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SCAVENGING OF HYPOCHLOROUS ACID AND OF MYOGLOBIN-DERIVED OXIDANTS BY THE CARDIOPROTECTIVE AGENT MERCAPTOPROPIONYLGLYCINE

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Mercaptopropionylglycine (MPG) has a marked cardioprotective action in several model systems of ischaemia-reoxygenation injury. Suggested mechanisms of action include scavenging of hydroxyl radical and of hypochlorous acid and reacting with an oxidant formed by reaction of myoglobin with H_2O_2, thereby slowing lipid peroxidation stimulated by myoglobin-H_2O_2 mixtures. This oxidant seems not to be singlet O_2 or hydroxyl radical. Studies *in vitro* show that scavenging of hypochlorous acid is a feasible mechanism of cardioprotective action for MPG *in vivo* in ischaemia/reperfusion systems to which neutrophil-mediated injury contributes. However, the poor ability of MPG to inhibit lipid peroxidation stimulated by myoglobin/H_2O_2 mixtures and its ability to increase iron ion release from myoglobin in the presence of a large excess of H_2O_2 suggests that MPG is unlikely to protect the myocardium by interfering with oxidants produced by the myoglobin-H_2O_2 system.

KEY WORDS: Myoglobin, hydrogen peroxide, mercaptopropionylglycine, ischaemia/reperfusion.

INTRODUCTION

Reperfusion of myocardium after a period of hypoxia or ischaemia results in further injury to the tissue and oxygen-derived species are thought to be involved in this "reoxygenation injury".[1-5] Superoxide radicals (O_2^-) and H_2O_2 are generated upon reoxygenation: potential sources of these species[1-4] include xanthine oxidase (although the role of this enzyme has been disputed[4,5]), disrupted mitochondrial electron transport chains and the activation of neutrophils in the reoxygenated tissue.[3] Activated neutrophils produce not only O_2^- and H_2O_2, but also the powerful oxidant hypochlorous acid, HOCl.[6]

It has been suggested that reoxygenation energy produced by O_2^- and H_2O_2 is due to their conversion into highly-reactive hydroxyl radicals, $\cdot OH$,[1-3] a process that requires catalytic transition metal ions. Iron ions are the most likely catalyst of such reactions *in vivo*.[7] A possible source of iron ions in reoxygenation injury is myoglobin,[8] since the haem ring of this protein breaks down to release iron ions upon exposure

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of the protein to a large excess of H_2O_2.[9] In addition, the first product of the reaction of myoglobin with H_2O_2 is a ferryl (Fe(IV)) species, whose formation is accompanied by production of an oxidant that can lead to stimulation of lipid peroxidation.[10-14] It has been suggested that this oxidant is $OH^{\cdot}$ and that formation of it contributes to reoxygenation injury.[13-14]

A wide variety of pharmacological agents has been claimed to offer variable degrees of protection against reoxygenation injury: they include Ca^{2+}-blockers, antioxidants (such as superoxide dismutase, catalase or dimethylsulphoxide) and inhibitors of eicosanoid synthesis.[1-3,14] For example, the thiol compound mercaptopropionylglycine (MPG) is a powerful protector against the prolonged depression of contractile function ("stunning") seen after brief ischaemia, followed by reoxygenation, in dog heart.[15] MPG has also been reported to decrease infarct size after longer periods of ischaemia followed by reoxygenation.[16,17]

The actions of MPG seem unrelated to haemodynamic effects, and it has been proposed that the protective action of MPG is due to free radical scavenging.[15,17] Consistent with this view, MPG was found to be very effective in preventing the trapping of radicals by the spin-trap PBN in a dog model of myocardial "stunning".[15] The ESR spectrum of the trapped radicals did not allow their identification. Studies *in vitro* showed that MPG does not inhibit peroxidation stimulated by addition of iron ions to liposomes or rat-liver microsomes.[15] MPG was found to be only a weak scavenger of $O_2^{\cdot-}$ and H_2O_2, but a very efficient scavenger of $\cdot OH$.[15] In the present paper, we explore two other mechanisms that could account for the protective action of MPG: scavenging of HOCl, and protection against myoglobin/H_2O_2-dependent lipid peroxidation.[13,14]

MATERIALS AND METHODS

Reagents

Sodium hypochlorite (NaOCl) and elastase (pig pancreatic) were purchased from BDH Chemicals Ltd. All other reagents, including MPG and arachidonic acid, were from Sigma Chemical Corp. α_1-Antiproteinase (human) was type A9024. Solutions of MPG and ascorbic acid were made up fresh immediately before use. HOCl was obtained when required by adjusting NaOCl to pH 6.2 with dil. H_2SO_4.[19] Horse-heart myoglobin was purified as described in.[9] Rat-liver microsomes were prepared by differential pelleting and washing as described in.[20] Lipid peroxidation was measured by the thiobarbituric acid (TBA) test.[20]

Methods

Measurements of elastase and α_1-antiproteinase were carried out as in[19]. Degradation of myoglobin by excess H_2O_2 was calculated by the Whitburn equations as explained in.[9] The ferrozine assay for non-haem iron was carried out as in.[21] All other details of methods are given in Table and Figure legends.

