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Myoglobin protects against endothelial cell membrane damage associated with hydrogen peroxide or xanthine/xanthine oxidase

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Oxymyoglobin at 'physiological' concentrations of 20–100 micromolar protected cultured endothelial cells from damage by xanthine/xanthine oxidase or by hydrogen peroxide. Metmyoglobin also provided a degree of protection, but apomyoglobin was ineffective. Protection was enhanced in the presence of ascorbate (0.01–1 mM). Myoglobin may have a physiological role in the protection of muscular tissue from ischaemia/reperfusion-induced damage.

Myoglobin; Hydrogen peroxide; Reperfusion injury; Cytotoxicity; Endothelial cell

1. INTRODUCTION

Oxygen-containing free radicals have been extensively discussed as potential intermediates in tissue damage associated with reperfusion after ischaemia. There is general agreement that externally generated free radicals cause damage [1,2], but results of experiments designed to detect free radical generation under ischaemia-reperfusion conditions or to show protection against injury by free radical scavenging agents have been less consistent [3–5]. Myoglobin is a ubiquitous muscle protein present in particularly high concentration (up to 300 micromolar) in cardiac or 'red' skeletal muscle. Its classical role is to facilitate intracellular oxygen transport at low oxygen tensions. As a heme protein, myoglobin will also interact with hydrogen peroxide and oxygen radical generating systems in a variety of ways [6–8]; it is unclear from a-priori reasoning whether these interactions are protective, irrelevant or actually harmful. We used cultured endothelial cells, which are known to be sensitive to oxygen free radical-induced damage, as a marker system to investigate whether physiological concentrations of myoglobin would enhance or protect against damage induced by xanthine/xanthine oxidase or hydrogen peroxide.

2. MATERIALS AND METHODS

Hydrogen peroxide was diluted from 30% stock solution (Sigma). Hydrogen peroxide concentration was measured by $KMnO_4$ titration or by horseradish peroxidase catalysed oxidations [9]. Superoxide anion (plus hydrogen peroxide) was generated by mixing 0.4 mM xanthine (sodium salt, Sigma) with 0.04 U/ml xanthine oxidase (Grade III, Sigma). Catalase (from bovine liver), superoxide dismutase (from bovine liver), horseradish peroxidase and L-ascorbic acid were purchased from Sigma.

Metmyoglobin (type III, from horse heart) and apomyoglobin were purchased from Sigma and purified on Sephadex G50 before use. Sigma apomyoglobin may be toxic to endothelial cells, but the toxicity was abolished by dialysis against Hanks solution. Toxicity was not seen with apomyoglobin prepared by acid–acetone precipitation [10] in our laboratory. Oxymyoglobin was prepared by adding an excess of sodium dithionite (Sigma) in the presence of air, and purified on a Sephadex G50 column. All commercially obtained proteins were assayed for contaminating catalase activity, but this is impracticable for oxy- or metmyoglobin since they have intrinsic 'catalase-like' activity In order to be sure that the apparent protective effect of myoglobin was not in fact due to contaminating catalase, we therefore reconstituted metmyoglobin from apomyoglobin and hemin [10], neither of which exhibited catalase-like activity alone.

Optical densities were measured using an S110 spectrophotometer (WPA). The amounts of met-, oxy- and ferrylmyoglobin were determined by making measurements at 490, 560 and 580 nm and applying the following equations [6,7,11]:

$$[\text{Oxymyoglobin}] = 2.8 \cdot A_{490} - 127 \cdot A_{560}$$
$$[\text{Metmyoglobin}] = 146 \cdot A_{490} - 108 \cdot A_{560} + 2.1 \cdot A_{580}$$
$$[\text{Ferrylmyoglobin}] = -62 \cdot A_{490} + 242 \cdot A_{560} - 123 \cdot A_{580}$$

Endothelial cells isolated from bovine aorta were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. They were cloned at first subculture [12], subcultured to 15 mm diameter wells in multiwell trays and used at confluence, approximately 1.8×10^5 per well. Before use, culture medium was removed and the cells washed with Hanks' solution (without Phenol red). Reagents were added to the cells in the order: Hanks' solution; ascorbic acid (0.01–1 mM) or catalase (10^3 U/ml) or superoxide dismutase (100 U/ml); myoglobin (0.01–0.1 mM); xanthine/xanthine oxidase or hydrogen peroxide. All concentrations were final concentrations in a reaction volume of 1 ml. 8 replicates were used per experiment. Unless otherwise stated, incubation with hydrogen peroxide or xanthine/xanthine oxidase was for 4 h at 37°C.

