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The Occurrence of Oxidative Stress During Reperfusion in Experimental Animals and Men

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Summary. **Reperfusion is the prerequisite for the ischemic myocardium to recover its metabolic and mechanical function. However, reperfusion after a prolonged period of ischemia in the experimental animal may exacerbate, or at least accelerate, the occurrence of ischemic injury, whilst in humans at the least it is not beneficial. This entity has been called reperfusion damage, since much of the damage is believed to be caused by events occurring at the moment of reperfusion rather than by changes occurring during ischemia. The existence of reperfusion damage, however, has been questioned, and evidence in favour of the concept is sparse. At the moment the molecular events occurring at the time of reperfusion are not completely understood, and the relative importance of several proposed deleterious mechanisms is not yet established. One of the most fashionable ideas for the cause of reperfusion damage is that the function of cell membrane is modified by oxygen radicals generated at the moment of reperfusion. Evidence in favour of and against this hypothesis is described in detail in the present article.**

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Reperfusion of heart muscle, after more than 60 minutes of ischemia, is associated with the release of the intracellular component, reduction of contractility, influx of calcium, disruption of the cell membrane, and eventual necrosis of at least a portion of the tissue [1]. This entity has been called reperfusion damage and the early literature has been reviewed by Hearse [2]. Since then, the existence of reperfusion damage has been questioned and doubts exist as to whether reperfusion causes further injury or just an acceleration of the manifestation of the damage caused by ischemia.

The cause of this reaction is apparently multifactorial and with the advent of angioplasty and thrombolytic treatment for acute myocardial infarction has become a key issue with important clinical implications.

It is clear that the greatest benefit of thrombolysis in terms of mortality is observed in those patients who receive thrombolytic treatment within 1 to 2 hours of the onset of chest pain [3]. Left ventricular function, however, does not always improve [4]. Benefits of later thrombolysis are fewer and, in any case, are present to approximately 4 to 6 hours [3]. The molecular events occurring on reperfusion may be responsible for the ventricular impairment present after early reperfusion or for the lack of benefits after late reperfusion [5,6].

A rapid increase of oxygen tension occurs at the time of reperfusion, and recent data indicate that the toxic effects of oxygen, mainly via reactive oxygen reduction intermediates, might contribute to the reperfusion damage.

The present article will first give a brief review of the occurrence of and protection against the oxygen intermediates in the heart. Evidence for the involvement of the oxygen intermediates in the molecular events occurring during reperfusion in experimental animals and in humans will then be discussed.

Occurrence of Reactive Oxygen Reduction Intermediates

A free radical is an atom, ion, or molecule with one or more unpaired electrons: This configuration leads to an increased reactivity with other molecules. The amount of reactivity depends on the facility with which a species can accept electrons (is reduced) or donate them (is oxidized).

Molecular oxygen is relatively nonreactive because of its unusual structure, including two unpaired electrons with parallel electrons spin. The majority of organic compounds that might react with oxygen contain paired electrons. The simultaneous insertion of two such paired electrons into a molecule of oxygen

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would violate the rules of quantum mechanics. This restriction explains why ordinary molecular oxygen is relatively nonreactive.

Incoming electrons prefer to enter the orbitals one at a time, thus leading to the formation of reduced oxygen intermediates or oxygen free radicals.

The reduction of oxygen to water in the myocardium proceeds by two pathways. The mitochondrial cytochrome oxidase reduces 95% of oxygen to water by tetravalent reduction without the production of any intermediates. The remaining 5% of oxygen proceeds by a univalent pathway in which several intermediates are produced, such as superoxide anions ($O_2^{\cdot}$), hydrogen peroxides (H_2O_2), and hydroxyl radical ($\cdot OH$), where the dot ($\cdot$) represents an unpaired electron and signifies a free radical.

The superoxide anion is comparatively unreactive and in the physiologic condition is converted by dismutation to hydrogen peroxide. This is less reactive, longer lived, and more lipophilic than the superoxide anion, and it can diffuse considerable distances from its site of generation. The major danger of increased tissue concentration of hydrogen peroxide is the production of hydroxyl radical by the Haber Weiss or Fenton reaction. The hydroxyl radical has a short half-life, but is extremely reactive and will rapidly interfere with unsaturated fatty acid side chains, resulting in lipid peroxidation.