RESULTS

Actions of Ascorbate and MPG on the Myoglobin System

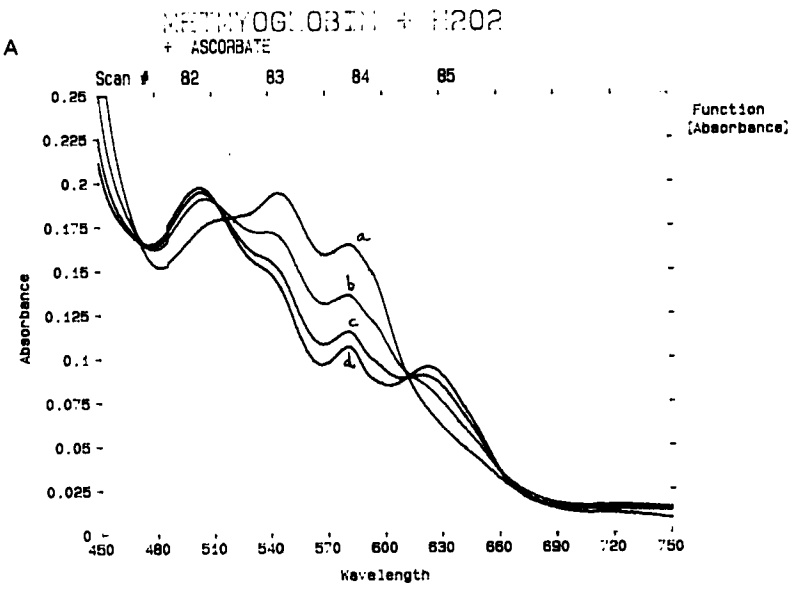
As expected,[9,10-14] incubation of purified horse-heart metymyoglobin with a 3-fold molar excess of H_2O_2 caused spectral changes characteristic of Fe(IV)-myoglobin (ferryl myoglobin) formation as shown in Figure 1A. Ferryl myoglobin has been reported to oxidize ascorbic acid.[11,22,23] In agreement with this, addition of 1 mM ascorbate to the reaction mixture caused a loss of ferryl myoglobin and the appearance of myoglobin (III) (Figure 1A). As expected,[23] we found that this reduction was followed by a further reduction of myoglobin(III) to myoglobin(II), which combined with O_2 to form oxymyoglobin (Figure 1B). These two successive reactions were evidenced by the time-course at 580 nm (an absorption peak for both myoglobin (IV) and oxymyoglobin), which showed a rapid decrease during the first 6–8 min and then a slow increase. It was further confirmed by the time course at 630 nm [λ_{max} of myoglobin(III)], which increased in the period 0–8 min and then decreased slowly (data not shown). Use of the Whitburn equations[9,10] showed that no haem breakdown was taking place under these reaction conditions.

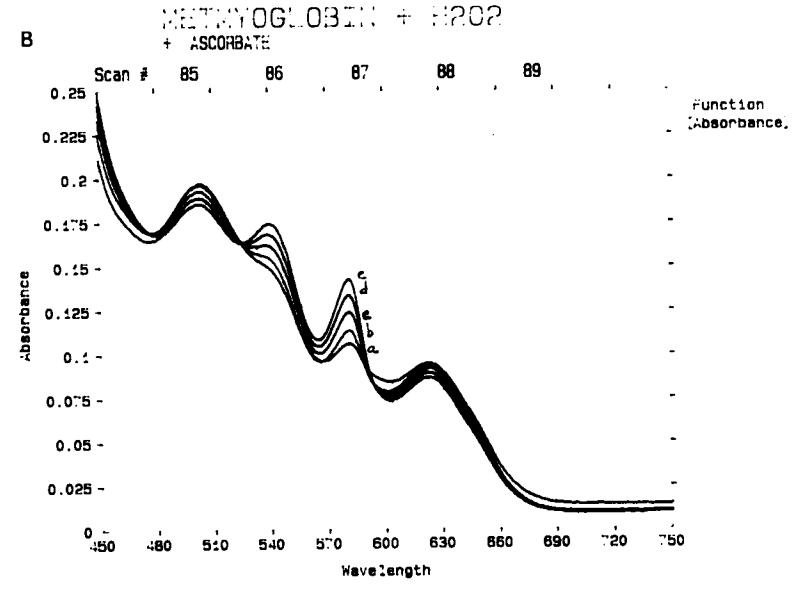
Mixtures of metmyoglobin and H_2O_2 caused peroxidation of arachidonic acid or of rat liver microsomes at pH 7.4, as measured by the thiobarbituric acid test. Table I shows some representative data for microsomes and Table II for arachidonic acid. Peroxidation was greatest when the lipid was present in the reaction mixture before H_2O_2 was added to the myoglobin: it was much less when the lipid was added 5 minutes after myoglobin and H_2O_2 had been allowed to react (Table II). Ferryl myoglobin formation was complete well within 3 minutes (data not shown), and was stable in the reaction mixture for at least 10 minutes. Peroxidation of lipid by myoglobin-H_2O_2 was not inhibited by scavengers of singlet O_2 (histidine) or of ·OH (histidine, phenylalanine, mannitol, formate).

