Volume 294, number 1,2, 38–42 FEBS 10435 December 1991

The modulation of ferryl myoglobin formation and its oxidative effects on low density lipoproteins by nitric oxide

Gareth Dee[2], Catherine Rice-Evans[1], Sharmin Obeyesekera[2], Shokoufeh Meraji[2], Michael Jacobs[3] and K. Richard Bruckdorfer[2]

[1]*Division of Biochemistry, United Medical and Dental Schools of Guy's and St Thomas's Hospitals, Lambeth Palace Road, London, SE1 7EH, UK* and *Departments of* [2]*Biochemistry and* [3]*Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, UK*

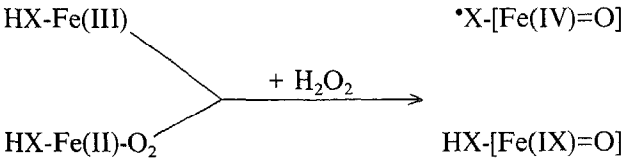
Received 17 September 1991

This study has investigated the interactions between nitric oxide and haem protein radicals. The results demonstrate that nitric oxide interacts with activated ferrylmyoglobin species with reduction to metmyoglobin, but the extent and duration of the reduction depends on the relative concentrations of nitric oxide and hydrogen peroxide. Ferryl myoglobin has a much greater relative potential for oxidising polyunsaturated fatty acid side chains in low density lipoproteins than in cell membranes. The peroxidative response can be modulated by nitric oxide: ferryl myoglobin-mediated peroxidation of LDL may be enhanced or suppressed by nitric oxide depending on the relative concentrations of NO and hydrogen peroxide.

Ferryl myoglobin species; Metmyoglobin; LDL; Lipid peroxidation; Nitric oxide

1. INTRODUCTION

Previous work has shown that ruptured cardiac myocytes under oxidative stress generate free radicals, the identity of which can be assigned to ferryl myoglobin species [1,2]. The activation of myoglobin by hydrogen peroxide to ferryl myoglobin proceeds through the oxidation by two oxidising equivalents. For metmyoglobin, the iron is converted to the iron(IV)-oxo state, one oxidising equivalent above that in metmyoglobin, and one oxidising equivalent is on a tyrosine residue on the surface of the globin protein [3–5]. After several minutes, this radical form decays to the iron(IV)-oxo ferryl myoglobin species. For oxymyoglobin, the iron(II) is directly oxidised to the iron(IV)-oxo ferryl state.



It has long been known since the early studies of Keilin and Hartree that nitric oxide forms a tight complex with a number of haem proteins, although there is a study reporting oxidation of haemoglobin to methaemoglobin [6].

A property of the endothelium is its ability to release factors which cause the relaxation of arterial smooth muscle, one of which is now known to be the radical nitric oxide [7]. NO acts by binding to the haem moiety of soluble guanylate cyclase stimulating the production of cyclic GMP and inducing relaxation [8]. NO activity is strongly inhibited by haemoglobin or myoglobin because of its strong reaction with haem [9]. NO also inhibits platelet function and therefore may play an important protective role against thrombosis and vasospasm. We have recently shown that oxidised LDL inhibits the formation or release of NO from endothelial cells [10].

In this study we have investigated the ability of nitric oxide to modulate the oxidation of LDL mediated by ferryl myoglobin species, as well as the direct effects of the nitric oxide on the haem protein radical species itself. The results demonstrate the ability of nitric oxide to reduce ferryl myoglobin to the met form, presumably by electron-donation; furthermore the ability of nitric oxide to modulate ferryl myoglobin-mediated oxidation of low density lipoproteins depends on the relative concentrations of nitric oxide to hydrogen peroxide.

Correspondence address: C. Rice-Evans, Division of Biochemistry, United Medical and Dental Schools of Guy's and St Thomas's Hospitals, Lambeth Palace Road, London, SE1 7EH, UK

2. MATERIALS AND METHODS

Myoglobin and haemoglobin were obtained from Sigma, Poole, Dorset, UK; nitric acid was from Cambrian Gases Ltd., Milton Keynes, UK.