In addition, some compounds react spontaneously with oxygen, they autoxidize. Virtually all autoxidations result in the formation of reactive oxygen reduction intermediates. Autoxidation of adrenaline [8], pyrogallol [9], and many other compounds leads to the formation of the superoxide radical. The superoxide radical is released when the methemoglobin is formed from oxyhemoglobin [10]. Some oxidases also form superoxide, the most important in the heart being xanthine oxidase, which oxidizes hypoxanthine and xanthine to uric acid [11]. The microsomal cytochrome P_{450} system releases superoxide [12]. There are also indications that superoxide is formed as a byproduct during prostaglandin synthesis [13]. Upon activation of leukocytes (polymorphonuclears, monocytes, macrophages, eosinophils) large amounts of superoxide are released [14,15]. It follows that the superoxide and secondary products subsequently formed are of great importance for the killing ability of the cells, but might also lead to damage in surrounding tissue.

Myocardial Defense Mechanisms Against Oxygen Free Radicals

Nature has provided the heart with a number of systems protecting against oxygen toxicity and has organized its metabolism so as to minimize the formation of oxygen intermediates. These mechanisms are schematically illustrated in Figure 1. The superoxide radical is dismutated to hydrogen peroxide by superoxide dismutases (SOD) localized either in the mitochondrial matrix (Mn-SOD) or in the cytosol (CuZn-SOD) [11]. Intracellular concentrations of hydrogen peroxide are also kept low by its divalent reduction to water, driven mainly by glutathione peroxidases (the catalase activity in the heart being very low), which catalyzes the reaction between reduced glutathione and hydrogen peroxide, forming oxidized glutathione [16].

The hydroxyl radical reacts so rapidly with compounds that no direct enzymic scavenging is possible. Low-molecular weight compounds like ascorbate and reduced glutathione will provide protection by reacting with hydroxyl radical [17].

In membranes and lipoproteins, the lipid-soluble alpha-tocopherol (vitamin E) protects against peroxidation of polyunsaturated fatty acids, acting as a chain breaker [18]. Finally, free iron concentrations are kept low by binding to proteins. Intracellular iron is bound to ferritin, and extracellular iron is bound to transferrin and neutrophil-derived lactoferrin. In the pathologic condition, however, the buffering effect of these proteins may be altered, causing the release of free iron.

Interestingly, we have shown [17,19,20] that isch-

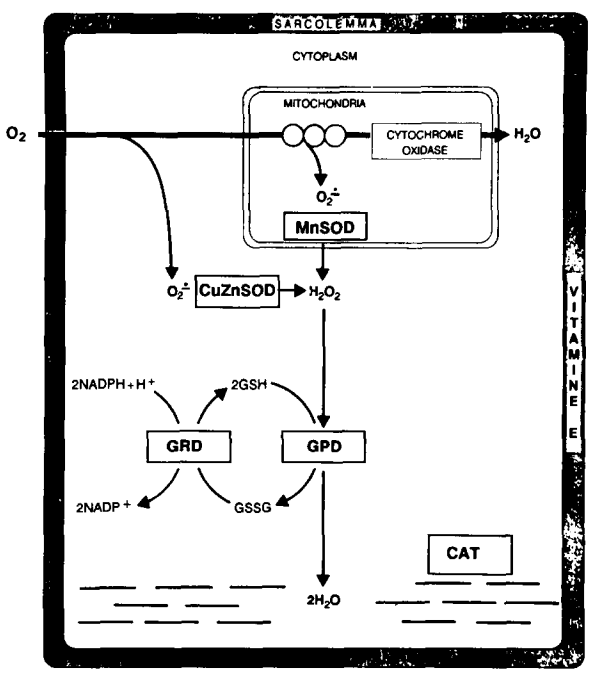

Fig. 1. *Schematic representation of the myocardial defense mechanisms against oxygen free radical.*

emia causes a severe reduction of SOD activity and a depauperation of GSH, thus making the myocardium more vulnerable to the deleterious effects of oxygen free radicals that might be produced on reperfusion. During ischemia there is also a depletion of naturally occurring antioxidants, such as alpha-tocopherol and ascorbate [21].