Ascorbate has previously been shown to inhibit metmyoglobin/H_2O_2-induced peroxidation of erythrocyte membranes.[23] Peroxidation of microsomes (Table I) or of arachidonic acid (Table II) in our system was also almost completely inhibited by ascorbic acid. By contrast, ascorbic acid rapidly accelerated peroxidation if myoglobin was replaced by Fe^{3+} ions (Table I) as would be expected.[24] Whereas ascorbate protected efficiently against peroxidation ([23], Tables I and II), MPG had no effect on the rate of myoglobin/H_2O_2-dependent peroxidation of arachidonic acid. With microsomes it had only a small inhibitory action until concentrations greater than 200 μM were added. MPG had no inhibitory action on peroxidation in the presence of Fe^{3+} ions or of Fe^{3+} and ascorbic acid (Table I).

When MPG was added to the myoglobin-H_2O_2 system, the spectral changes were complex. Myoglobin (IV) disappeared, being probably reduced to myoglobin (III) as evidenced by a decrease in absorbance in the 500–600 nm region and an increase in the 610–650 nm range. In Figure 1C, scans were recorded at 0, 3, 6, 9 and 12 min after addition of MPG. Then a change in the isosbestic point appears (Figure 1D), and finally, another reaction characterized by an increase in absorbance in the 520–590 nm region and around 615 nm (Figure 1E). The final spectrum obtained (at 48 min) has some similarities with that of porphyrin, suggesting an alteration of the haem structure.

When metmyoglobin is incubated with a large molar excess of H_2O_2, haem degradation takes place[9] and iron ion release can be detected by the ferrozine method (Table III). As expected,[23] ascorbate prevented this iron release. By contrast, MPG at





