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The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs

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The interaction of myoglobin with H_2O_2 leads via a two-electron oxidation process to the formation of ferryl myoglobin. Metmyoglobin is more readily activated than oxymyoglobin to the ferryl states, which are capable of inducing peroxidative damage to membranes. E.p.r. and optical spectroscopic studies show that the thiol-containing compounds *N*-(2-mercaptopropionyl)glycine and *N*-acetylcysteine and the trihydroxamate desferrioxamine attenuate these processes by reducing the ferryl myoglobin species to metmyoglobin, with the formation of thiyl radicals and the desferrioxamine nitroxide radical respectively. Biochemical investigations of the potential for myoglobin in ruptured myocytes to be involved in radical generation, when under oxidative stress, and of the nature of the resulting species, were also undertaken. E.p.r. spectroscopic studies revealed the formation of a radical species which is capable of inducing membrane lipid peroxidation. The interaction of the thiol compounds and desferrioxamine with components of myocardial tissue under these conditions results in the generation of thiol-derived radical species and the desferrioxamine nitroxide radical respectively. These data, along with those obtained using optical spectrocopy, support the assignment of the identity of the radical species generated from the myocytes as the ferryl myoglobin radical.

INTRODUCTION

Reperfusion injury to the myocardium, as may occur in unstable angina, myocardial stunning and certain therapeutic intervention techniques (such as coronary angioplasty, thrombolysis and coronary bypass surgery), is associated with myocardial dysfunction. Many studies have proposed that the occurrence of these contractile and rhythmic disturbances is due, at least in part, to the formation of reactive oxygen species (Braunwald & Kloner, 1982; Hearse, 1987; Lucchesi, 1990). The myocyte contains abundant superoxide dismutase, but relatively low levels of glutathione peroxidase (Mills, 1960) and little catalase (Lawrence & Burk, 1978), and impaired function of these intracellular antioxidants during the ischaemic phase may predispose the myocardium to further injury upon reperfusion by decreasing the ability of the cell to remove active oxygen species. Thus the myocardium is prone, under these conditions, to the deleterious effects of radical formation and the transient localized generation of H_2O_2. It is now recognized that myoglobinaemia, arising from the rupture of myocytes, is one of the earliest detectable markers of acute myocardial infarction (Drexel *et al.*, 1983).

The interaction of myoglobin with H_2O_2 leads via a two-electron oxidation process to the formation of ferryl myoglobin (George & Irvine, 1952; Whitburn *et al.*, 1982). Myoglobin in the +3 oxidation state (ferric or met state) is activated to a ferryl form which has been characterized as a radical species in which the haem iron is one oxidizing equivalent above that of metmyoglobin, and one oxidizing equivalent is on the globin moiety:

$$HX{-}Fe^{III} + H_2O_2 \rightarrow {}^{\bullet}X{-}[Fe^{IV}{=}O] + H_2O$$

E.p.r. spectroscopy has demonstrated that oxidation of the globin occurs ultimately at a tyrosine residue, resulting in the formation of a tyrosine phenoxyl radical; this species is postulated to react subsequently with oxygen to give a tyrosine peroxyl radical (Davies, 1990, 1991). Studies have shown that both of these species are accessible to components in bulk solution, i.e. they are located on the surface of the protein (Tew & Ortiz de Montellano, 1988; Davies, 1990, 1991). The ferryl species of myoglobin can react with membranes (Kanner & Harel, 1985; Rice-Evans *et al.*, 1989; Turner *et al.*, 1990), lipoproteins (Bruckdorfer *et al.*, 1990) and electron-donating antioxidants (Rice-Evans *et al.*, 1989; Rice-Evans & Okunade, 1989), and recent evidence suggests that it is the peroxyl radical that reacts with membranes (E. S. R. Newman, C. A. Rice-Evans & M. J. Davies, unpublished work).