Damage to target endothelial cells was assessed by two methods:

(i) Tritiated adenine release assay: endothelial cells were loaded with [U-^{3}H]adenine by incubation for 24 h in culture medium containing 0.037MBq/ml [^{3}H]adenine (Amersham), washed three times with

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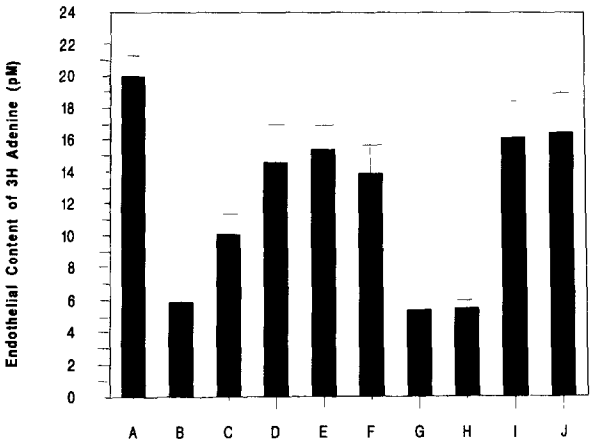

Fig. 1. Effect of oxymyoglobin on endothelial integrity measured as content of tritiated adenine (pM per 10^5 cells, bars=S.D.) after treatment with xanthine/xanthine oxidase. (A) Control; (B) plus xanthine 0.4 mM and xanthine oxidase 0.04 U/ml; (C) xanthine/xanthine oxidase + 10 micromolar oxymyoglobin; (D) xanthine/xanthine oxidase + 20 micromolar oxymyoglobin; (E) xanthine/xanthine oxidase + 50 micromolar oxymyoglobin; (F) xanthine/xanthine oxidase + 100 micromolar oxymyoglobin; (G) xanthine/xanthine oxidase + 100 micromolar apomyoglobin; (H) xanthine/xanthine oxidase + superoxide dismutase; (I) xanthine/xanthine oxidase + catalase; (J) xanthine/ xanthine oxidase + superoxide dismutase + catalase.

Hanks solution, then incubated for 4 h with xanthine/xanthine oxidase or hydrogen peroxide. Cells were again washed three times with Hanks, lysed in 2 N NaOH, and the lysate counted using Biofluor (NEN) scintillant and a Tri-Carb 1500 liquid scintillation analyzer.

(ii) Terminal adenine uptake assay [11]. Unlabelled endothelial cells were first treated with hydrogen peroxide and other reagents as described above. The endothelial cell monolayer was washed three times with Hanks' solution and incubated for a further hour with Hanks solution containing 5 mM glucose and 0.037 MBq/ml [^{3}H]adenine. Cells were again washed three times, and then lysed and counted as described above.

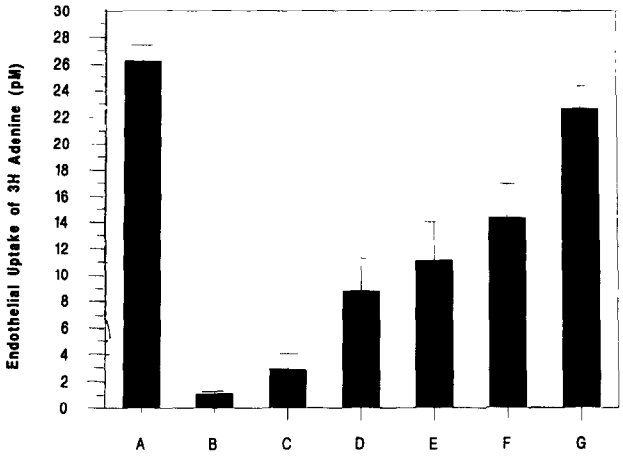

Fig. 3. Effect of oxymyoglobin on endothelial function as assessed by terminal adenine uptake assay. Cells were first treated with 1 mM hydrogen peroxide and then incubated with [^{3}H]adenine. Adenine uptake expressed as pM per 10^5 cells. (A) Normal control; (B) hydrogen peroxide 1 mM; (C–F) hydrogen peroxide 1 mM with oxymyoglobin 10, 20, 50 and 100 μM, respectively; (G) hydrogen peroxide + catalase 10^3 U/ml.

