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THE GLUTATHIONE STATUS OF PERFUSED RAT HEARTS SUBJECTED TO HYPOXIA AND REOXYGENATION: THE OXYGEN PARADOX

V.M. DARLEY-USMAR,* V.O'LEARY and D. STONE

Biochemical Sciences, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, UK.

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Langendorff perfused rat hearts subjected to 30 min hypoxia followed by 20 min reoxygenation and the levels of the oxidised and reduced forms of glutathione measured. No change in the concentration of oxidised glutathione was detected in reoxygenated hearts when compared to normoxic controls. In contrast hearts exposed to oxidative stress in the form of H_2O_2 showed elevated levels of both oxidised glutathione (GSSG) and the glutathione-protein mixed disulphide. These results suggest that if oxidants do contribute to cell damage on reoxygenation of the hypoxic myocardium then their action is local and not through overwhelming of the cells antioxidant defences.

KEY WORDS: Glutathione, Oxygen Paradox, Reperfusion Damage, Oxidative Stress.

INTRODUCTION

When blood flow to an area of the myocardium is restricted during ischaemia and then restored either naturally or by clinical intervention a porportion of the cells are irreversibly damaged as a conseuqence of reperfusion.[1-3] Experiments with isolated perfused hearts have led to the conclusion that the reintroduction of oxygen plays a central role in the processes responsible for the cell damage that occurs on reperfusion and consequently this phenomenon has become known as the "oxygen paradox".[2,4,5] It is well known that the reduced oxygen metabolites, superoxide and hydroxyl radical and H_2O_2 can damage proteins and mebranes and so cause cell death[6-7] and it has been suggested that they may be involved in causing the cell damage characteristic of the oxygen paradox.[9-14] In support of this hypothesis it has been shown that allopurinol, the inhibitor of the superoxide and H_2O_2 generating enzyme xanthine oxidase, decreases cell damage when hypoxic rat hearts are reoxygenated.[12,14]

The best studied examples of cell death caused by oxidative stress involve catalytic generation of oxidants by redox cycling xenobiotics or exposure of the cell to oxidants such as H_2O_2 or other peroxides.[15-19] As protection against oxidative stress the cell contains both antioxidants and anitoxidant enzymes and it has been shown that the antioxidants are consumed when the cell is exposed to oxidants of the type described above. 15-19 For example, the oxidation of the intracellular antioxidant glutathione to either GSSG or a protein-glutathione mixed disulphide (protS-SG) can be detected when liver cells are exposed to oxidants.[15,16,19] The precise mechanisms which lead to cell death after exposure to oxidants are still unclear but it has been suggested that

* Author to whom correspondence should be addressed.

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elevated levels of oxidised glutathione, particularly protS-SG, may play an important role.[15]

In the present study we have examined the possibility that oxidants generated on reoxygenation of the hypoxic rat myocardium may cause cell damage by a process similar to that which is thought to occur in conditions of oxidative stress i.e. by overwhelming of the cells antioxidant defences and perturbation of the protein thiol oxidation state. It has been established that perfused hearts are able to detoxify externally applied oxidants using the glutathione-glutathione peroxidase system and that the GSSG formed is actively transported from the cell.[17] Furthermore, others have demonstrated that hypoxia-reoxygenation results in a decrease in the heart GSH content, suggesting that some oxidative stress might occur under these conditions, although in these studies no change in GSSG content could be detected and the protS-SG content was not determined.[11] In the present study we have measured the tissue and perfusate contents of GSH, GSSG and protS-SG in hearts subjected to hypoxia-reoxygenation under conditions where cell lysis occurs. In order to establish that the heart is able to form GSSG and protS-SG under conditions of oxidative stress, we have examined the effect on the glutathione status of the heart after exposure of the organ to H_2O_2.