In view of the emerging appreciation of the role of oxidative stress in the pathogenesis of coronary arterial disease, biochemical investigations of the involvement of the myoglobin from ruptured cardiac myocytes in radical generation, the nature of the oxidizing species and their ability to peroxidize membranes were undertaken. The incorporation into these systems of the compounds desferrioxamine, *N*-(2-mercaptopropionyl)glycine (NMPG) and *N*-acetylcysteine (NAC), currently under investigation as potentially therapeutic interventions (Bolli *et al.*, 1989; Reddy *et al.*, 1989) in myocardial reperfusion injury, was also studied in view of their potential for attenuating tissue damage.

MATERIALS AND METHODS

Myoglobin (ferric form, horse heart, type III), thiobarbituric acid (TBA), malonaldehyde bis(dimethylacetal) 1,1,3,3-methoxypropane derivative, NAC, NMPG, ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine] and Sephadex were all purchased from Sigma Chemical Co. DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide), from Aldrich Chemical Co., was purified before use as described previously (Buettner & Oberley, 1978).

Abbreviations used: DMPO, 5,5-dimethyl-l-pyrroline-*N*-oxide; TBA, thiobarbituric acid; NMPG, *N*-(2-mercaptopropionyl)glycine; NAC, *N*-acetylcysteine.

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Desferrioxamine was a gift from Ciba–Geigy (Basel). All other chemicals were of AnalaR grade and were supplied by BDH Chemicals.

Isolated cardiac myocytes were prepared at the Sherrington School of Physiology, St Thomas's Hospital. Isolated, Ca^{2+}-tolerant, ventricular myocytes from male New Zealand White rabbits were prepared by proteolytic disruption of Langendorff perfused hearts according to the method of Fry *et al.* (1984) with minor modifications (Ward & Warley, 1990): the intact heart was perfused in the Langendorff mode with a 'low-calcium' Hepes-buffered physiological saline (pH 7.3) (containing 146 mM-Na^+, 3.5 mM-Mg^{2+}, 5 mM-Hepes, < 1 μM-Ca^{2+}, 10 mM-creatine, 20 mM-taurine, 5.6 mM-glucose, 0.3 mM-NaH_2PO_4, 0.1 mM-EGTA and 4 mM-K^+) in order to flush the coronary vasculature of blood. After 5 min the heart was then perfused with a similar solution containing in addition a mixture of proteolytic enzymes [collagenase type I (1 mg/ml; Worthington); protease (0.1 mg/ml; Sigma)] and 200 μM-Ca^{2+}, but omitting EGTA, in order to degrade the extracellular matrix and separate the intercellular junctions. Perfusion was followed by further enzymic treatment, together with mechanical disruption of the tissues.

The myocytes in Hepes buffer were separated from contaminating erythrocytes by Percoll density-step centrifugation. Myocyte suspension (2 ml) was layered on top of 2 ml of a Percoll solution (48% in Hepes buffer) and centrifuged for 20 min at 1600 rev./min. The myocytes remaining on top of the Percoll were then removed, resuspended (2 × 5 ml) in standard Hepes buffer (pH 7.4) and centrifuged at 3000 rev./min for 10 min. The myocytes were lysed by sonication (MSE PG464 Sonicator) at full power for 45 s. The total haem protein content was assessed, after removing the membranous fraction by centrifugation at 20000 rev./min for 20 min, by measurement of the absorbance of the supernatant at 414 nm after subtraction of the background absorbance at 700 nm. The latter wavelength was chosen as it is remote from any known haem absorption band.

Myoglobin solutions for model studies were made up in 5 mM-phosphate-buffered saline, pH 7.4. Purification of metmyoglobin was conducted by addition of equimolar potassium ferricyanide and subsequent separation using a Sephadex G-15-120 column. This method consistently yielded a 95% metmyoglobin preparation, with the balance being present in the oxy form.

