loss of haem is stoichiometric with the detection of non-haem iron. In the case of metmyoglobin as pro-oxidant, where the LDL oxidation was much less extensive, no non-haem iron was detected after 90 min of exposure to LDL (data not shown). This former finding supports the contention that, on oxidation by ferryl myoglobin, the oxidised LDL attacks the haem protein, destabilising the haem ring and releasing the iron. The pre-treatment of LDL with the monohy-

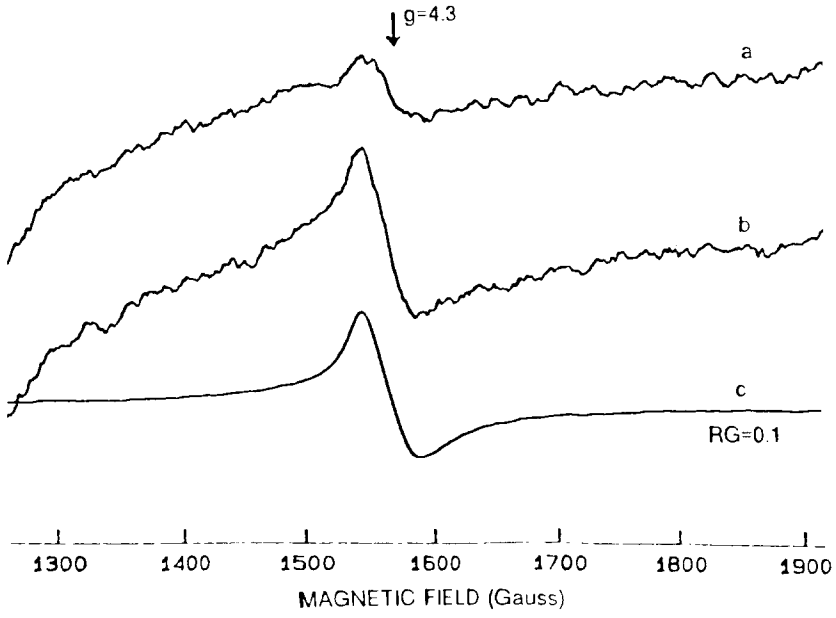


Fig. 3. Low-temperature EPR spectrum showing iron release from myoglobin in the presence of LDL and ferryl myoglobin. Samples were frozen at the times indicated. DFO was added to chelate the released iron for identification. (Trace a) MetMb + $H_2O_2$ + DFO + LDL, time = 5 s; (trace b) MetmMb + $H_2O_2$ + LDL; DFO was added at the end of incubation at time = 90 min; (trace c) 50 $\mu$M ferrioxamine (at one-tenth the gain of (a) and (b), thus depicting 5 $\mu$M non-haem iron). Reaction concentrations: [MetMb] 20 $\mu$M, [$H_2O_2$] 25 $\mu$M, [DFO] 100 $\mu$M, [LDL] 0.25 mg/ml. EPR conditions: Sweep time 3G/s; microwave power 20 mW; microwave frequency 9.37 GHz; modulation amplitude 10 G; time constant 330 ms; signal is average of 2 scans; temperature 30 K. Spectra (a) and (b) are displayed at 10-times the gain of (c).

droxamate compound prior to exposure to ferryl myoglobin prevents the oxidation of LDL. Thus the haem ring is protected from destabilisation, and the release of iron is prevented.

## 4. DISCUSSION

Our previous studies suggested that ruptured myocytes under oxidative stress generate ferryl myoglobin radicals which damage the lipids of membranes [10]. In addition, haem proteins from ruptured erythrocytes have been shown to oxidise LDL in vitro via activation to the ferryl state [14], and to modify its recognition properties affording uptake by macrophages. This can be prevented by enhancing the antioxidant status of the LDL [25].

The results reported here show that oxidised LDL, oxidatively modified by interaction of the LDL with ferryl myoglobin, can interact with the myoglobin destabilising the haem ring and inducing iron release. In the presence of hydroxamate compounds, the haem ring is protected and iron release does not occur due to their hydrogen-donating antioxidant properties. It has been reported by other workers that at levels of hydrogen peroxide in excesss of the haem protein, with prolonged exposure, destabilisation of the haem ring occurs and iron may be released [11]. This work demonstrates that under conditions of interaction between myoglobin and hydrogen peroxide (in the absence of LDL) in which destabilisation of the haem ring of the ferryl myoglobin does not occur, the additional presence of oxidised LDL augments the peroxide level and the haem ring is disrupted.