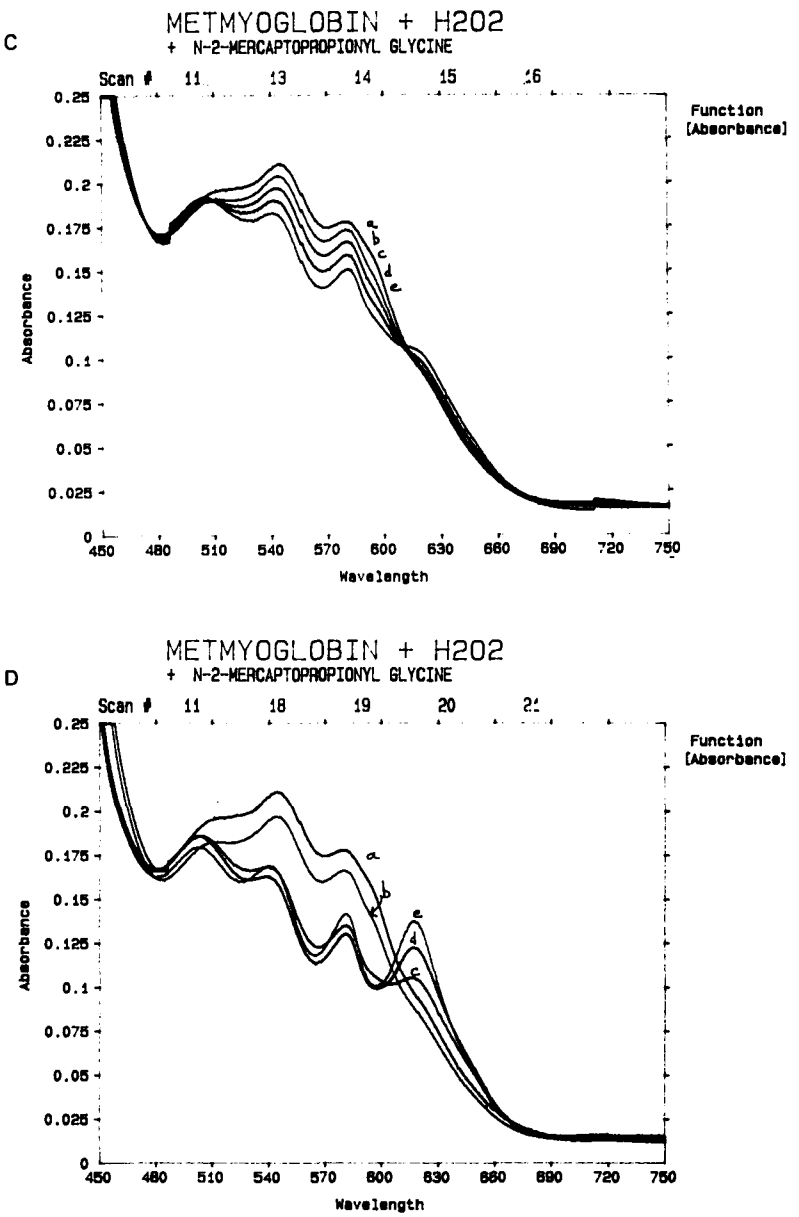


FIGURE 1. Spectral changes on incubating metmyoglobin with H_2O_2: the effects of ascorbate and MPG. Reaction mixtures contained, in a total volume of 1 ml, the following reagents at the final concentrations stated: 25 mM KH_2PO_4-KOH buffer pH 7.4, 25 μM metmyoglobin, 75 μM H_2O_2 and, where indicated, 1 mM ascorbate or 1 mM MPG at 37° C. 1A Spectra represent myoglobin (IV) [a] and then the mixture immediately (b), 3 minutes (c) and 6 minutes (d) after addition of ascorbate. 1B Spectra at 6 min (a), 12 min (b), 18 min (c), 24 min (d) and 30 min (e) after ascorbate addition. 1C Spectra were recorded immediately (a) and 3 min (b), 6 min (c), 9 min (d) and 12 min (e) after addition of MPG. 1D Spectra recorded at 0 min (a), 6 min (b), 12 min (c), 18 min (d) and 24 min (e) after addition of MPG 1E Spectra recorded at 30 min (a), 36 min (b), 42 min (c) and 48 min (d) after addition of MPG.

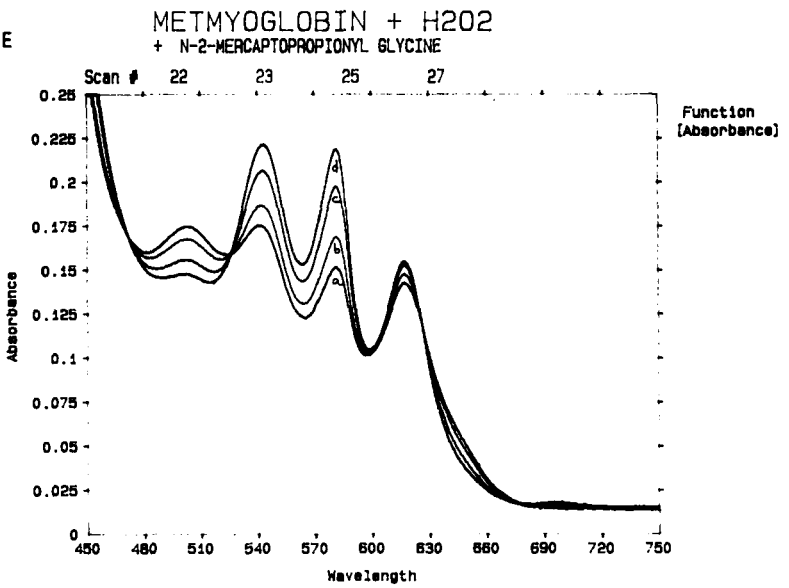


FIGURE 1. (continued). Spectral changes on incubating metmyoglobin with H_2O_2: the effects of ascorbate and MPG. Reaction mixtures contained, in a total volume of 1 ml, the following reagents at the final concentrations stated: 25 mM KH_2PO_4-KOH buffer pH 7.4, 25 μM metmyoglobin, 75 μM H_2O_2 and, where indicated, 1 mM ascorbate or 1 mM MPG at 37°C. 1A Spectra represent myoglobin (IV) [a] and then the mixture immediately (b), 3 minutes (c) and 6 minutes (d) after addition of ascorbate. 1B Spectra at 6 min (a), 12 min (b), 18 min (c), 24 min (d) and 30 min (e) after ascorbate addition. 1C Spectra were recorded immediately (a) and 3 min (b), 6 min (c), 9 min (d) and 12 min (e) after addition of MPG. 1D Spectra recorded at 0 min (a), 6 min (b), 12 min (c), 18 min (d) and 24 min (e) after addition of MPG 1E Spectra recorded at 30 min (a), 36 min (b), 42 min (c) and 48 min (d) after addition of MPG.

low concentrations ($< 200\,\mu$M) had no effect, whereas high concentrations of MPG actually reproducibly enhanced iron release (Table III).