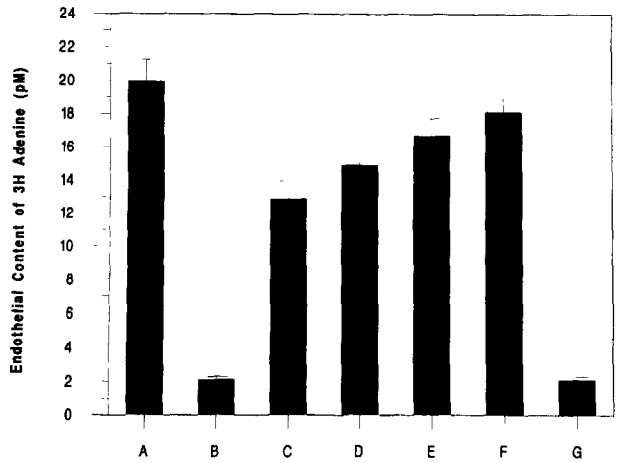

Fig. 2. Effect of ascorbate on endothelial protection measured as [^{3}H]adenine content by oxymyoglobin in the presence of 1 mM hydrogen peroxide. (A) Normal control; (B) 1 mM hydrogen peroxide; (C) hydrogen peroxide 1 mM + oxymyoglobin 100 μM; (D) C + ascorbate 0.01 mM; (E) C + ascorbate 0.1 mM; (F) C + 1 mM ascorbate; (G) ascorbate 1 mM.

Endothelial damage was also monitored by phase contrast microscopy after incubation with 0.1% Trypan blue.

3. RESULTS

Oxymyoglobin or metmyoglobin reduced tritiated adenine release from preloaded cells exposed to hydrogen peroxide or xanthine/xanthine oxidase in a dose-related manner (Fig. 1, Table I). Catalase was protective, but superoxide dismutase was not. Apomyoglobin had no protective effect. Adding ascorbic acid enhanced

Table I

Effect of oxymyoglobin, metmyoglobin and ascorbic acid on endothelial cells adenine retention after exposure to 1 mM hydrogen peroxide

Conditions	Adenine content (pM/10^5 cells)	Standard deviation
Control	20	0.9
Hydrogen peroxide	2.2	0.5
Hydrogen peroxide plus:		
10 μM Oxymyoglobin	3.8	0.6
10 μM Metmyoglobin	4.1	0.8
20 μM Oxymyoglobin	7.1	0.5
20 μM Metmyoglobin	7.1	1.1
20 μM Oxymyoglobin + 1 mM ascorbate	16.6	0.7
100 μM Oxymyoglobin	13.0	0.8
100 μM Metmyoglobin	12.9	0.9
100 μM Apomyoglobin	2.3	0 6
100 μM Oxymyoglobin + 1 mM ascorbate	16.1	0.8
Catalase 1,000 U/ml	19.4	1.1