MATERIALS AND METHODS

Male Wistar rats were killed by cervical dislocation, their hearts excised and placed in ice-cold Krebs bicarbonate buffer containing glucose (11 mM) and heparin (150 U/ml). The aorta was attached to a stainless steel cannula and a non-recirculating Langendorff perfusion was performed at a constant flow rate of 6 ml/min at 382C.[4] Buffer was gassed by passing through a coil of silastic tubing inside a gassing jar. All experiments included a 5 min equilibration period with buffer containing 11 mM glucose and gassed with 95% O_2, 5% CO_2. After 5 min the gas was changed to 95% N_2, 5% CO_2 and the buffer changed to glucose-free Krebs bicarbonate so that the heart received hypoxic (nitrogen gassed) and glucose deficient buffer at the same time. Reoxygenation was initiated by switching the gas from 95% N_2, 5% CO_2 to 95% O_2, 5% CO_2 in the same buffer. After perfusion the heart was trimmed of extraneous tissue, dried in an oven at 120°C for 18h and weighed. For glutathione measurements, hearts were freeze-clamped after reoxygenation using metal tongs pre-cooled in liquid N_2. The perfusate from the heart was passed through a combined filter and bubble trap and its absorbance was recorded at 214 nm using a flow through optical cell. Reoxygenation damage was calculated by dividing the area under the trace at 214 nm over a 20 min period after the re-introduction of oxygen by the dry weight of the heart in mg.[20]

Hearts exposed to H_2O_2 were perfused for a total of 40 min with oxygen-gassed buffer. Following the initial equilibration period with buffer containing glucose (11 mM) the hearts were perfused for a further period of 35 min with glucose-free buffer. H_2O_2 was added to the perfusion medium 15 min after the start of the experiment, using an infusion pump, to give a final concentrations of 200 μM, 300 μM or 400 μM over a 13 min period. Nine minutes after termination of H_2O_2 infusion the hearts were freeze-clamped using metal tongs pre-cooled in liquid N_2.

Glutathione, in its reduced, oxidised and mixed disulphide forms, was measured in samples of freeze-clamped tissue by HPLC.[16,21,22] Perfusate samples were collected on ice and the GSH and GSSG concentrations measured spectrophotometrically.[23,24]

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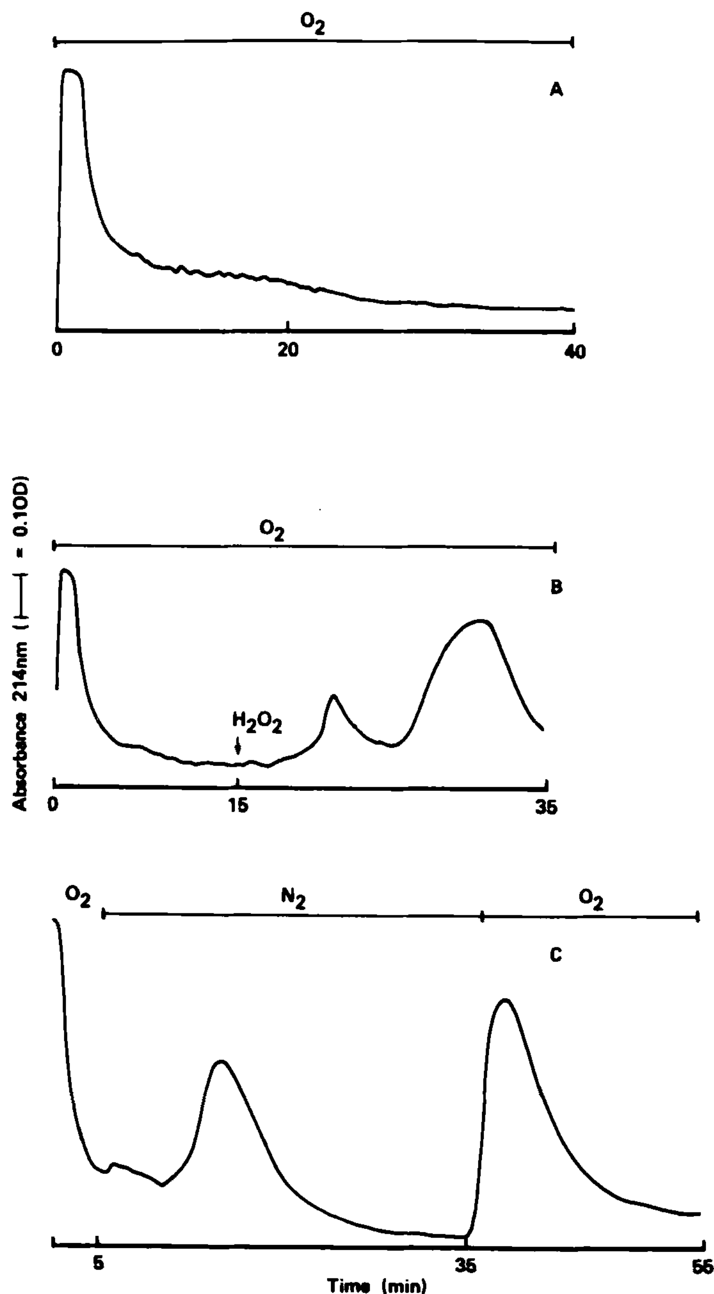