Scavenging of Hypochlorous Acid

One of the important targets attacked by HOCl *in vivo* is α_1-antiproteinase, the major circulating inhibitor of proteolytic enzymes such as elastase.[6] α_1-Antiproteinase is very rapidly inactivated by HOCl, losing its elastase-inhibitory capacity.[6,19,25] Thus a good test for physiologically-relevant scavenging of HOCl by a compound is to see whether that compound, as the concentrations that are achieved *in vivo*, can protect α_1-antiproteinase against inactivation by HOCl.[19,26,27] HOCl was used directly in these experiments rather than being generated by the myeloperoxidase/H_2O_2/Cl^- system, because thiols have complex effects on the activity of peroxidase enzymes.[28-30]

Table IV (second line) shows that α_1-antiproteinase inhibited elastase: a concentration sufficient just to inhibit completely was used. Treatment of the α_1-antiproteinase with 60 μM HOCl almost completely abolished its elastase-inhibitory activity (Table IV, third line). MPG, tested at concentrations up to 120 μM, had no effect on the activity of elastase itself, or on the ability of α_1-antiproteinase to inhibit elastase (data not shown). However, MPG was able to protect α_1-antiproteinase against inactivation by HOCl (Table IV). Thus 120 μM MPG protected completely.

TABLE I

Peroxidation of rat liver microsomes by myoglobin-H_2O_2. Reaction mixtures contained, in a final volume of 1.0 ml, 0.5 ml of phosphate/saline buffer (5 mM Na_2HPO_4-NaH_2PO_4, 0.15 M NaCl, pH 7.4), 0.35 mg of microsomal protein, 25 μM metmyoglobin and 75 μM H_2O_2 and were incubated for 5 min at 37°C. Peroxidation was started by adding metmyoglobin + H_2O_2, except in the starred experiment below, when microsomes were added 5 min after mixing the other reagents, followed by a further 5 min incubation. In each case, the values were obtained against a blank containing all the reagents except H_2O_2. Peroxidation was measured by the TBA test.[20] Compounds were added to give the final concentrations stated in the reaction mixture.

Addition to reaction mixture	Extent of peroxidation A_{532}
None	0.166
None (omit myoglobin or H_2O_2)	0.000
*None (microsomes added to reaction mixture 5 min after mixing other reagents)	0.071
MPG 50 μM	0.168
100 μM	0.123
200 μM	0.094
500 μM	0.082
750 μM	0.079
1 mM	0.085
Ascorbate (1 mM)	0.016
Histidine (10 mM)	0.140
Sodium formate (20 mM)	0.144
Mannitol (20 mM)	0.125
Fe^{3+} (100 μM), omit myoglobin	0.029
Ascorbic acid (1 mM), omit Mb	0.013
Fe^{3+} (100 μM) + ascorbic acid (1 mM), omit Mb	0.226
Fe^{3+} (100 μM) + MPG (1 mM), omit Mb	0.031
Fe^{3+} + ascorbic acid + MPG (1 mM), omit Mb	0.220

DISCUSSION

MPG is a powerful cardioprotective agent in several ischaemia/reoxygenation model systems.[15-17] How does it act? Its reactions with O_2^- and H_2O_2 are slow and unlikely to account for its ability to protect against reoxygenation injury.[15] MPG is a powerful scavenger of ·OH, but ·OH reacts equally fast with most biological molecules.[18] In the present paper, we have investigated two other potential cardio-protective mechanisms. Firstly, MPG, at concentrations that are achieved *in vivo*,[15] is an extremely powerful scavenger of HOCl (Table IV), an agent known to do severe damage to the myocardium.[31] It is interesting to note that two other cardioprotective agents, dimethylthiourea[32] and N-acetylcysteine[33], have also been shown to be powerful scavengers of HOCl.[34,35] In the myocardial stunning model used by Bolli *et al.*,[15] neutrophils do not contribute to the tissue injury[2] because of the brief ischaemic period and so the protective action of MPG cannot be due to scavenging of HOCl derived from neutrophils. By contrast, these cells make substantial contributions to injury after longer periods of ischaemia,[3] and the ability of MPG to protect against such damage[16,17] could well be mediated by removal of HOCl. Our data therefore show that HOCl scavenging is a feasible mechanism of protection by MPG *in vivo* in situations involving prolonged ischaemia associated with necrosis.