Calculation of the relative proportions of the different forms of myoglobin present in the purified myoglobin (Mb) reaction mixtures was performed by applying the Whitburn equations (Whitburn *et al.*, 1982):

$$[MbO_2] = 2.8A_{490} - 127A_{560} + 153A_{580}$$

$$[Mb^{III}] = 146A_{490} - 108A_{560} + 2.1A_{580}$$

$$[\text{Ferryl Mb}] = -62A_{490} + 242A_{560} - 123A_{580}$$

Similar calculations could not be carried out on the ruptured myocytes due to the presence of other absorbing species (e.g. cytochromes). All incubations of myoglobin with H_2O_2 were conducted at ambient temperature. H_2O_2 solutions were prepared in phosphate-buffered saline.

Solutions of desferrioxamine, NAC and NMPG were prepared in distilled water. Final concentrations in the reaction mixture for optical spectroscopic studies were 500 μM for desferrioxamine and 400 μM for the thiols. For the e.p.r. experiments, higher concentrations were used, as shown in the relevant Figures. Additions of these compounds were made in minimum volumes and the final pH of the solution was not affected.

Visible spectra were recorded on a Beckman DU-70 recording spectrophotometer. Haem destruction was assessed as the amount of non-haem iron released into the reaction medium by use of the ferrozine assay (Ceriotti & Ceriotti, 1980). The reaction between H_2O_2 and the thiol compounds was investigated by determining the amount of unreacted thiol remaining at a given time point using the thiol assay of Haest *et al.* (1978).

Human haemoglobin-free erythrocyte membranes were prepared from normal fresh erythrocytes according to the procedure of Dodge *et al.* (1963). Membrane concentration was assessed by assaying protein (Lowry *et al.*, 1951). Incubations of activated myoglobin with membranes were conducted at a final membrane concentration of 0.5 mg/ml in a shaking water bath at 37 °C. Overall lipid peroxidation was assessed by application of the TBA assay (Walls *et al.*, 1979), with the absorbance of the chromophore, measured at 532 nm, corrected for background absorbance at 580 nm, due to possible contributions from haem proteins. Standards were run simultaneously under the same conditions utilizing malondialdehyde prepared by acid hydrolysis of the bis(dimethylacetal) derivative.

E.p.r. spectra of samples contained in a standard aqueous cell were recorded at room temperature 70 s after mixing, using a Bruker ESP300 spectrometer equipped with 100 kHz modulation and a Bruker ER035M gaussmeter for field calibration. Hyperfine coupling constants (*a*) were measured directly from the field scan. Where necessary, spectra were scanned repeatedly at fixed time intervals to obtain data on the stability of the radical adducts. Signal intensities were determined by measurement of peak-to-peak line heights on spectra recorded with the use of identical spectrometer settings.

RESULTS

Radical formation in isolated cardiac myocytes

Treatment of ruptured cardiac myocytes (final [haem] = 3 μM) with H_2O_2 (10-fold molar excess) resulted in formation of a highly oxidizing species capable of initiating peroxidation of myocyte membranes, as indicated by an increase in the formation of TBA-reactive compounds (over control values) of 1.9 nmol/10^6 cells. Investigation of the responses of activated ruptured myocyte lysate by H_2O_2 by e.p.r. spin trapping using the spin trap DMPO (30 mM) resulted in the detection of broad weak spin-adduct signals, suggesting that radical formation does occur under these conditions, but provided insufficient information to identify these species (Fig. 1*a*).

On inclusion of desferrioxamine (1 mM) in the activated myocyte system, a 9-line e.p.r. signal was observed (Fig. 1*b*). The parameters of this signal (a_N 0.780 mT, a_{2H} 0.628 mT) are identical to those previously determined (Davies *et al.*, 1987*a*) for the desferrioxamine nitroxide radical; this signal is therefore assigned to this species. Incorporation of the spin trap DMPO into this system produced the same spectrum, with no other trapped radicals observed. When NMPG (1 mM) or NAC (1 mM) was incorporated into this system, signals assignable to DMPO spin adducts were detected (Fig. 1*c*); omission of either the thiol compound or the spin trap resulted in the loss of these species. (Thus thiyl radical adducts are not seen arising from endogenous glutathione released from the ruptured myocytes.) The parameters of these signals (a_N 1.520 mT, a_H 1.520 mT for NMPG and a_N 1.521 mT, a_H 1.681 mT for NAC) are consistent with the previous data for thiyl radical adducts to this spin trap (Davies *et al.*, 1987*b*), and are therefore assigned to the NMPG and NAC thiyl adducts respectively. These results are consistent with the formation of an oxidizing species by the H_2O_2-activated myocyte system which can react with either desferrioxamine or thiol compounds.