Results are mean of 8 replicates

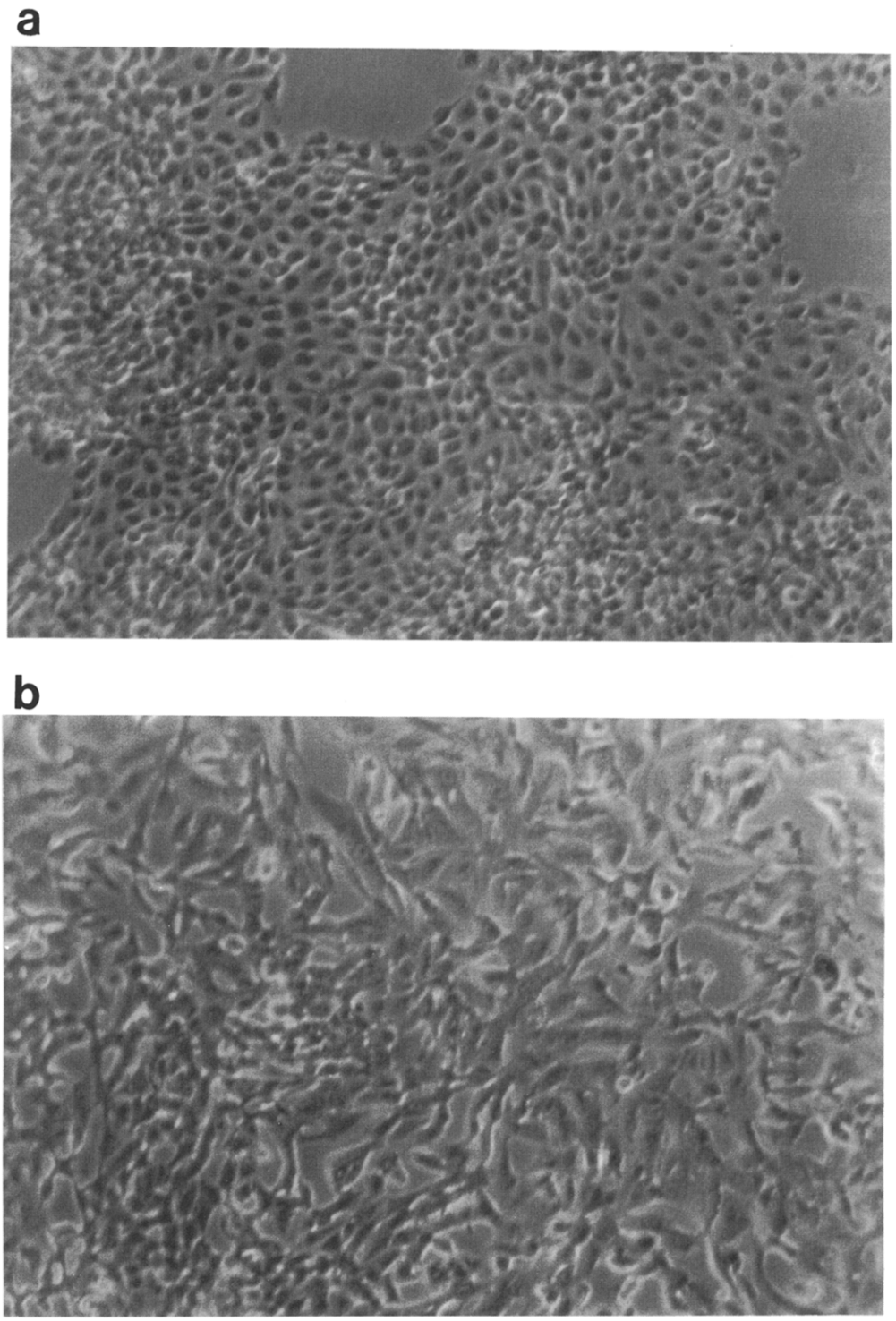

Fig. 4. Phase contrast micrographs (magnification ×100) showing cytotoxic effect of 1 mM hydrogen peroxide (a) and protection in the presence of 100 micromolar oxymyoglobin (b).

the protective effect of met- or oxymyoglobin, but ascorbic acid was ineffective in the absence of myoglobin (Fig. 2). These results were replicated using a terminal adenine uptake assay (Fig. 3). Protection by myoglobin was also confirmed by phase contrast microscopy (Fig. 4). Native myoglobin and myoglobin reconstituted from apomyoglobin and hemin had identical effects. Ascorbate did not potentiate the protective effect of catalase.