FIGURE 1 Perfusate absorbance (214 nm) for control hearts, hearts perfused with 400 μM H202 and hearts subjected to hypoxia and reoxygenation. Hearts were perfused with Krebs-bicarbonate buffer and the perfusate monitored continuously at 214 nm. 1A: control hearts perfused with O_2 gassed buffer for 45 min. 1B: hearts perfused with O_2-gassed buffer and then exposed to 400 μM H_2O_2 after a 15 min equilibration period. H_2O_2 exposure lasted for 13 min. 1C: Hearts were perfused for a 5 min equilibration period followed by 30 min hypoxia and 20 min reoxygenation.

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RESULTS

Heart Perfusions

Rat-hearts were perfused under three sets of conditions; control (oxygenated buffer), hypoxia followed by reoxygenation, and in the presence of H_2O_2 (oxygenated buffer). Figure 1A shows the 214 nm absorbance for a control heart perfused for 40 min with O_2. After the initial high absorbance due to blood washed out from the heart has fallen no further increase occurs. Figure 1C shows the 214 nm absorbance with time for a heart made hypoxic for 30 min and then reoxygenated for 20 min. After 8 min of hypoxia there is an increase in 214 nm absorbance which is not, however, due to cell lysis as it contains no creatine kinase.[20] On the reintroduction of oxygen there is an immediate increase in absorbance which we have shown is due to the appearance in the perfusate of the cytosolic contents.[20] It is this response which is thought to be analogous to the early phase of reperfusion damage in the ischaemic heart.[2,4] Perfusion of hearts with increasing concentrations of H_2O_2 led to increased perfusate absorbance indicating increased cell damage (data not shown). Figure 1B shows a typical result obtained by perfusing an oxygenated heart with 400 μM H_2O_2 15 min after the start of the experiment. The absorbance at 214 nm increases soon after the introduciton of H_2O_2 and is biphasic.

Glutathione measurements in perfused hearts

Glutathione as the reduced (GSH) oxidised (GSSG) and mixed disulphide (protS-SG) forms was measured in freeze-clamped samples of hearts perfused under control conditions (40 min normoxic perfusion), with H_2O_2, and after 30 min hypoxia followed by 20 min reoxygenation. The control hearts showed a high intracellular GSH to GSSG ratio (Table 1) with values in good agreement with those reported in the literature.[17] The protS-SG content of control hearts was extremely low. Hearts which had been subjected to hypoxia and reoxygenation showed a significant decrease in the amount of intracellular GSH but no significant change in either GSSG or protS-SG when compared to normoxic controls (Table 1). In contrast, perfusion of hearts with 200 μM, 300 μM or 400 μM H_2O_2 resulted in an increase in protS-SG and the intracellular protS-SG content of these hearts correlated well with the extent of cell damage as judged by the release of 214 nm aborbing material (Figure). Compared with

TABLE 1
Intracellular and total glutathione in hearts perfused with H202 or subjected to hypoxia and reoxygenation.

Treatment	GSH	GSSG (nmol/gm wet weight)	ProtS-SG	total GSH	
control	1099 ± 40	24 ± 5	7.2 ± 1.3	1213 ± 42	n = 6
H_2O_2	576 ± 131*	33 ± 4	74.0 ± 10***	1289 ± 147	n = 3
hypoxia-reoxygenation	616 ± 123**	36 ± 12	4.5 ± 0.9	1097 ± 133	n = 5

Rat hearts were perfused with O_2-gassed buffer for 45 min (control), 400 μM H_2O_2, or 30 min hypoxia followed by 20 min reoxygenation before freeze clamping. Glutathione content was determined by the HPLC method.[21] Total glutathione was calculated by summation of the different forms shown above together with any glutathione rleased into the perfusate and is expressed in terms in terms of GSH. Results are reported as the mean ± the s.e.m. and "n" denotes the number of experiments. Significance of the results was determined by the Student "t" test: * = $p < 0.01$, ** = $p < 0.005$, *** = $p < 0.001$.