In order to determine whether this species is the ferryl myoglobin radical, visible spectroscopic studies of the myocyte lysate were undertaken. Addition of a range of concentrations of H_2O_2 to the myocyte lysate resulted in a shift in the Soret peak

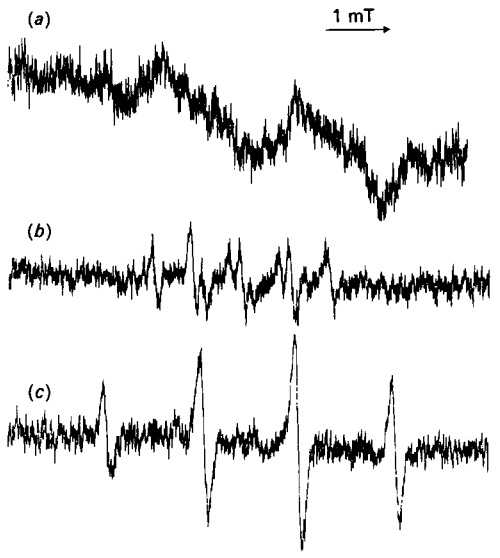

Fig. 1. E.p.r. spectra of cardiac myocytes exposed to H_2O_2 in the presence of the spin trap DMPO

(*a*) Myocyte myoglobin (3.3 μM), H_2O_2 (33 μM) and DMPO (30 mM) in phosphate buffer, pH 7.4. (*b*) As (A), with desferrioxamine (1 mM); spectrum assigned to the desferrioxamine nitroxide radical. (*c*) as (*b*), except with 1 mM NMPG in the place of desferrioxamine; spectrum assigned on the basis of its hyperfine coupling constants (a_N 1.520 mT, a_H 1.520 mT) to the NMPG thiyl radical adduct to DMPO. In both (*b*) and (*c*) the spectrometer gain was half that of (*a*).

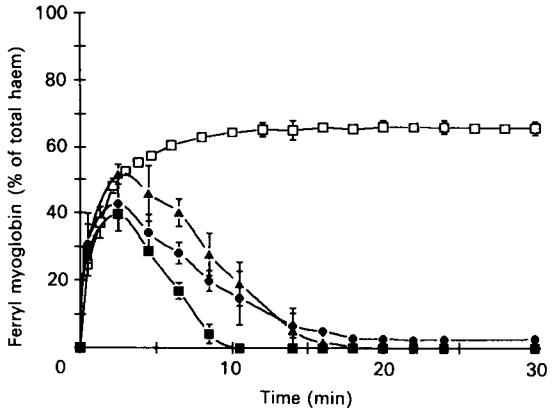

Fig. 2. Formation of ferryl myoglobin, as a function of time, on activation of metmyoglobin by H_2O_2

Ferryl myoglobin is expressed as percentage of total haem: □, metmyoglobin (20 μM) and H_2O_2 (25 μM) in phosphate buffer, pH 7.4; ■, +desferrioxamine (500 μM) added immediately before H_2O_2; ●+NMPG (400 μM); ▲ +NAC (400 μM). Values are means ± S.D. ($n = 6$).

from 412 nm, which characterizes the majority of the haem as oxymyoglobin with the remainder as metmyoglobin (approx. 5%), to 415 nm, consistent with the formation of a new oxidized species. Under these conditions there was no change in the total haem concentration, and release of non-haem iron was not detected. In order to characterize this process further, we have undertaken studies on purified myoglobin in model chemical systems.