There are two potential mechanisms by which myoglobin may promote the peroxidation of LDL. Firstly, ferryl myoglobin can initiate peroxidation of the polyunsaturated fatty acid side chains by hydrogen abstraction. Secondly, in the absence of hydrogen peroxide, metmyoglobin amplifies the propagation of the LDL peroxidation by catalysing the decomposition of trace amounts of preformed lipid hydroperoxides within the LDL, according to the mechanisms for haem-mediated decomposition of lipid hydroperoxides (Eqn. 1–3) [26].

$$LOOH + HX\text{-}Fe^{3+} \rightarrow LO^{\cdot} + HX\text{-}[Fe^{IV} = O]^{2} + H^{+} \quad (1)$$

$$LOOH + HX\text{-}Fe^{3+} \rightarrow LOO^{\cdot} + HX\text{-}Fe^{2+} + H^{+} \quad (2)$$

$$LOOH + HX\text{-}Fe^{2+} \rightarrow LO^{\cdot} + HX\text{-}Fe^{3+} + OH^{-} \quad (3)$$

The alkoxyl and peroxyl radicals and the ferryl species formed as the haem iron species recycle, are capable of re-initiating further LDL peroxidation, although only a small proportion of the metmyoglobin cycling through the ferryl state is spectroscopically detectable by this mechanism.

The ability of DFO and NMHH to function as hydrogen donors [9,10,23,27–30], independently of their iron-chelating properties, and to protect the haem protein from destabilisation by reducing the initiating spe-

cies, may be an important contributor to the mechanism of the hydroxamate-mediated inhibition of ferryl myoglobin-induced oxidation of LDL. The hydroxamates may also inhibit the propagation process by acting as chain-breaking antioxidants and intercepting the propagation phase of LDL oxidation by H donation to alkoxyl or peroxyl species. The results suggest that, when in excess, the monohydroxamate is a more efficient inhibitor of ferryl myoglobin formation and hence its effectiveness in inhibiting lipid peroxidation, because of its scavenging of the initiator. DFO has a lower rate constant than NMHH for interaction with the ferryl myoglobin species [23] and thus is not so effective in directly reducing the latter; however, at hydroxamate concentrations well below that of myoglobin, DFO suppresses the ferryl myoglobin species more effectively than it reduces lipid peroxidation, suggesting that DFO is not acting as a chain-breaking antioxidant at these low concentrations. In fact, NMHH is more effective in inhibiting metmyoglobin-mediated LDL oxidation than DFO and is thus a more efficacious chain-breaking antioxidant. Importantly, since the hydroxamates possess the dual activities of antioxidant and iron-chelating properties, they also have the potential to inhibit the formation of hydroxyl radicals, which may be generated on release of iron from the haem protein by oxidised LDL. It is possible, in vivo, that haem proteins, leaking from ruptured cells, may be capable of enhancing the oxidation of LDL which has penetrated the endothelium of the coronary vessels. This may occur by haem protein-mediated decomposition of LDL hydroperoxides which have already been oxidised by, for example, contact with neighbouring cells or the enzymatic activity of lipoxygenases. As the level of LDL oxidation increases on prolonged interaction with the haem protein, iron release may occur. Previous studies [31] have reported the destruction of haem and the release of iron interaction of LDL with haemin, an oxidative denaturation product of haemoglobin, but only in the presence of excessive hydrogen peroxide levels (10 or 20-fold molar excesses). At these concentrations the presence of oxidised LDL is not necessary to drive the iron release. Recent analyses of the contents of human atherosclerotic lesions [1] have suggested that this is a potentially pro-oxidant environment with the possible availability of iron and copper. Our findings suggest a potential mechanism whereby iron may be derived from myoglobin released from ruptured cells in advanced lesions.

Table I

The percentage inhibition of myoglobin-mediated LDL oxidation by desferrioxamine (DFO) and *N*-methyl-*N*-hexanoyl hydroxamate (NMHH)

| | % decrease in $A_{532}-A_{580}$/mg LDL protein | *n* |
|---|---|---|
| Ferryl myoglobin | | |
| 100 $\mu$M DFO | 94 ± 3 | 8 |
| 100 $\mu$M NMHH | 95 ± 2 | 8 |
| 10 $\mu$M DFO | 60 ± 5 | 4 |
| 10 $\mu$M NMHH | 90 ± 6 | 4 |
| Metmyoglobin | | |
| 100 $\mu$M DFO | 55 ± 12 | 6 |
| 100 $\mu$M NMHH | 89 ± 5 | 8 |
| 10 $\mu$M DFO | 30 ± 5 | 4 |
| 10 $\mu$M NMHH | 58 ± 6 | 4 |

Hydroxamates were added to LDL (0.25 mg/ml) prior to addition of metmyoglobin (20 $\mu$M) ± hydrogen peroxide (25 $\mu$M) and incubated for 1.5 h at 37°C. Concentrations stated are final concentrations.

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