Oxymyoglobin and methyoglobin were prepared in fresh solution as described previously [11]. Spectroscopic evaluation of the haem oxidation states, expressed as percentages of the total myoglobin, were calculated according to the Whitburn algorithms [12] from visible spectra obtained with a Beckman DU 64 spectrophotometer fitted with Quant 1 software.

Published by Elsevier Science Publishers B.V.

The ferryl iron-oxo form of myoglobin is not distinguishable from the ferryl myoglobin tyrosyl radical species by visible spectroscopy, but its presence has been shown under the experimental conditions used [13].

Haemoglobin-free erythrocyte membranes were prepared from normal fresh erythrocytes by the procedure of Dodge et al. [14] and used at final concentrations of 0.5 mg protein/ml. LDL was prepared from fresh plasma of human volunteers (with informed consent) in a Kontron 2070 ultracentrifuge (Kontron Instruments Ltd, Watford, Herts, UK) fitted with an MSE (Scientific Instruments, Crawley, Sussex, UK) vertical rotor (8×35 ml) according to the method of Chung et al. [15], for 2.5 h at 59 000 rpm and then the LDL fraction re-centrifuged for 10 h in a Kontron angle rotor at 50 000 rpm to remove minor protein contaminants. The concentration of the protein was determined [16] and the LDL were used at final concentrations of 0.5 mg protein/ml.

2.1. *Nitric oxide*

NO gas was prepared in stock solutions in helium-flushed buffer [7] and dispensed in gas-tight syringes.

2.2. *Measurement of lipid peroxidation*

The membranes or lipoproteins were incubated in the presence of metmyoglobin (20 μM) and hydrogen peroxide (25 μM, 100 μM) with or without the incorporation of NO (25 μM, 600 μM) at time points 2 or 15 min after the addition of H_2O_2 to the mixture of myoglobin and membranes or LDL. The extent of lipid peroxidation was assessed by the thiobarbituric acid assay [17], with the absorbance of the chromophore, measured at 532 nm, corrected for background absorbance at 580 nm due to possible contribution from haem proteins. Standards were run simultaneously under the same conditions utilising malondialdehyde prepared by acid hydrolysis of the bis(dimethyl acetal) derivative.

3. RESULTS AND DISCUSSION

Activation of oxymyoglobin by a 5-fold molar excess of hydrogen peroxide induced the formation of ferryl myoglobin up to 46% at 15 min; this level increased slightly over 5 min, 34% oxymyoglobin remaining (Fig. 1a). The addition of excess NO (600 μM) 15 min after addition of H_2O_2 caused an immediate suppression of ferryl myoglobin and oxidation of the majority of the remaining oxymyoglobin, with a resultant increase in metmyoglobin (b). Five minutes after adding NO (d), the amount of metmyoglobin had diminished extensively with reactivation to the ferryl form to the same extent as that observed in the non-supplemented system (c). This indicates that the effect of a single bolus of NO is only transitory even at such an excess concentration. However, when oxymyoglobin was activated by a lower concentration of H_2O_2 (25 μM) a lower level of ferryl myoglobin was observed after 15 min which increased to 40% after a further 5 min. The transitory nature of the suppressive effects of the NO (25 μM) were more obvious (Fig. 2).

Activation of metmyoglobin with a five-fold molar excess of hydrogen peroxide (100 μM) induced the formation of ferryl myoglobin to the extent of 74% at 15 min (Fig. 3). This position was totally reversed by the addition of 600 μM NO 15 min after the H_2O_2, when >90% of the ferryl myoglobin reverted to the met form. After 5 min, the ferryl species reappeared extensively as