Characterization of the model system

Addition of a 1.25-fold molar excess of H_2O_2 to metmyoglobin (20 μM) resulted in a rapid loss of spectral characteristics attributable to metmyoglobin, with the concomitant appearance of ferryl myoglobin with a characteristic shift in the Soret band. Visible spectroscopic techniques do not distinguish the radical and non-radical species (King & Winfield, 1963). Thus the Soret peaks of metmyoglobin and oxymyoglobin (which occur at 410 and 414 nm respectively) are replaced by that of ferryl myoglobin at 420 nm. By applying the Whitburn equations, the proportions of oxy, met and ferryl myoglobin may be calculated; the formation of the ferryl myoglobin species (Fig. 2) reached a plateau at 10 min and constituted 65% of the total haem. Activation of oxymyoglobin with a 20-fold molar excess of H_2O_2 resulted in a slower, less extensive, formation of the ferryl species, with this reaching a plateau at 42% of the total haem (results not shown). Addition of desferrioxamine (500 μM) to the activated met- and oxy-myoglobin shifted the Soret peak back to 410 nm, consistent with the reduction of the ferryl form, by the electron-donating properties of desferrioxamine, to metmyoglobin.

When similar reaction mixtures containing NMPG or NAC (both 400 μM; added at the time of activation) were investigated, a significant suppression in the extent of ferryl formation was seen (Fig. 2). In marked contrast with the system in the absence of the thiol compounds, the proportion of total haem in the ferryl states in the presence of these compounds attained a peak value at 2.5 min after activation, and progressively decreased thereafter.

Comparison of the effects of NAC with those of NMPG shows that the latter is the more effective at suppressing the maximum extent of formation of the ferryl species. On the other hand, whereas NMPG is never able to suppress formation completely, NAC gives total suppression by 20 min. Desferrioxamine is the most effective at both limiting the maximal ferryl myoglobin level attained (38% inhibition) and achieving complete suppression of the ferryl form (by 10 min). When the thiol compounds were added to metmyoglobin in the absence of H_2O_2, NMPG was shown to decrease the proportion of metmyoglobin and to increase the proportion of oxymyoglobin present (result not shown). NAC does not show this effect.

Addition of a 2-fold molar excess of H_2O_2 to metmyoglobin in the presence of the spin trap DMPO resulted in the immediate detection by e.p.r. of an anisotropic (i.e. with considerably broadened high field lines) DMPO adduct signal with hyperfine coupling constants of approx. a_N 1.45, a_H 0.83 mT (Fig. 3*a*). This signal is assigned, as previously (Davies, 1990, 1991), to a DMPO adduct of a tyrosine peroxyl radical located on the surface of the protein at tyrosine-103. When similar reactions were carried out in the presence of either of the thiol-containing compounds, this DMPO adduct signal was replaced by strong signals from further DMPO radical adducts (Fig. 3). These signals, which decay rapidly, are qualitatively identical, though more intense, than those observed in the ruptured myocyte system, and are likewise assigned to the thiyl radical adducts.

Addition of NMPG to metmyoglobin in the absence of H_2O_2 resulted in the slow formation of an identical thiyl radical adduct signal; this was not observed with NAC. Reaction of metmyoglobin with H_2O_2 in the presence of desferrioxamine and DMPO resulted in the formation of the desferrioxamine nitroxide radical (with parameters identical to those from the myocyte system) and the loss of the signal from the tyrosine peroxyl radical adduct.