Sources of Free Radicals During Myocardial Ischemia and Reperfusion

Oxygen free radicals are normally produced even in the aerobic myocardium, which is able to handle and neutralize them by the activity of the above-mentioned defense mechanisms. During ischemia, and particularly during reperfusion, the production of oxygen free radicals might increase above the neutralizing capacities of the myocardium that are impaired. Under these conditions, radicals could be generated from mitochondria, myocardial cell membrane, endothelial cells, and white cells.

During ischemia, the components of the mitochondrial electron transport chain become reduced, allowing an increase of electron leakage from the respiratory chain, which, in turn, will react with residual molecular oxygen, leading to the formation of superoxide radicals. Reperfusion will reenergize the mitochondria, but electron egress through cytochrome oxidase will be reduced because of the lack of ADP, again causing the formation of oxygen free radicals. There is evidence of increased production of reduced oxygen intermediates from heart mitochondria harvested after ischemia, and particularly after reperfusion [22–25]. Interestingly, production from the mitochondria is the only source of oxygen free radicals that has never been questioned.

Free radicals may be generated within membranes, in association with the arachidonic acid cascade and with autooxidation of catecholamines. During ischemia, the calcium-mediated activation of phospholipases increases the release of arachinodate [26]. During this period, noradrenaline is accumulated in the extracellular space of the ischemic area, and there is a large, sudden release of noradrenaline on reperfusion of the myocardium. The precise role of these two systems in oxygen free radicals production, however, is not yet known.

In the capillary endothelial cell, the enzyme xanthine dehydrogenase during ischemia is converted in the oxidase form, which catalyzes the conversion of hypoxanthine and xanthine to uric acid, using oxygen as an electron acceptor. On reperfusion, the delivered oxygen can be reduced by this system, producing oxygen free radicals. In addition, there is evidence that allopurinol, an inhibitor of xanthine oxidase, protects the myocardium against reperfusion damage [27–29]. However, allopurinol could be protective by mechanisms other than inhibition of the enzyme [30], and not all the studies with this compound are positive [31–33]. Recently it has been shown that distribution of xanthine oxidase varies widely, the rabbit, pig, and human myocardium having essentially no activity [34–36], and yet, these species are not immune to reperfusion injury. In addition, conversion into the true oxidase form in the heart is rather low [37], making it very unlikely that xanthine oxidase is an important source of free radicals during early reperfusion, when the majority of damage occurs.

Neutrophils, when activated, generate several types of oxygen free radicals, which are relevant to the defense against bacterial infection and inflammatory reactions, such as acute myocardial infarction [38]. Thus the recruited neutrophils to the infarct site might damage the myocardium, producing oxygen free radicals. Removal of white cells from the blood does reduce infarct size [39], but neutrophils are absent from many preparations in which oxidative damage during reperfusion has been demonstrated. Furthermore, doubts exist as to whether neutrophils are activated at the time of reperfusion or relatively later, when damage has already occurred [6].

A schematic representation of the possible sources of oxygen free radicals during myocardial ischemia followed by reperfusion is shown in Figure 2.

Demonstration of Oxygen Free Radical Production During Ischemia and Reperfusion

Direct measurement of free radical species has been limited primarily by the instability of these oxygen metabolites. One primary technique has been used, electron paramagnetic resonance (EPR) spectroscopy, a system employed for many years to identify and characterize free radicals in simple chemical systems with or without the use of spin trap agents. Direct spectroscopy (EPR) has been used to analyze frozen myocardium, whilst EPR spectroscopy with spin adducts has been used to analyze coronary effluent.

Conflicting results have been obtained. Zweier et al. [40] identified a spectral signal similar to superoxide oxygen-centered free radicals, which increased significantly during 10 minutes of ischemia, and even more significantly during the first 10 seconds of reperfusion. However, Luber and associates [41], using the same EPR technique in a similar model of ischemia and reperfusion, failed to confirm the presence of such