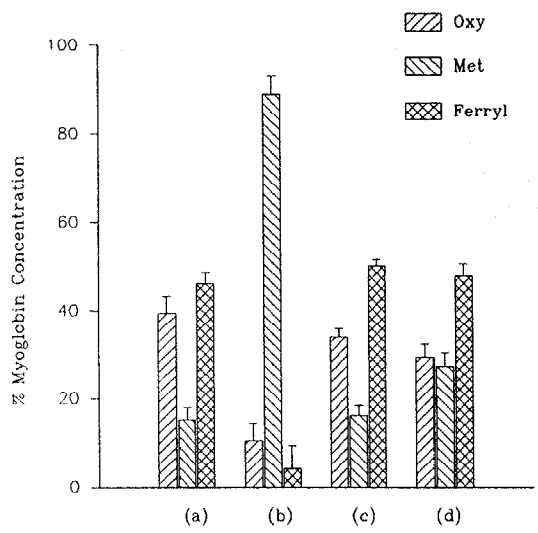


Fig. 1. The influence of nitric oxide on ferryl, met and oxy myoglobin levels formed from oxymyoglobin (20 μM)/hydrogen peroxide (100 μM). Absorption spectra were obtained immediately after the addition of nitric oxide (600 μM) (b) or buffer (a) to ferryl myoglobin 15 min post-activation. Readings were taken again after a further 5 min, (d) and (c), respectively. The percentages of the myoglobin forms were determined using the Whitburn equations (see section 2). All measurements were performed in triplicate and results are the means ± SEM of 3 experiments.

observed with oxymyoglobin. When a lower concentration of H_2O_2 (25 μM) was used, this reversal did not occur within the 5-min period (Fig. 4), but if 25 μM instead of 600 μM NO was added, then the reduction of the ferryl myoglobin was less and the subsequent reversal to ferryl was almost complete within this time (Fig. 5). The effects observed thus depend on the presence of a relative excess of peroxide to reactivate the metmyoglobin, formed on reduction of the ferryl species

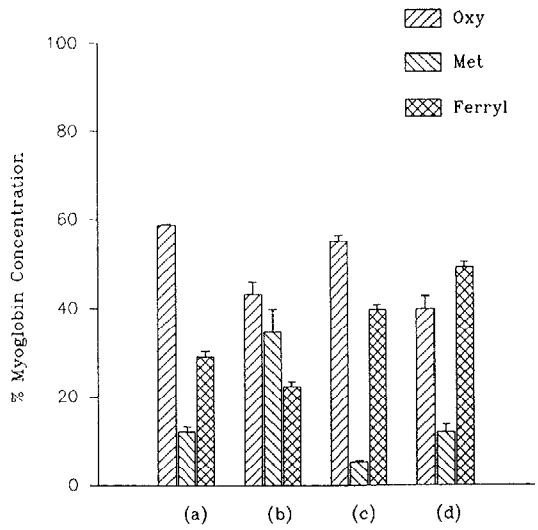


Fig. 2. As Fig. 1 except that the final concentrations of hydrogen peroxide and of nitric oxide were 25 μM.

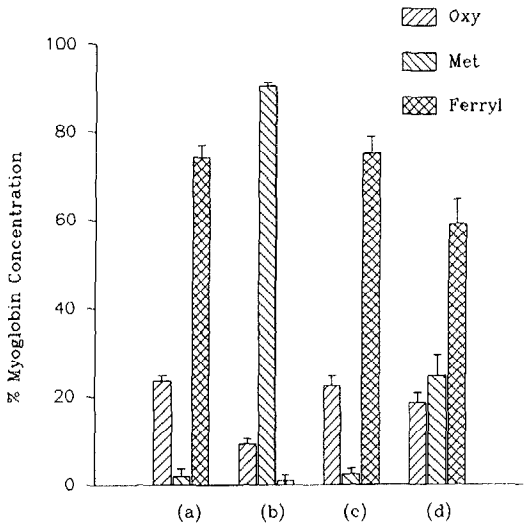


Fig. 3. The influence of nitric oxide on the formation of ferryl myoglobin from metmyoglobin (20 μM) in the presence of hydrogen peroxide. Experiments were conducted as described in the legend to Fig. 1, except that 100 μM hydrogen peroxide and 600 μM nitric oxide were applied.

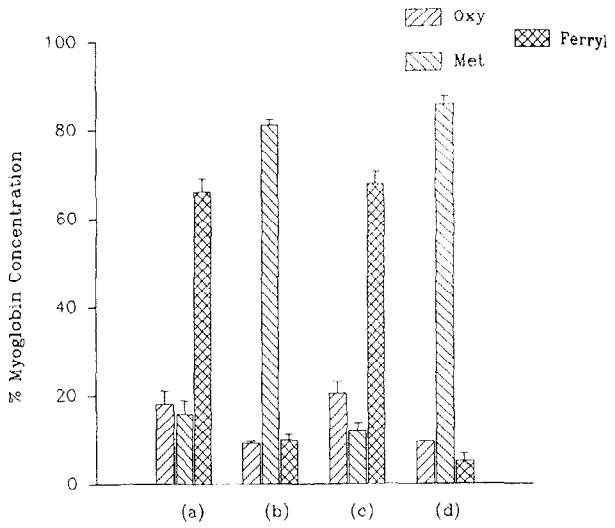


Fig. 4. As Fig. 3 except that the final concentration of hydrogen peroxide was 25 μM.