Suppression of membrane peroxidation by electron-donating drugs

Activation of metmyoglobin (20 μM) by H_2O_2 (1.25-fold molar excess) in the presence of haemoglobin-free erythrocyte mem-

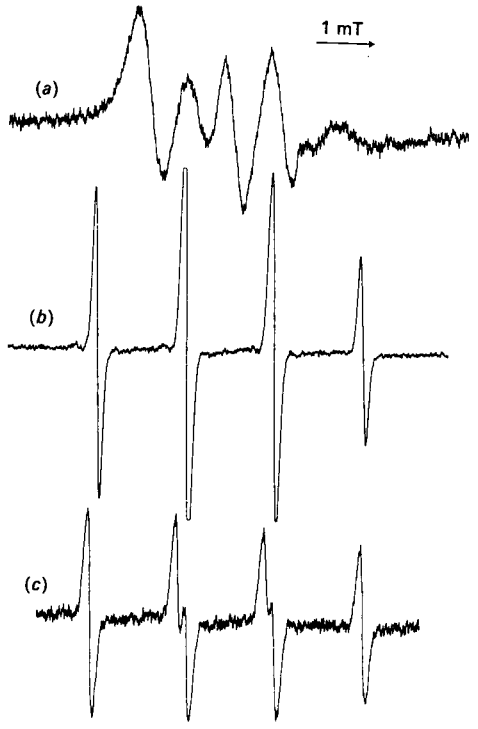

Fig. 3. E.p.r. spectra of the reaction of metmyoglobin (33 μM) with H_2O_2 (66 μM) in the presence of the spin trap DMPO (22 mM)

(*a*) Complete system; spectrum assigned to the DMPO adduct of a tyrosine peroxyl radical located on the surface of the protein (see text for hyperfine coupling constants). (*b*) Complete system with the addition of 1 mM-NMPG; spectrum assigned on the basis of its hyperfine coupling constants (a_N 1.520 mT, a_H 1.520 mT) to the NMPG thiyl radical adduct to DMPO. Spectrometer gain × 0.25. (*c*) Complete system with the addition of NAC; spectrum assigned on the basis of its hyperfine coupling constants (a_N 1.521 mT, a_H 1.681 mT) to the NAC thiyl radical adduct to DMPO.

Table 1. Ability of activated ferrylmyoglobin to stimulate membrane lipid peroxidation, and inhibition by electron-donating compounds

Values are expressed as nmol of TBA-reactive compounds/mg of membrane protein above control values and are means ± S.D. (n = 4) (3 h incubation at 37 °C).

Additions	TBA-reactive/compounds (nmol/mg of membrane protein)
Metmyoglobin (20 μM) + H_2O_2	0.65 ± 0.04
(25 μM) + membranes (0.5 mg/ml)	(A_{532} 0.07)
+ Desferrioxamine (500 μM) (added before H_2O_2)	0.05 ± 0.02
+ NMPG (400 μM) (added before H_2O_2)	0.40 ± 0.05
+ NMPG (400 μM) (omitting H_2O_2)	0.24 ± 0.01

branes (0.5 mg of membrane protein/ml) induced a significant stimulation of lipid peroxidation at 3 h, to 0.65 ± 0.04 nmol/mg of protein (Table 1) above control values, as previously reported (Rice-Evans *et al.*, 1989). The presence of 500 μM-desferrioxamine (added at the time of myoglobin activation) caused a marked suppression (94% at 3 h) of lipid peroxidation. The inhibitory effect of NMPG was less marked (38% at 3 h). Paradoxically, it was found that the addition of the thiol compound alone (in the absence of H_2O_2) resulted in a significant stimulation of lipid peroxidation above control values (Table 1).

DISCUSSION

There are several clinical settings in which the myocardium is exposed to transient ischaemia, including evolving myocardial infarction, myocardial stunning and coronary thrombosis. On reperfusion the sudden re-introduction of normotensive molecular oxygen may be detrimental to the previously ischaemic myocardium, leading to sub-optimal myocardial salvage. E.p.r. spectroscopic studies (Arroyo *et al.*, 1987; Bolli *et al.*, 1989) have confirmed the involvement of free radicals in myocardial stunning both in animal models of coronary occlusion *in vivo* as well as in isolated heart studies; furthermore, infusion of antioxidants, such as NMPG and desferrioxamine, at specific time points before and after reperfusion, shows that maximal recovery of contractile function and inhibition of free radical production occurs when the infusion is started just before reflow.

