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# OXYGEN AND REPERFUSION DAMAGE: AN OVERVIEW

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Reperfusion of the ischaemic myocardium is associated with a number of oxygen dependent processes which can damage cells. The role of oxidants in mediating reperfusion damage will be discussed.

KEY WORDS: Free radicals, reperfusion damage, calcium, oxygen paradox



## INTRODUCTION

Oxygen performs an essential role in eukaryotic cells by providing an electron sink for mitochondrial electron transport, a process in which it is fully reduced to water. In contrast, partial reduction of oxygen leads to the formation of the oxidants superoxide, hydrogen peroxide or the hydroxyl radical which can damage proteins, membranes and nucleic acids. The cell generates these oxidants during normal oxidative metabolism and is protected from their effects by a number of endogenous antioxidants. The effectiveness of the cell's antioxidants in preventing oxidative damage is constrained by a number of factors and, under conditions of severe oxidative stress, can be exceeded. If this occurs it results in oxygen dependent modification of cell function which may lead to cell death.

One example of oxygen-induced cell damage is the response of the ischaemic myocardium to reperfusion. It has been shown that reperfusion is associated with a number of dysfunctions which impair cardiac recovery after ischaemia. These include such diverse phenomena as reperfusion arrhythmias,[1,2] stunned myocardium,[3,4] the oxygen paradox[5,6] and the development of infarction.[7–9] Each of these abnormalities occurs after different periods of ischaemia. For example myocardial stunning results from reperfusion following short periods of ischaemia[3] and does not involve tissue necrosis whereas the necrosis associated with infarct development only occurs after much longer periods of ischaemia.[7–9] In this brief overview we will discuss each of these different aspects of reperfusion damage and consider the evidence relating to the role, origin, identity and site of action of oxidants produced after reperfusion of the ischaemic myocardium.

## MYOCARDIAL STUNNING AND REPERFUSION ARRHYTHMIAS

If hearts are made ischaemic for short periods (15–20 min) and then reperfused little or no necrosis occurs but the function and metabolic competence of the heart is found

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to be impaired.[3] This is known as myocardial "stunning". A role for the oxidants hydrogen peroxide and superoxide in causing this effect has been inferred from the finding that a combined treatment of superoxide dismutase (SOD) and catalase considerably enhanced functional recovery.[10] Interestingly this functional recovery was not reflected in either elevated ATP levels in the reperfused heart or improvement in hemodynamic parameters.[4] The observation that the antioxidants SOD plus catalase, which cannot penetrate cells, exert a beneficial effect suggests that the stunned myocardium arises from the effects of oxidants acting through a compartment accessible to macromolecules. Depletion of circulating neutrophils by antiserum had no effect either on contractile function or wall thickening caused by myocardial stunning.[11] It is unlikely therefore that oxidants originating from the neutrophil contribute to this type of reperfusion damage. The mechanism by which oxidants may produce deleterious effects may be direct (e.g. by oxidative modification of proteins) or perhaps by interaction with EDRF (endothelium relaxing factor) which is now known to be the free radical nitric oxide (NO).[12] This important mediator of vascular tone is destroyed by superoxide which is known to be produced by the heart on reperfusion.[2,12] The removal of superoxide by SOD may result in a greater effective concentration of NO with a consequential beneficial effect on restoration of flow to the ischaemic area.

In addition to stunning, reperfusion can cause other functional abnormalities such as reperfusion arrhythmias which can be inhibited by antioxidants added to the perfusate.[1,2] In these studies the antioxidants found to be anti-arrhythmic were, as in the case of the stunned myocardium, unable to enter the cells.

The results with both myocardial stunning and reperfusion arrhythmias indicate that oxidants are released into the lumen of the vascular bed on reperfusion. Recent experiments using esr have been successful in detecting these radicals by spin trapping.[13] In addition it has been shown that the spin traps are themselves anti-arrhythmic.[14] The observations that these radicals have been detected in isolated buffer-perfused hearts indicate that they originate from the myocyte or endothelial cell.

## THE RESPONSE OF HYPOXIC MYOCARDIUM AND ISOLATED MYOCYTES TO REOXYGENATION

If Langendorff-perfused rat hearts are made hypoxic for periods of 20–30 min and then reoxygenated, gross physical disruption of the myocardium occurs and is accompanied by the appearance of creatine kinase activity in the perfusate.[5,6] The resulting reoxygenation damage can be quantitated by monitoring the perfusate at 214 nm (Figure 1). It has been established that this phenomenon is dependent on the reintroduction of oxygen to the hypoxic myocardium.[5]