by the nitric oxide, thus generating the ferryl species. Where the nitric oxide is in large excess, the ferryl myoglobin does not reform.

However, an important consideration of the mechanism of action of nitric oxide in these systems is its rate of decomposition in an aqueous environment. The 'half-life' of the biological response of nitric oxide has been determined to be ca. 30 s [7], giving apparently sufficient time for it to reach the biological target. Otherwise, in aqueous solution nitric oxide decomposes with the eventual formation of stable end products nitrite and nitrate [18]. As reported earlier, incorporation of nitrite in place of nitric oxide in the studies reported here produced similar results but after a much prolonged time for reaction [19] indicating a mirroring of the response seen with NO but with a very much slower rate of reaction. This suggests that nitric oxide is more likely to be the reactive component in these systems, even though nitrite may also have some significant effects in vivo.

Exposure of LDL and erythrocyte membranes (0.25 mg/ml) to ferryl myoglobin, formed from metmyoglobin (20 μM), activated by 100 μM hydrogen peroxide, induced a much greater extent of oxidation of LDL after 1.5 h (Table I). At these concentration ratios

Table I

Effects of activated myoglobin on the peroxidation of erythrocyte membranes and LDL. Erythrocyte membranes (0.5 mg protein) and LDL (0.25 mg protein in order that the amount of lipid substrate is more equivalent) were incubated for 1.5 h in the presence or absence of 20 μM myoglobin and 100 μM H_2O_2 and the peroxidation of these substrates assayed. The results are expressed as means ± SEM of at least 3 experiments each measured in triplicate or quadruplicate.

	Lipid peroxidation TBA-reactive compounds (nmol/mg protein)	
Additions	Membranes	LDL
MetMb + H_2O_2	***1.66 ± 0.02 (n=5)	***23.2 ± 6.4 (n=5)
MetMb	**0.40 ± 0.03 (n=5)	*4.5 ± 2.3 (n=4)
Control	0.40 ± 0.02 (n=5)	2.1 ± 0.02 (n=7)

*P<0.05; **P<0.01; ***P<0.001, compared to control.

Table II

The effects of activated myoglobin (20 μM) on the peroxidation of LDL (0.25 mg/ml protein)

Additions to LDL		Lipid peroxidation TBA-reactive compounds (nmol/mg protein)		
		0 h	0.5 h	1.5 h
No additions		2.2 ± 0.3	2.9 ± 0.5	6.1 ± 1.2
Myoglobin		2.1 ± 0.4	4.4 ± 0.3	4.5 ± 3.5
1:5, Mb/H_2O_2				
− NO		2.6 ± 0.5	10.3 ± 1.4	20.3 ± 0.9
+ NO	25 μM	2.3 ± 0.4	15.7 ± 3.3	24.4 ± 4.2
	100 μM	2.4 ± 0.3	19.8 ± 1.7	27.4 ± 2.4
	600 μM	2.5 ± 0.4	22.5 ± 2.5	27.1 ± 2.4
1:1.25, Mb/H_2O_2				
− NO		1.3 ± 0.2	10.6 ± 1.7	23.7 ± 2.4
+ NO	25 μM	1.4 ± 0.4	10.9 ± 2.8	24.4 ± 3.3
	100 μM	1.6 ± 0.5	13.2 ± 4.2	24.9 ± 3.2
	600 μM	1.8 ± 0.4	5.2 ± 0.6	13.3 ± 2.0