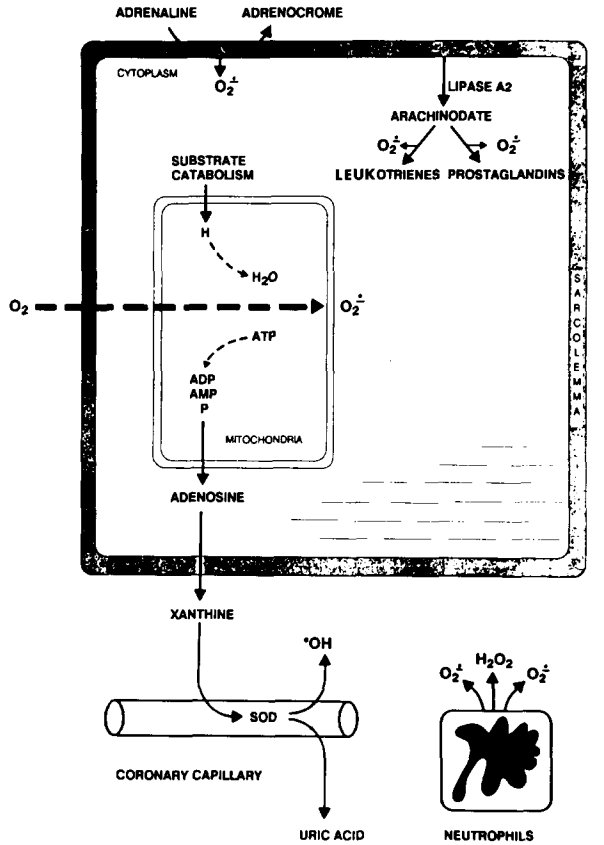

Fig. 2. Schematic representation of the sources of oxygen free radicals during reperfusion of the ischemic myocardium.

oxygen-derived free radical species and doubted the importance of free radical generation during postischemic reperfusion. Following the original report of Zweier et al. [40], many other authors using different spin trap agents have reported that superoxide or its derivative radicals can be demonstrated in the reperfused isolated hearts [42–44] or in vivo in animals as much as 3 hours after reperfusion [45]. However, electron spin resonance spectra reported by other investigators did not produce the same conclusion [46,47]. Additionally, Nakazawa et al. [48], performing experiments with either isolated rat and rabbit hearts, or open-chest canine hearts subjected to ischemia and reperfusion, have pointed out that direct-ERP spectroscopy results need to be analyzed with caution, since artifactual radicals are misleading problems that are common to this method. In particular, the superoxide and nitrogen-centered radicals that are commonly detected have been shown to be artificially produced by pulverization of the frozen samples. Interestingly, these authors identified the radicals native to the myocardium as coenzyme Q_{10}, suggesting that the mitochondria might be an important site of production of these radicals. Therefore, the studies employing the EPR technique tend to suggest, but not univocally, that free radicals are produced in significant amounts during reperfusion. The presence of blood in the system is not a prerequisite for oxygen free radical production, suggesting a possible direct myocardial formation of these toxic species. At the moment, however, it is not possible to determine which radicals are produced under these circumstances.

Evidence of oxygen free radical production also comes from experiments in which the occurrence of oxidative stress has been measured. This will be the subject of the next section.

Occurrence of Oxidative Stress During Ischemia and Reperfusion in the Experimental Setting

Oxidative stress is a condition in which oxidant metabolites can exert their toxic effect because of increased production of, an altered cellular mechanism of protection, or both [49–51]. Oxidative stress is evidenced by an increase of the product of lipid peroxidation and by an increase of tissue content and myocardial release of oxidized glutathione (GSSG) [52,53].

The most frequently utilized assay of lipid peroxidation is the measurement of malondialdehyde with thiobarbituric acid. Several positive results employing this method have provided evidence for the occurrence of oxidative stress during reperfusion in experimental animals [54–56]. However, this method lacks specificity, and many doubts remain about the interpretation of these results [57]. Lipid peroxidation does not necessarily prove that peroxidation itself is a primary mechanism of damage, as it may be a secondary phenomenon in tissue already damaged by other means [58]. When more accurate technology (HPLC) has been employed for malondialdehyde measurements, no sign of lipid peroxidation could be detected, after either early or late reperfusion of the severely ischemic heart [59]. In addition, we have been unable to demonstrate an increase in tissue content of the other products of lipid peroxidation, such as conjugated dienes, hydroperoxides, and Shiff's bases during reperfusion of the irreversibly damaged myocardium [60].