During the course of the incubation, oxymyoglobin

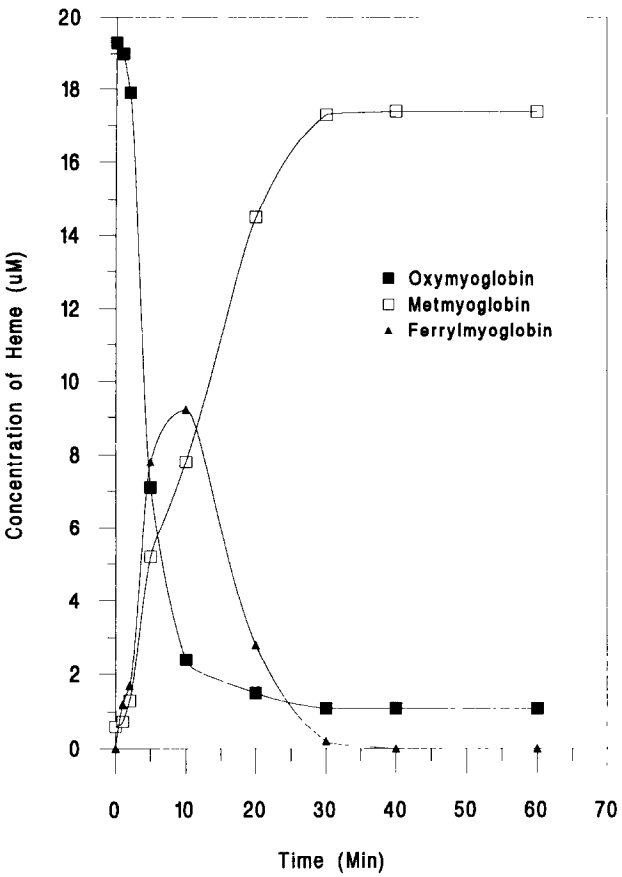

Fig. 5. Effect on myoglobin of incubation with xanthine 0.4 mM and xanthine oxidase 0.04 U/ml at 25°C. Concentrations (micromolar) 1 min after mixing were oxymyoglobin 19.0, metmyoglobin 0.6, ferrylmyoglobin 1.2, total (heme) 20.7. After 60 min concentrations were oxymyoglobin 1.1, metmyoglobin 17.4, ferrylmyoglobin 0.2, total (heme) 18.7.

was converted to metmyoglobin with the intermediate formation of ferrylmyoglobin (Fig. 5). Calculated loss of heme under these conditions was approximately 10%.

4. DISCUSSION

The interactions of myoglobin and haemoglobin with hydrogen peroxide have been extensively studied. Previous workers have tended to emphasise the potential role of heme proteins in generating, rather than scavenging, oxygen-containing radicals, either directly or through the release of free iron [14,15]. More recently, Giulivi and Davies described the 'comproportionation' of ferrylhemoglobin and oxyhemoglobin as a potential antioxidant and free radical scavenging mechanism [16]. A similar reaction between oxy- and ferrylmyoglobin had earlier been described by Whitburn [6]. Walters, Kennedy and Jones [17] described the oxidation of myoglobin in isolated myocytes by 15-hydroperoxy-5,8,11,13 eicosatetraenoic acid, but considered this as a marker of oxidative stress rather than a protective mechanism. Galaris, Cadenas and Hochstein [18] described redox 'cycling' of myoglobin and ascorbate and speculated that this might act as a defence mechanism, but did not demonstrate this effect in a biological system. More recently, Ozawa and Koreswka [19] described a non- specific oxidase activity of myoglobin after exposure to hydrogen peroxide. We have found that oxy- or metmyoglobin (but not apomyoglobin) markedly accelerate the decay of the ESR signal of the hydroxyl radical adduct of 5,5' dimethyl-1-pyrroline-*N*-oxide (DMPO), and have tentatively ascribed this to further oxidation to non-paramagnetic products [20].

In our system we found that both oxy- and metmyoglobin in 'physiological' concentrations provide partial protection against endothelial membrane damage caused by external oxygen free radical generating systems and hydrogen peroxide. We chose endothelial cells as targets because they are known to be particularly sensitive to oxygen free radical-mediated damage [21] and because they do not contain myoglobin.

A non-specific radical scavenging effect can be ruled out by the lack of efficacy of apomyoglobin. The transient generation of ferrylmyoglobin, and subsequent conversion to metmyoglobin, under the conditions we used would be compatible with a peroxide-scavenging 'comproportionation' reaction as part of the mechanism in the case of oxymyoglobin, but would not explain the protective effect of metmyoglobin alone. In the presence of myoglobin hydrogen peroxide will oxidise ascorbate [18]. Ascorbate probably reacts with ferrylmyoglobin as well as with metmyoglobin.

Provided the supply of ascorbate (or equivalent reducing agent) is adequate, the net effect will be protective. The reactions involved have been well reviewed by Arduini and Hochstein [22], although we would place a different emphasis on the likelihood of MB ($Fe^{4+}OH$) oxidising a low molecular weight electron donor rather than important structural components of the cell (Fig. 6)

The mechanism of the protective effect in the absence of ascorbate is less clear, and may involve sacrificial auto-oxidation with loss of the heme group. Potential toxicity resulting from the release of iron from free heme [11,23] might explain why, even in high concentrations, the protection offered by oxy- or metmyoglobin alone was less complete than with catalase.