It is well known that both reoxygenation and reperfusion are associated with $Ca^{2+}$ uptake by the myocardium and we have confirmed this by measuring total tissue $Ca^{2+}$ in freeze clamped hearts reoxygenated after different periods of hypoxia (Table 1). Calcium uptake increased progressively as the period of hypoxia was lengthened (Table 1). If both reoxygenation damage and $Ca^{2+}$ uptake were directly related it would follow that the ratio of reoxygenation damage: $Ca^{2+}$ uptake should be constant. As can be seen from Table 1 this is not the case since the ratio increases

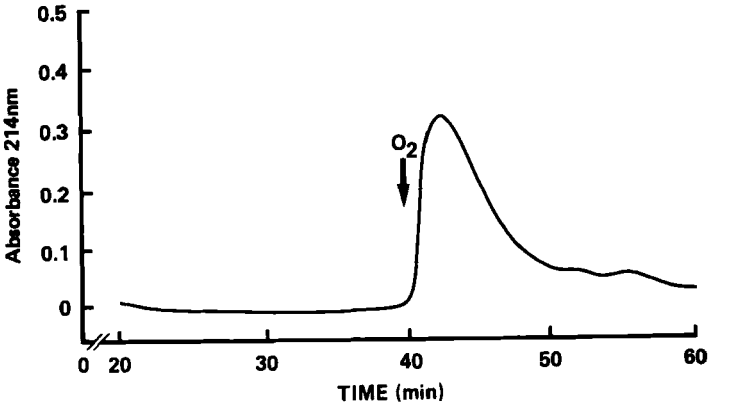


FIGURE 1 Effect of Reoxygenation of the Hypoxic Rat Heart on Perfusate Absorbance (214 nm). Hearts were perfused for 40 min under hypoxic conditions, without glucose, and for a further 20 min after the reintroduction of oxygen. The perfusate was monitored continuously at 214 nm using a flow-through cell (path length 0.2 cm). A representative trace of the perfusate absorbance over the last 20 min of the hypoxic period and the reoxygenation period is shown.

TABLE I
Calcium uptake and damage on reoxygenation.

| Perfusion Conditions | $Ca^{2+}$ Content on reoxygenation $\mu$mole/gm w.w. | Reoxygenation damage (R.D.) | R.D./$Ca^{2+}$ |
|---|---|---|---|
| Control 40-0 | 2.11 ± 0.13 (n = 7) | – | – |
| 20-H-20-0 | 3.31 ± 0.32 (n = 5) | 32 ± 3 (n = 4) | 10 ± 1 (n = 4) |
| 28-H-20-0 | 4.56 ± 0.23 (n = 6) | 143 ± 9 (n = 5) | 31 ± 1 (n = 5) |
| 40-H-20-0 | 5.97 ± 0.34 (n = 7) | 472 ± 42 (n = 12) | 76 ± 11 (n = 7) |

Hearts were perfused under the following conditions: 40-0: 40 min oxygenated buffer. 20-H, 28-H, 40-H: 20, 28 and 40 min hypoxia followed by 20 min reoxygenation (20-0). After reoxygenation hearts were freeze clamped with liquid $N_2$ cooled tongs and prepared for estimation of total tissue $Ca^{2+}$ by atomic absorption.[29] Reoxygenation damage was measured as described in.[30] Results are reported as the mean ± s.e.m. and the number of experiments are shown in parenthesis.

dramatically with increasing reoxygenation damage. This result also implies that $Ca^{2+}$ uptake may in fact precede cell lysis.

The perfused heart contains a number of cell types and during hypoxia individual cells will be exposed to varying degrees of hypoxia. In addition the response of the hypoxic myocardium to reoxygenation may be affected by alterations in flow. For these reasons we have studied a model of hypoxia-reoxygenation in isolated myocytes. In order to study $Ca^{2+}$ uptake the cells were brought to isotopic equilibrium with a medium containing 1 mM $^{45}Ca^{2+}$. The cells were made hypoxic for 40 min and subsequently reoxygenated. Throughout this procedure samples of the cells could be taken, washed, and the $^{45}Ca^{2+}$ content determined.

Under these conditions any net increase in total cell $Ca^{2+}$ should be reflected in an increase in $^{45}Ca^{2+}$ content. Figure 2A shows that making the cells hypoxic had little effect on total cell $Ca^{2+}$ but reoxygenation after 40 min result in 1.5–1.7 fold increase. Unlike the perfused hearts, however, and in agreement with results from other laboratories,[15] this was not associated with any oxygen dependent loss of creatine kinase from the cells (results not shown).

## OXIDATIVE STRESS, REOXYGENATION DAMAGE AND CALCIUM UPTAKE

We have demonstrated two oxygen dependent processes, $Ca^{2+}$ uptake and cell lysis, in the Langendorff-perfused hearts which occur on reoxygenation and which are both dependent on the length of the preceding period of hypoxia (Table 1).[5] These processes may occur as a consequence of the effects of oxidants produced extracellularly or from oxidation of intracellular membranes or proteins. In the first case we would predict that antioxidants which are unable to enter the cell would be protective and in the second that intracellular antioxidants such as glutathione would show evidence of oxidation.