More precise evidence of oxygen free radical damage comes from experiments in which oxidative stress has been measured by determining glutathione status. The scheme illustrated in Figure 3 represents the glutathione reduction-oxidation cycle in the heart.

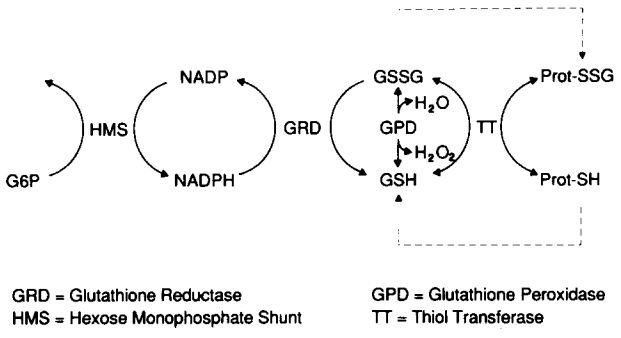

Fig. 3. *Schematic representation of glutathione metabolism at myocardial level.*

The hexose monophosphate shunt (HMPS) produces, through glucose-6-phosphate oxidation, the reducing equivalents (NADPH) for the action of glutathione reductase. Reduced glutathione (GSH) is then utilized by GSH peroxidases, but it is also in dynamic equilibrium with all cellular sulfhydryl groups. In fact, glutathione-mixed disulfides with proteins constitute an important part of total cellular glutathione, and the entire equilibrium is regulated by thiol transferases. There is much evidence proving that glutathione plays an important role in myocardial metabolism [61,63]. In the heart, glutathione predominantly occurs intracellularly in concentrations of 1.1 μM [64]. More than 95% of cardiac glutathione is in the form of GSH, the GSH/GSSG ratio of aerobic myocardium being over 50 [18]. Among other functions, glutathione is a key factor in the detoxification of electrophilic metabolites and reactive oxygen intermediates. As the determinant of the sulfhydryl/disulfide ratio [65], glutathione modulates the activity of a number of enzymes, and it may be involved in the transport of amino acids across the cell membrane [66]. Furthermore, GSH as a cosubstrate of glutathione peroxidase (Figure 1) plays an essential protective role against oxygen free radicals and prevents peroxidation of membrane lipids, the activity of superoxide dismutase in the heart being nearly four times less than in the liver, and the catalase activity being extremely low [65]. This protective mechanism results in an increased formation of intracellular oxidized glutathione (GSSG) that is actively transported across the cell membrane, so that its intracellular concentration is kept low.

Thus, changes of glutathione status and the increased formation and release of GSSG from the cell reflects glutathione peroxidase activity and indicates an inability of the cell to produce reducing equivalents for GSH resynthesis, and it is considered a sensitive and specific index of myocardial oxidative stress [16,17,66–70].

To investigate the possible role of oxygen free radicals in reperfusion injury, we have determined the effects of ischemia on the activity of mitochondrial and cytosolic superoxide dismutase, and of glutathione peroxidase and glutathione reductase, the two major lines of defense against oxygen free-radical production. In addition, we have also measured the tissue GSH/GSSG ratio as an index of oxidative stress. These experiments have been performed using isolated and perfused rabbits hearts, as previously described [17,19,20], and the data are shown in Figures 4–6. Figure 4 shows that the reduction of coronary flow to 1 ml/min induced a rapid decline of developed pressure, with contractile activity completely ceasing 9 minutes after the onset of ischemia. Resting pressure began to rise progressively 20 minutes after the onset of ischemia. Ninety minutes of ischemia specifically reduced the activity of mitochondrial Mn-SOD, whilst the same period of ischemia did not affect the activity of the cytosolic CuZn-SOD, or of glutathione peroxidase or glutathione reductase (Figure 5). We have no information on mitochondria function in these experiments. However, it is possible that the residual flow of 1 ml/min maintained some degree of mitochondrial respiration. Figure 5, however, shows that ischemia induced a reduction in the myocardial GSH/GSSG ratio. This was mainly due to a consistent reduction of tissue content of GSH, GSSG being unchanged (Figure 6).