A second protective mechanism, proposed by Mitsos *et al.*,[13] is the ability of MPG

TABLE II

Peroxidation of arachidonic acid by myoglobin-H_2O_2. Reaction mixtures contained, in a final volume of 1 ml, 50 mM NaH_2PO_4-NaOH buffer pH 7.3, 100 μM diethylenetriaminepentaacetic acid to bind any released iron ions, 0.4 mM arachidonic acid, 0.5 mM H_2O_2 and 50 μM myoglobin. They were incubated at 30° C for 10 minutes. Peroxidation was measured by the TBA test.[20] MPG and other compounds were added to give the final concentrations stated.

Reagent added to reaction mixture	Final concentration (mM)	Extent of peroxidation A_{532}
None	-	0.455
None (omit H_2O_2)	-	0.044
None†	-	0.279
Ascorbate	1.0	0.142
MPG	0.05	0.423
	0.1	0.424
	0.2	0.446
	0.5	0.519
	0.75	0.547
	1.0	0.550
Histidine	1.0	0.455
	5.0	0.455
Phenylalanine	10.0	0.465
Mannitol	20.0	0.453
Sodium formate	20.0	0.444

†Arachidonic acid added to the reaction mixture 5 min after mixing the other reagents, followed by 10 min incubation.

to react with the oxidizing species produced by exposure of myoglobin to H_2O_2 and so to inhibit lipid peroxidation stimulated by this species (Tables I and II). The nature of the oxidant produced when myoglobin reacts with H_2O_2 is uncertain. In order for it to stimulate peroxidation of microsomes and fatty acids, it must presumably leave the haem pocket. The oxidant has been suggested to be ·OH,[12] but this is unlikely to be the case since it does not degrade the sugar deoxyribose,[9] which is destroyed by ·OH at a fast rate (rate constant $3.1 \times 10^9\ M^{-1}\ s^{-1}$[36]) and peroxidation induced by it is not prevented by ·OH scavengers such as histidine or mannitol (Table II).

MPG did not inhibit peroxidation stimulated by "free" iron salts (15; Table I). As

TABLE III

Iron ion release from myoglobin by H_2O_2. Effects of ascorbic acid and MPG. Reaction mixtures contained, in a final volume of 1.0 ml, 50 μM metmyoglobin and 750 μM H_2O_2 in 25 mM KH_2PO_4-KOH buffer pH 7.4 and were incubated for 1 hour at 37° C. Reactions were started by adding H_2O_2. After incubation, an aliquot of the mixture was sampled for the ferrozine assay.[21] Results shown are the mean of 3 separate assays which differed by less than ± 5%. All concentrations quoted are the final concentrations in the reaction mixtures.

Addition to reaction mixture	Iron ions released
None	9.3
Ascorbic acid (1 mM)	0.0
MPG 100 μM	9.5
200 μM	9.2
500 μM	7.8
1 mM	8.1
1.5 mM	11.9
2 mM	13.9

TABLE IV

Inactivation of α_1-antiproteinase by hypochlorous acid: effect of mercaptopropionylglycine (MPG). α_1-Antiproteinase (0.2 mg/ml), HOCl (60 μM) and MPG (if any) were incubated in a final volume of 1.0 ml in phosphate-buffered saline pH 7.4 (full details in[19]) at 37° C for 30 minutes. Then 2 ml of phosphate-buffered saline and 0.05 ml of elastase were added, followed by further incubation at 37° C for 20 min. This allows any α_1-antiproteinase still active to inhibit elastase. (Any HOCl remaining is diluted out to the point at which it cannot affect elastase itself). The remaining elastase activity was then measured by adding elastase substrate,[19] which is hydrolysed by elastase to give an increase in A_{410}. Concentrations of scavengers added were those present in the first (1.0 ml) reaction mixture; scavengers and α_1-antiproteinase were mixed together before adding HOCl. Control experiments showed that none of the substances tested themselves affected elastase activity or interfered with the ability of α_1-antiproteinase to inhibit elastase. Results are the means of duplicate experiments that agreed to within 8%.

Additional to first reaction mixture	Elastase activity in final reaction mixture A_{410}/sec^{-1}
Buffer only	6.38×10^{-3}
α_1-antiproteinase	0
α_1-antiproteinase + HOCl	6.07×10^{-3}
α_1AP + HOCl + 24 μM MPG	3.88×10^{-3}
α_1AP + HOCl + 48 μM MPG	1.98×10^{-3}
α_1AP + HOCl + 72 μM MPG	1.48×10^{-3}
α_1AP + HOCl + 90 μM MPG	9.1×10^{-4}
α_1AP + HOCl + 120 μM MPG	0

previously reported,[23] ascorbic acid reacts with ferryl myoglobin and protects against peroxidation induced by myoglobin-H_2O_2 mixtures, a report confirmed in this study using a different lipid substrate (microsomes in Table I). However, physiologically-achievable MPG concentrations (100–200 μM range [15]) had no effect (arachidonic acid) or only a partial inhibitory effect (microsomes) against peroxidation, casting doubt on whether this can be an important protective effect *in vivo*.