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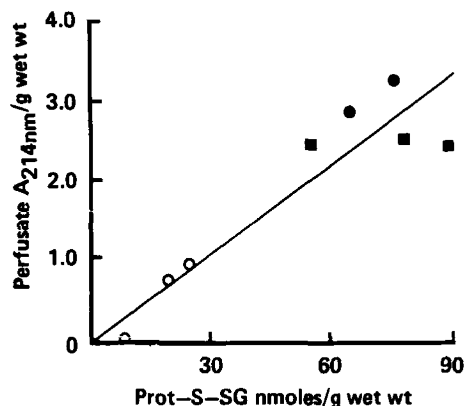

FIGURE 2 Correlation of damage induced by H_2O_2 with concentration of protS-SG. Hearts were perfused for a total of 35 min with O_2 gassed buffer and the perfusate monitored continuously at 214 nm. 15 min after the start of the experiment hearts were exposed to either 200 μM H_2O_2 = ○, 300 μM H_2O_2 = ●, or 400 μM H_2O_2 = ■, for a total of 13 min. At the end of the experiment hearts were freeze clamped with tongs cooled in liquid N_2 and the protS-SG determined as described in the materials and methods. The damage to the heart was estimated from the 214 nm absorbance trace with time but was normalised to the wet weight of the heart. Each point describes the protS-SG an damage for a single heart. The line shows the best fit to the points with a correlation coefficient 'r' of 0.91 which was significant at a confidence level of $p < 0.0025$ as determined by the Student "t" test.

controls, hearts perfused with 400 μM H_2O_2 showed an approximately ten fold increase in portS-SG content, a significant reduction in intracellular GSH content and no significant change in GSSG content (Table 1).

The amounts of GSH and GSSG were also measured in the perfusate of the hearts perfused under the same three sets of conditions. Very low amounts of GSH were found in the perfusate from control hearts (0.25 ± 0.05 nmol/ml, mean + s.e.m., n = 4). As expected in view of the cell lysis which occurs both with H_2O_2 treatment and following exposure to hypoxia and reoxygenation, glutathione was present in the perfusate from both these groups of hearts. In the perfusate of hearts subjected to hypoxia and reoxygenation, the glutathione was present predominantly in the reduced form (GSH:GSSG = 3.5 ± 6:1) while in contrast the perfusate of hearts treated with H_2O_2 contained GSSG but no detectable GSH. ProtS-SG could not be detected in the perfusate of any group. The total glutathione, (tissue plus perfusate) for all three groups of hearts was the same within experimental error (Table 1). This result implies that the decrease in GSH seen after hpoxia-reoxygenation or H_2O_2 treatment is mainly due to loss of glutathione to the perfusate.

DISCUSSION

Glutathione is one of the key anti-oxidants used by the cell to combat the effects of oxidative stress and therefore it is not surprising that there is a close correlation between oxidative stress and increased levels of oxidised glutathione.[15,16] In the liver, it has been shown that oxidative stress induced by either peroxides or xenobiotics causes an increase in the level of GSSG which is either exported from the cell or reacts with protein thiols to produce protS-GS.[15,16,19] We have shown that when the perfused

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heart is exposed to H_2O_2 a similar response occurs and that the amount of protS-SG formed increases with increasing oxidant stress. This effect is also accompanied by a progressive increase in cell damage as determined by an increase in the 214 nm absorbance of the perfusate. These results demonstrate that oxidative stress can result in cell damage in the heart and that the changes in the glutathione status that accompany this damage are similar to those reported for liver.

Reoxygenation of the hypoxic myocardium cases extensive cell lysis resulting in loss of the cytosolic contents to the perfusate.[2,4] It has been suggested by several groups that this damage may be caused by oxidants such as superoxide and H_2O_2 generated on reoxygenation.[9-14] If these oxidants are important in this process then they must originate from an intracellular source since externally applied cellular impermeant anti-oxidants such as superoxide dismutase, catalase and mannitol are without effect on reoxygenation damage[20,25] (V. Darley-Usmar unpublished data).

The data reported in the present studies suggest that if oxidants are produced intracellularly on reoxygenation, then the amounts of these oxidants are not sufficient to change the overall glutathione status of the cell. Thus, in contrast to the effects of H_2O_2 treatment, hypoxia and reoxygenation had no effect on the protS-SG or GSSG levels and although the latter treatment resulted in a fall in the GSH level this could be accounted for by the loss of GSH to the perfusate on reoxygenation.

These results demonstrate therefore that reoxygenation after hypoxia does not cause overall oxidative stress comparable to that produced by exposure of the heart to oxidants. These results do not exclude the possibility that the local generation of oxidants on reoxygenation leads to mechanical dysfunction but do imply that the effects are localised. Indeed there is good evidence from ESR studies that free radicals are produced on reoxygenation and that these radicals have arrthymogenic properties and compromise heart function.[12,26]

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