In our model superoxide dismutase had no effect either on the formation of ferrylmyoglobin or on cell damage. This accords with other reports that hydrogen peroxide, or more probably hydroxyl radicals, are the crucial species, and that the conversion of superoxide anion to hydrogen peroxide is not rate limiting [24,25]. The initial concentration of hydrogen peroxide we used in some of our experiments, 1 mM, is large in comparison with concentrations used by some other authors, but hydrogen peroxide concentration falls rapidly under the incubation conditions used, and we wished to model the effects of sudden exposure to large hydrogen perox-

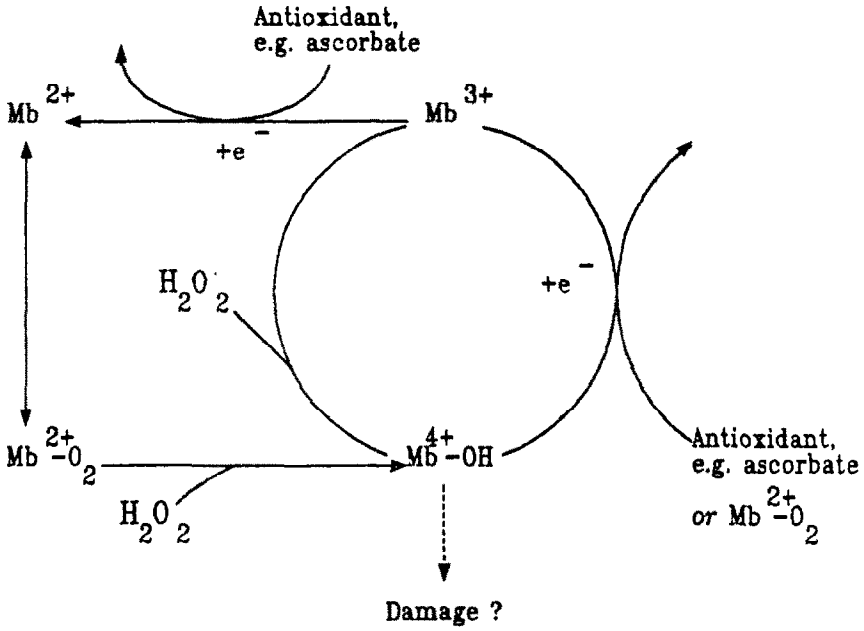

Fig. 6. Suggested reaction schemes for myoglobin. Metmyoglobin (Fe^{3+}), oxymyoglobin and deoxymyoglobin (Fe^{2+}) can all be oxidized to ferrylmyoglobin (Fe^{4}) by hydrogen peroxide. Ferrylmyoglobin may comproportionate with oxymyoglobin, or may be reduced to metmyoglobin by reacting with a low molecular weight electron donor such as ascorbate.

ide concentrations as a possible consequence of reperfusion after ischaemia. Continuous production of hydrogen peroxide, whether by xanthine/xanthine oxidase or by activated polymorphs, is more damaging to cells even when steady-state concentrations are much lower; we found that myoglobin was protective under both sets of conditions. Endothelial cells are capable of producing nitric oxide, and it has been suggested that peroxynitrite formed from superoxide and nitric oxide is a particularly damaging species [26]. Heme proteins are good scavengers of nitric oxide, and this is another potential mechanism for protection.

Myoglobin in our system clearly has an overall protective effect on endothelium in the presence of oxidative stress, and was indeed necessary for the protective effect of biological antioxidants such as ascorbate. This is despite its facilitation of hydrogen peroxide mediated oxidative reactions: possibly because myoglobin selectively catalyses oxidation of 'expendable' small molecules rather than important membrane components. Myoglobin occurs physiologically in cell types which are continually exposed to extremes of ischaemia and reoxygenation, and has been highly conserved in evolution. The possibility that, exceptionally, it may act as a 'double-edged sword' cannot be discounted, but we suggest that it may have a physiological role in protection against oxygen free radical-mediated damage

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