The reaction of MPG with ferryl myoglobin is not simple. One-electron oxidation of MPG may result in formation of reactive sulphur-containing radicals that might attack and damage myoglobin,[37,38] thus accounting for the complex spectral changes observed (Figure 1). It is interesting to note that MPG, unlike ascorbate (23; Table III) was not able to protect against the haem destruction resulting from exposure of myoglobin to a large excess of H_2O_2:[9] indeed, it reproducibly stimulated iron ion release (Table III). It thus seems unlikely that prevention of myoglobin-dependent oxidative membrane damage could be a major mechanism of action of MPG *in vivo*.

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References

1. J.M. McCord (1985) Oxygen-derived free radicals in post ischemic tissue injury. *New England Journal of Medicine*, **312**, 159–163.
2. R. Bolli (1988) Oxygen-derived free radicals and postischemic myocardinal dysfunction ('stunned myocardium'). *Journal of the American College of Cardiology*, **12**, 239–249.
3. P.J. Simpson and B.R. Lucchesi (1987) Free radicals and myocardial ischaemia and reperfusion. *Journal of Laboratory and Clinical Medicine*, **110**, 13–30.

4. B. Halliwell (1989) Superoxide, iron, vascular endothelium and reperfusion injury. *Free Radical Research Communications*, **5**, 315–318.
5. J.P. Kehrer (1989) Concepts related to the study of reactive oxygen and cardiac reperfusion injury. *Free Radical Research Communications*, **5**, 305–314.
6. S.J. Weiss (1989) Tissue destruction by neutrophils. *New England Journal of Medicine*, **320**, 365–376.
7. B. Halliwell and J.M.C. Gutteridge (1988) Iron as a biological prooxidant. *ISI Atlas of Science, Biochemistry*, **1**, 48–52.
8. K.I. Nomoto, N. Mori, T. Shoji and K. Nakamura (1987) Early loss of myocardial myoglobin detected immunohistochemically following occlusion of the coronary artery in rats. *Experimental and Molecular Pathology*, **47**, 390–402.
9. A. Puppo and B. Halliwell (1988) Formation of hydroxyl radicals in biological systems. Does myoglobin stimulate hydroxyl radical formation from hydrogen peroxide? *Free Radical Research Communications*, **4**, 415–422.
10. K.D. Whitburn (1987) The interaction of oxymyoglobin with hydrogen peroxide: the formation of ferrylmyoglobin at moderate excesses of hydrogen peroxide. *Archives of Biochemistry and Biophysics*, **253**, 419–430.
11. S. Harel and J. Kanner (1985) Muscle membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin. *Journal of Agricultural and Food Chemistry*, **33**, 1188–1192.
12. D. Galaris, D. Mira, A. Sevanian, E. Cadenas and P. Hochstein (1988) Co-oxidation of salicylate and cholesterol during the oxidation of metmyoglobin by H_2O_2. *Archives of Biochemistry and Biophysics*, **262**, 221–231.
13. S.E. Mitsos, D. Kim, B.R. Lucchesi and J.C. Fantone (1988) Modulation of myoglobin-H_2O_2-mediated peroxidation reactions by sulfhydryl compouns. *Laboratory Investigation*, **59**, 824–830.
14. D. Galaris, E. Cadenas and P. Hochstein (1989) Glutathione-dependent reduction of peroxides during ferryl- and met-myoglobin interconversion: a potential protective mechanism in muscle. *Free Radical Biology and Medicine*, **6**, 473–478.
15. R. Bolli, M.O. Jeroudi, B.S. Patel, O.I. Aruoma, B. Halliwell, E.K. Lai and P.B. McCay (1989) Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. *Circulation Research*, **65**, 607–622.
16. S.E. Mitsos, T.E. Askew, J.C. Fantone, S.L. Kunkel, G.D. Abrams, A. Schork and B.R. Lucchesi (1986) Protective effects of N-2-mercaptopropionylglycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. *Circulation*, **73**, 1077–1086.
17. S.E. Mitsos, J.C. Fantone, K.P. Gallagher, K.M. Walden, P.J. Simpson, G.D. Abrams, M.A. Schork and B.R. Lucchesi (1986) Canine myocardial reperfusion injury: protection by a free radical scavenger, N-2-mercaptopropionylglycine. *Journal of Cardiovascular Pharmacology*, **8**, 978–988.
18. B. Halliwell (1990) How to characterize a biological antioxidant. *Free Radical Research Communications*, **9**, 1–31.
19. M. Wasil, B. Halliwell, D.C.S. Hutchison and H. Baum (1987) The antioxidant action of extracellular fluids. *Biochemical Journal*, **243**, 219–223.
20. G.J. Quinlan, B. Halliwell, C.P. Moorhouse and J.M.C. Gutteridge (1988) Action of lead(II) and aluminium(III) ions on iron-stimulated lipid peroxidation in liposomes, erythrocytes and rat liver microsomal fractions. *Biochimica et Biophysica Acta*, **962**, 196–200.
21. P. Carter (1971) Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (Ferrozine). *Analytical Biochemistry*, **40**, 450–458.
22. J. Kanner and S. Harel (1985) Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. *Archives of Biochemsitry and Biophysics*, **237**, 314–321.
23. C. Rice-Evans, G. Okunade and R. Khan (1989) The suppression of iron release from activated myoglobin by physiological electron donors and by desferrioxamine. *Free Radical Research Communications*, **7**, 45–54.
24. E.D. Wills (1969) Lipid peroxide formation of microsomes. The role of non-haem iron. *Biochemical Journal*, **113**, 325–332.
25. R.A. Clark, P.J. Stone, A.E. Hag, J.D. Calore and C. Franzblau (1981) Myeloperoxidase-catalyzed inactivation of α_1-protease inhibitor by human neutrophils. *Journal of Biological Chemistry*, **256**, 3348–3353.
26. M. Wasil, B. Halliwell, C.P. Moorhouse, D.C.S. Hutchison and H. Baum (1987) Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by some anti-inflammatory drugs. *Biochemical Pharmacology*, **36**, 3847–3850.
27. O.I. Aruoma, M. Wasil, B. Halliwell, B.M. Hoey and J. Butler (1987) The scavenging of oxidants by sulphasalazine and its metabolites. *Biochemical Pharmacology*, **36**, 3739–3742.