The work described in this present paper has shown that ruptured cardiac myocytes, in response to oxidative stress, are capable of inducing peroxidative damage to membranes. E.p.r. spectroscopic studies of the myocyte cytosolic fractions exposed to H_2O_2 do not provide definitive evidence as to the nature of the radical generated. However, the addition of radical scavengers resulted in the detection of radicals derived from the scavengers, confirming the presence of an oxidizing species. Addition of the thiol-containing antioxidants NMPG or NAC (in the presence of DMPO) gives signals from the corresponding DMPO–thiyl radical adducts, and addition of desferrioxamine generates the characteristic 9-line signal from the desferrioxamine nitroxide radical (Hartley *et al.*, 1990).

Recent observations in a variety of systems support the ability of the hydroxamate moiety of desferrioxamine to act as an electron donor, independently of its activity as an iron chelator, through its ability to denate hydrogen atoms or electrons to a variety of systems (Davies *et al.*, 1987*a*; Morehouse *et al.*, 1987; Harel & Kanner, 1987; Rice-Evans *et al.*, 1989). In addition, the ability of desferrioxamine to intercept the propagation phase of peroxidizing lipids has also been reported (Rice-Evans *et al*, 1989; Darley-Usmar *et al.*, 1989), with the concomitant formation of the desferrioxamine nitroxide radical (Hartley *et al.* 1990).

Possible identities of the initiating species on exposure of ruptured myocytes to oxidative stress include high-oxidation-state haem proteins, and superoxide and hydroxyl radicals. No signals from the well-characterized superoxide or hydroxyl radical adducts to DMPO were detected in either the ruptured myocyte or purified metmyoglobin systems, suggesting that neither of these species is being generated to a significant extent. Furthermore, no significant haem destruction or iron release was detected within 3 h under the conditions of these studies. Optical spectroscopic investigations of the H_2O_2-treated myocyte lysate preparations indicate characteristic changes in the Soret region. These alterations and the results of the e.p.r. experiments concur with the interpretation that a ferryl myoglobin radical is formed. Similar behaviour has not been observed with activated mitochondrial cytochrome proteins, suggesting that these haem proteins are not the source of the oxidizing species observed in the myocyte preparation (Wikstrom *et al.*, 1981). Consistent with this interpretation, Walters *et al.* (1983) and Arduini *et al.* (1990) have detected, by visible spectroscopy, the formation of ferryl myoglobin in cardiac myocytes *in vitro* in response to oxidative stress imposed by hydroperoxides and in isolated ischaemic rat hearts respectively.

Thiol drugs such as NMPG have been shown to be effective in inhibiting free radical production and improving recovery of contractile function when infused just prior to reperfusion (Bolli *et al.*, 1989). The thiyl radical formed when these thiols act as

antioxidants either can be removed by mutual annihilation (bimolecular termination) or, under aerobic conditions, can react with oxygen to form the thiyl peroxy radicals ($RSOO^{\bullet}$; Monig *et al.*, 1987). This latter species shows considerable reactivity (Saez *et al.*, 1982), and may be involved in initiating deleterious modification of cellular constituents. The formation and subsequent reactions of this type of radical may be the reason for the relatively ineffective suppression of peroxidative degradation effected by NMPG, and the observation that metmyoglobin in the presence of NMPG (in the absence of H_2O_2), which we have shown to generate slowly the parent thiyl radical, is able to elicit a significant amount of lipid peroxidation. These observations are consistent with the recent work of Puppo *et al.* (1990), who reported the adverse effects of this compound caused by its ability to enhance iron release from myoglobin in the presence of a large excess of H_2O_2. Thus these workers conclude that NMPG is unlikely to protect the myocardium by interfering with oxidants produced by the activated myoglobin system.

The nature and precise origins of the radical species contributing towards reperfusion injury are still unclear. However, the work presented here has demonstrated, in a biochemical model system, that cardiac myocytes, under oxidative stress, can initiate radical generation and tissue damage, and that these processes may be a result of the formation of a ferryl myoglobin species.

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