Results are the mean ± SEM (n = 3), each individual assay being performed in triplicate.
(A_{532} 0.75 ≡ 7.5 nmol TBA-reactive compounds/mg protein)

of myoglobin and hydrogen peroxide in the presence of membranes for the incubation times applied, no significant iron release from myoglobin occurred as reported previously [11]. With LDL as the substrate, NO had no independent action as expected. However, the extent of the oxidation of LDL induced by ferryl species depended on the relative concentrations of H_2O_2 and NO used (Table II). With 100 μM H_2O_2 and 25, 100 or 600 μM NO (Table II), NO increased the oxidation when added 2 min (and 15 min; not shown) after the H_2O_2, reaching statistical significance ($P<0.01$) at the two higher concentrations of NO after 30 min. However, when the H_2O_2 concentration was lowered to 25 μM and that of the NO remained at 600 μM (Table II), the NO reduced the amount of oxidation and showed the protective effect observed, as with membranes as substrates [19]. If low concentrations of H_2O_2 (25 μM) and NO (25 or 100 μM) were used, no significant effects on the oxidation of LDL by ferryl myoglobin radicals were observed.

The most obvious result of this work is the demonstration that ferryl species of myoglobin are potent initiators of oxidation of LDL. This has previously been observed for membrane lipids [11,20] although the latter are relatively resistant to peroxidation compared with LDL.

It is also clear from the results that NO has a potent effect on the oxidation state of myoglobin.

In the presence of H_2O_2, both oxymyoglobin and metmyoglobin form ferryl species. NO reverses this process by reduction of ferryl to the met form. These effects are clearly reversible, the rate depending on the concentration of NO added. After a further 5 min, the effects of the NO persisted, if the initial concentration was high enough. However, in the presence of high concentrations of H_2O_2 which regenerate the ferryl species, the effect of NO was readily reversed. Interestingly, when oxymyoglobin was used with NO and H_2O_2, at certain concentrations, the amount of ferryl species increased after 5 min recovery from NO, compared to the initial levels 15 min post-activation in the absence of NO (Fig. 2).

In the presence of high concentrations of hydrogen peroxide (and therefore rapid regeneration of ferryl species), the NO actually increased the oxidation of LDL as measured by the thiobarbituric acid method. We have, as yet, no clear explanation of the process by which NO may increase oxidation. The lack of effect of nitric oxide and hydrogen peroxide on LDL in the absence of myoglobin precludes the straightforward explanation of the formation of an additional reactive species under conditions of excess available hydrogen peroxide. Further information is required concerning the reactions of nitric oxide with haem-containing proteins in the presence of H_2O_2. It is known that NO may react with the superoxide anion to form peroxynitrite anions which are toxic and degrade to form hydroxyl radicals and NO_2 [21]. There is no evidence that this process occurs in this system. As indicated earlier, nitrite may also have some slow action and the observed effects of NO may include some contribution of nitrite. Since the experiments may be of relevance in vivo, it was essential to perform them in the presence of oxygen. Further work is in progress to determine the relative contribution of nitrite and NO.

In the presence of pre-formed lipid hydroperoxides in the LDL, haem proteins such as metmyoglobin (and methaemoglobin) may also catalyse the propagation of lipid peroxidation (as shown in Table II), the formation of the initiating ferryl species is not necessary:

$$\text{LOOH} + \text{Fe}^{\text{III}}\text{-complex} \rightarrow \text{LO}^{\bullet} + [\text{Fe}^{\text{IV}} = \text{O complex}] + \text{H}^{+} \quad [22]$$

or

$$\text{LOOH} + \text{Fe}^{\text{III}}\text{-complex} \rightarrow \text{LOO}^{\bullet} + \text{Fe}^{2+}\text{-complex} + \text{H}^{+} \quad [23]$$

This means that met-haemproteins such as metmyoglobin and methaemoglobin may be capable of enhancing the oxidation of LDL which has penetrated the endothelium of coronary vessels and has already been minimally oxidised by contact with neighbouring cells. The reaction may be slower, but is not dependent on the simultaneous presence of H_2O_2. The release of NO from endothelial cells by macrophages and endothelial cells may encourage formation of metmyoglobin or methaemoglobin which is likely to produce the same effects. Interestingly, Kanner et al. distinguished the effects of metmyoglobin (a pro-oxidant) and myoglobin-NO (an anti-oxidant) in the oxidation of β-carotene [24].