We examined the effect of a number of exogenous antioxidants on reoxygenation damage in isolated myocytes and perfused hearts. It is well known that hydrogen peroxide can be (heterolytically) reduced by iron II to form the powerful oxidant the hydroxyl radical.[16] This reaction can be inhibited by chelation of the iron by desferrioxamine.[16] However, neither the iron chelator desferrioxamine, the hydroxyl radical scavenger mannitol nor a combination of SOD-catalase had any effect on reoxygenation damage (Table 2). No effect of SOD-catalase on the oxygen dependent $Ca^{2+}$ uptake was found in isolated myocytes (Figure 2B). We found, as have other, that the inhibitors of xanthine oxidase allopurinol or oxypurinol can decrease reoxygenation damage (Table 2). However, the action of these compounds cannot be uniquely ascribed to inhibition of superoxide and hydrogen peroxide from xanthine oxidase since they may also enhance purine salvage and appear to be equally effective in preparations which do not contain this enzyme.[17–19]

TABLE 2
Effect of radical scavengers on reoxygenation damage.

| Perfusion conditions | Antioxidant | Reoxygenation damage |
|---|---|---|
| 25-H-20-0 | none | 146 ± 14 (n = 14) |
| 25-H-20-0 | oxypurinol | 51 ± 4 (n = 7) |
| 25-H-20-0 | Desferrioxamine | 162 ± 6 (n = 4) |
| 30-H-20-0 | none | 187 ± 5 (n = 5) |
| 30-H-20-0 | SOD/catalase | 210 ± 22 (n = 7) |
| 35-H-20-0 | none | 283 ± 27 (n = 7) |
| 35-H-20-0 | Mannitol | 343 ± 62 (n = 4) |

Hearts were perfused with hypoxic buffer (H) for 25, 30 or 35 min before reoxygenation (0) for 20 min. The control groups were perfused with substrate free Krebs-bicarbonate buffer. Desferrioxamine (100 μM), oxypurinol (8 μM) or mannitol (11 mM) were added throughout the period of hypoxia and reoxygenation whereas SOD/catalase (100 U/ml) were added 2 min before reoxygenation. Reoxygenation damage was measured as described in.[30] Results are reported as the mean ± s.e.m. and the number of experiments are shown in parenthesis.

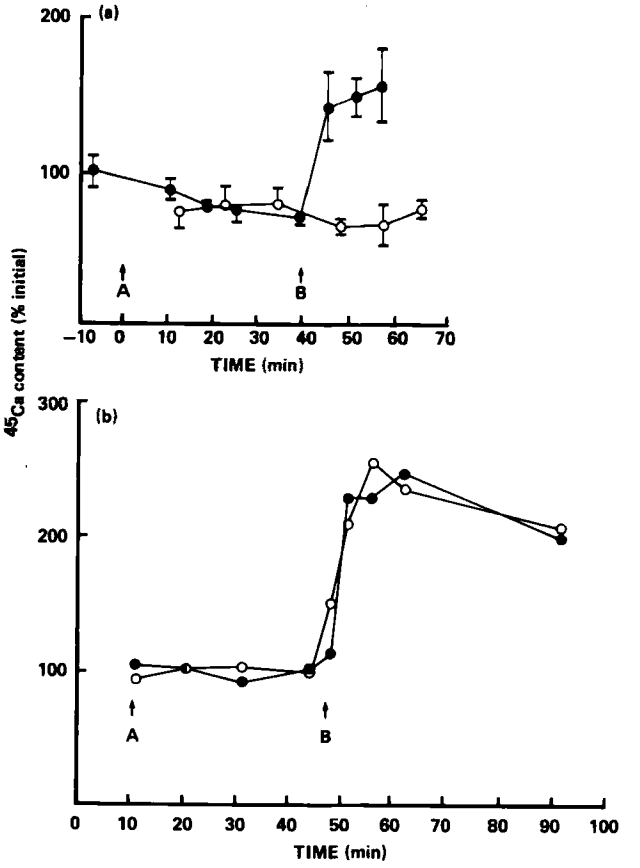


FIGURE 2 Reoxygenation induced calcium uptake in hypoxic myocytes: the effect of SOD-Catalase. Panel A: $^{45}Ca^{2+}$ loaded-myocytes were maintained under hypoxic conditions for 40 min from point A. At point B flasks were either reoxygenated (●) or maintained under hypoxia (○). Samples were removed at the times shown and the $^{45}Ca^{2+}$ content determined. Each point represents the mean ± SD for 5 experiments. Panel B: After 48 min hypoxia (point B) cells were reoxygenated in the absence (●) or presence (○) of 100 U/ml SOD-Catalase added 10 min before reoxygenation. Each point represents the mean for two experiments.