28. B.E. Svensson (1989) Synergism between substrate and non-substrate thiols in peroxidase-oxidase reactions. *Chemico-Biological Interactions*, **70**, 323–337.
29. N.R. Matheson (1982) The effect of antiarthritic drugs and related compounds on the human neutrophil myeloperoxidase system *Biochemical and Biophysical Research Communications*, **108**, 259–265.
30. R.A. Cuperus, H. Hoogland, R. Wever and A.O. Muijsers (1987) The effect of D-penicillamine on myeloperoxidase: formation of compound III and inhibition of the chlorinating activity. *Biochimica et Biophysica Acta*, **912**, 124–131.
31. R.C. Kukreja, A.B. Weaver and M.L. Hess (1989) Stimulated human neutrophils damage cardiac sarcoplasmic reticulum function by generation of oxidants. *Biochimica et Biophysica Acta*, **990**, 198–205.
32. R. Bolli, W.X. Zhu, C.J. Hartley, L.H. Michael, J.E. Repine, M.L. Hess, R.C. Kukreja and R. Roberts (1987) Alteration of dysfunction in the postischemic stunned myocardium by dimethylthiourea. *Circulation*, **76**, 458–468.
33. M.B. Forman, D.W. Puett, C.U. Cates, D.E. McCroskey, J.K. Beckman, H.L. Greene and R. Virmanti (1988) Glutathione redox pathway and reperfusion injury. *Circulation*, **78**, 202–213.
34. O.I. Aruoma, B. Halliwell, B.M. Hoey and J. Butler (1989) The antioxidant action of N-acetylcysteine. *Free Radical Biology and Medicine*, **6**, 593–597.
35. M. Wasil, B. Halliwell, M. Grootveld, C.P. Moorhouse, D.C.S. Hutchison and H. Baum (1987) The specificity of thiourea, dimethylthiourea and dimethylsulphoxide as scavengers of hydroxyl radicals. Their protection of α_1-antiproteinase against inactivation by hypochlorous acid. *Biochemical Journal*, **243**, 867–870.
36. B. Halliwell, J.M.C. Gutteridge and O.I. Aruoma (1987) The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals *Analytical Biochemistry*, **165**, 215–219.
37. M.D. Sevilla, M. Yan, D. Becker and S. Gillich (1989) ESR investigations of the reactions of radiation-produced thiyl and DNA peroxyl radicals: formation of sulfoxyl radicals. *Free Radical Research Communications*, **6**, 21–24.
38. O.I. Aruoma, B. Halliwell, J. Butler and B.M. Hoey (1989) Apparent inactivation of α_1-antiproteinase by sulphur-containing radicals derived from penicillamine. *Biochemical Pharmacology*, **38**, 4353–4357.

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