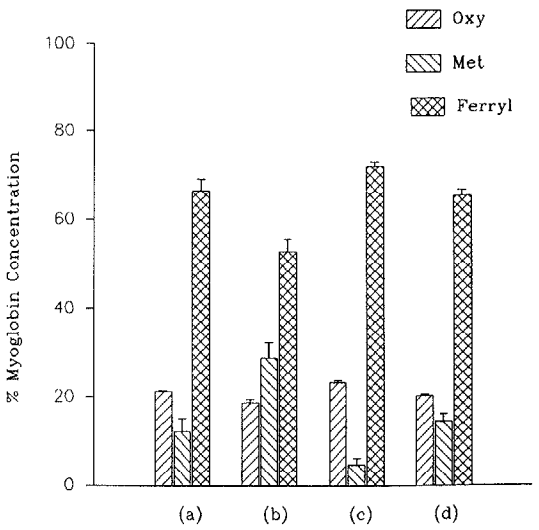


Fig. 5. As Fig. 3 except that the final concentrations of hydrogen peroxide and nitric oxide were 25 μM.

Evidence is not presented here that methaemoglobin has properties identical to that of metmyoglobin, but it has been shown to oxidixe sarcosomal membranes in the presence of H_2O_2 [20].

Acknowledgements: We acknowledge financial support from the Medical Research Council (G.D., S.O.), the Wellcome Trust (G.D.), The British Technology Group (C.R.E.) and the Peter Samuel Royal Free Fund (K.R.B., M.J.).

REFERENCES

[1] Turner, J.J.O., Rice-Evans, C., Davies, M. and Newman, E.S.R. (1991) Biochem. J. 277, 833–837.
[2] Turner, J.J.O., Rice-Evans, C., Davies, M. and Newman, E.S.R. (1990) Biochem. Soc. Trans. 18, 1056–1059.
[3] Tew, D. and Ortiz de Montellano, P.R. (1988) J. Biol. Chem. 263, 17880–17886.
[4] Davies, M.J. (1991) Biochim. Biophys. Acta, in press.
[5] Newman, E.S.R., Rice-Evans, C. and Davies, M.J. (1991) Biochem. Biophys. Res. Commun., in press.
[6] Doyle, M.P. and Hoekstra, J.W. (1981) J. Inorg. Biochem. 14, 351–358.
[7] Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nature 327, 524–526.
[8] Rapoport, R.M. and Murad, F. (1983) Circ. Res. 52, 352–357.
[9] Martin, W., Villani, G., Jothianandan, D. et al. (1985) J. Pharmacol. Exp. Ther. 233, 679–685.
[10] Jacobs, M., Plane, F. and Bruckdorfer, K.R. (1990) Br. J. Pharmacol. 100, 21–26.
[11] Rice-Evans, C., Okunade, G. and Khan, R. (1989) Free Rad. Res. Commun. 7, 45–54.
[12] Whitburn, K.D. (1987) Arch. Biochem. Biophys. 253, 419–424.
[13] Gibson, J.F., Ingram, D.J.E. and Nichols, P. (1958) Nature 181, 1398–1399.
[14] Dodge, J.T., Mitchdell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 10, 119–128.
[15] Chung, B.H., Wilkinson, T., Geer, J.C. and Segrest, J.P. (1980) J. Lipid Res. 221, 284–317.
[16] Markwell, M.A., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[17] Walls, R., Kumar, K.S. and Hochstein, P. (1979) Arch. Biochem. Biophys. 172, 463–468.
[18] Marletta, M.A., Tayeth, M.A. and Hevel, J.M. (1990) BioFactors 2, 219–225.
[19] Bruckdorfer, K.R., Dee, G., Jacobs, M. and Rice-Evans, C. (1990) Biochem. Soc. Trans. 18, 285–286.
[20] Kanner, J. and Harel, S. (1985) Arch. Biochem. Biophys. 237, 314–321.
[21] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B. (1990) Proc. Natl. Acad. Sci. USA 87, 1620–1624.
[22] Labeque, R. and Marnett, L. (1988) Biochemistry 27, 7960–7970.
[23] O'Brien, P.J. (1969) Can. J. Biochem. 47, 485–492.
[24] Kanner, J., Ben-Gera, I. and Berman, S. (1980) Lipids 15, 944–948.