In support of the hypothesis that oxidants might be produced within the cell it has been shown that the concentration of the intracellular antioxidant glutathione is decreased on reoxygenation of the hypoxic myocardium.[20] This result could, however, be interpreted as a direct consequence of the extensive cell lysis which occurs on reoxygenation (Figure 1). We have addressed this question in more detail by measuring both the oxidised and reduced forms of glutathione in hearts subjeced to hypoxia-reoxygenation.[21] In liver it has been shown that glutathione is predominantly in its reduced form (GSH) but after oxidative stress induced by either redox active drugs or organic peroxides there is a rapid rise in the concentrations of the oxidised forms GSSG and protS-SG.[22] In agreement with this result we found that protS-SG levels in perfused hearts were increased by more than ten-fold after exposure to hydrogen

peroxide.[21] However, we found no evidence for oxidation of glutathione in hypoxic and reoxygenated hearts in either the tissue or perfusate.[21]

It is important to know whether $Ca^{2+}$ uptake and cell lysis are a direct consequence of lesions at the sarcolemmal membrane as has been suggested.[23] In isolated myocytes we have shown that $Ca^{2+}$ uptake occurs without creatine kinase release indicating that gross sarcolemmal damage does not occur. It has also been shown that $Ni^{2+}$ inhibits the oxygen-dependent $Ca^{2+}$ uptake supporting the hypothesis that the sarcolemmal membrane is intact.[24] In the perfused hearts it is not possible to separate changes consequent on cell lysis from other responses such as $Ca^{2+}$ uptake which may precede it. Despite this several investigators have suggested that $Ca^{2+}$ uptake may precede any sarcolemmal membrane disruption[24,25] and our results would tend to support this view (Table 1).

It has been shown that the mitochondrial inhibitor cyanide can prevent reoxygenation damage, an observation which suggests that there is an involvement of mitochondrial electron transport in the processes leading to cell lysis.[6]

## MYOCARDIAL INFARCTION AND OXYGEN METABOLISM

If we assume that reoxygenation damage occurs in hearts which have been made partially ischaemic then after reperfusion a number of cells within the ischaemic zone will have died. The healing process is thought to involve the migration of neutrophils into the reperfused zone or indeed into ischaemic tissue where cell death has begun to occur.[7] The neutrophils, when activated, produce as a primary oxidant superoxide from which the oxidants hydrogen peroxide and hypochlorite are formed. It has been proposed that this inflammatory response leads to an increase in the area of dead tissue (infarct).[7] This hypothesis is supported by the finding that neutropenia, induced either chemically or immunologically, results in decreased infarct size in dogs.[8,26] Furthermore, a number of drugs such as nafazatrom and BW755C, which are known to inhibit neutrophil migration, have also been reported to give significant decreases in infarct size.[8,27] However these compounds, in addition to affecting neutrophil migration, are also antioxidants so their mechanism of action in this model cannot be interpreted unambiguously. Some studies have shown that superoxide dismutase, which can remove the primary oxidant of neutrophil origin (superoxide), decreases infarct size by about 50% compared to untreated control groups of animals implying a role for superoxide in causing cell damage.[9]

The evidence suggests that extracellular superoxide production in the reperfused myocardium contributes to cell death. The most likely source of the superoxide under these conditions is activated neutrophils. It is probable that some cell death also occurs as a result of the oxygen paradox; a process which is not affected by extracellular superoxide dismutase. The relative contributions of these two processes in-vivo is presently unknown but may vary between different models of ischaemia-reperfusion.

## SUMMARY

The ischaemic myocardium shows a number of distinct oxygen dependent responses to reperfusion. In the case of myocardial stunning and reperfusion arrhythmias there

appears to be a beneficial effect of scavenging radicals in the extracellular space. This result is supported by the finding that free radicals can be detected extracellularly after reperfusion. The source of these oxidants and site of action is as yet unclear. In contrast hypoxic myocytes shown an oxygen dependent $Ca^{2+}$ uptake on reoxygenation which is not affected by externally applied antioxidants. This $Ca^{2+}$ uptake may in turn lead to the cell lysis characteristic of the oxygen paradox in the perfused heart. As yet there is no compelling evidence to suggest that this aspect of reperfusion damage is due to oxidative stress. It appears more likely that mitochondrial electron transport and ion movement play a central role. In the open chested dog model of ischaemia reperfusion, in which the volume of infarcted tissue is measured, considerable evidence suggests that oxidants derived from activated neutrophils contribute to cell death. This is not however the sole mechanism for cell damage in this model since an inhibitor of mitochondrial $Ca^{2+}$ uptake, ruthenium red, can improve recovery after reperfusion.[28] We conclude that a number of mechanisms may contribute to the observed oxygen dependent dysfunctions which occur on reperfusion of ischaemic tissue.

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