Reperfusion induced a further increase of diastolic pressure, with almost no recovery of developed pressure. As indicated in Figure 6, on reperfusion there was also a significant increase of tissue GSSG from the ischemic value of 0.18 ± 0.02 nM/mg protein to 0.55 ± 0.02 nM/mg protein (Figure 6), resulting in a further decline of GSH/GSSG ratio (Figure 5). Figures 4 and 5 show that the readmission of coronary flow did not modify significantly the SOD, GRD, and GPD activities.

The alterations of mitochondrial SOD and of the GSH/GSSG ratio during reperfusion were coincident with important changes in the rate of release of GSH and GSSG, as shown in Figure 6. Ischemia did not significantly alter the rate of GSH or GSSG release, but on reperfusion there was a marked and sustained release of GSH and GSSG from the heart.

These results suggest that ischemia induces metabolic alterations capable of reducing the defense mechanisms against oxygen toxicity. The prime alteration seems to lie at the level of mitochondrial SOD, its activity being reduced by 50%. Under these conditions the readmission of molecular oxygen is likely to stimulate the production of oxygen radicals above the neutralizing capacity of mitochondrial SOD. Conse-

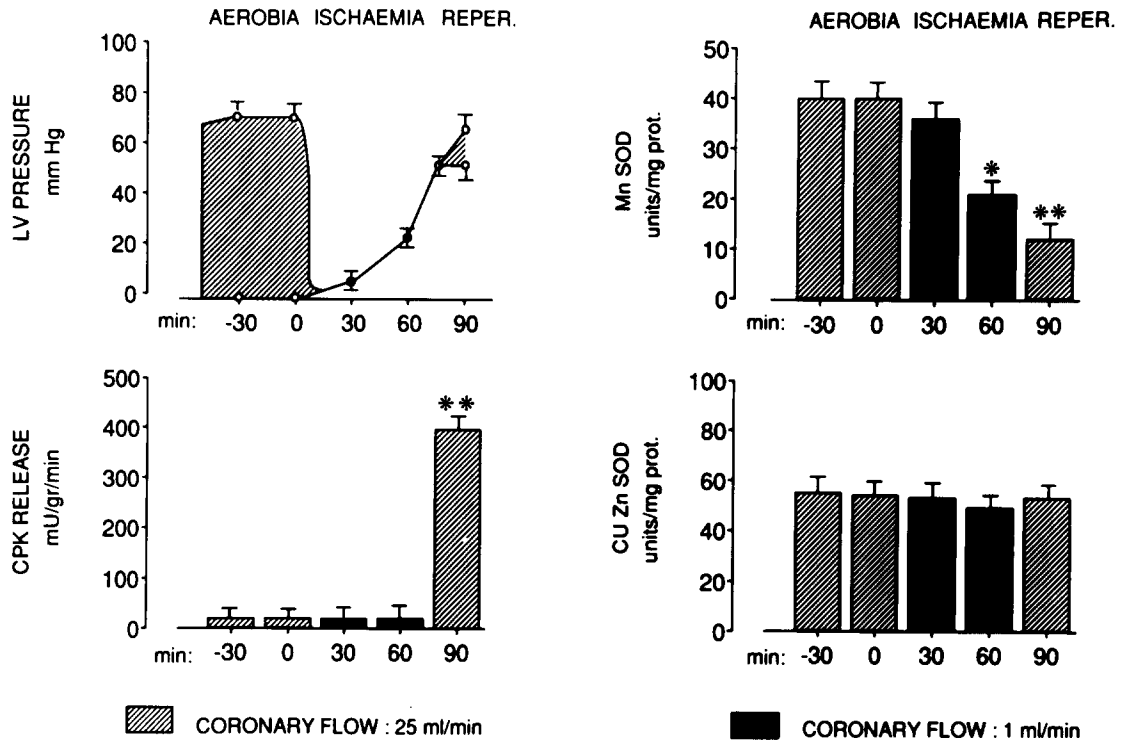

*Fig. 4. Effects of ischemia and reperfusion on left ventricular pressure, CPK release, and superoxide dismutase activity of isolated rabbit hearts. Determination of mitochondrial (Mn) and cytoplasmic (CuZn) SOD were made in the homogenate. Values are mean ± SE of six experiments. *p < 0.05; **p < 0.01 with respect to preischemic aerobic values.*

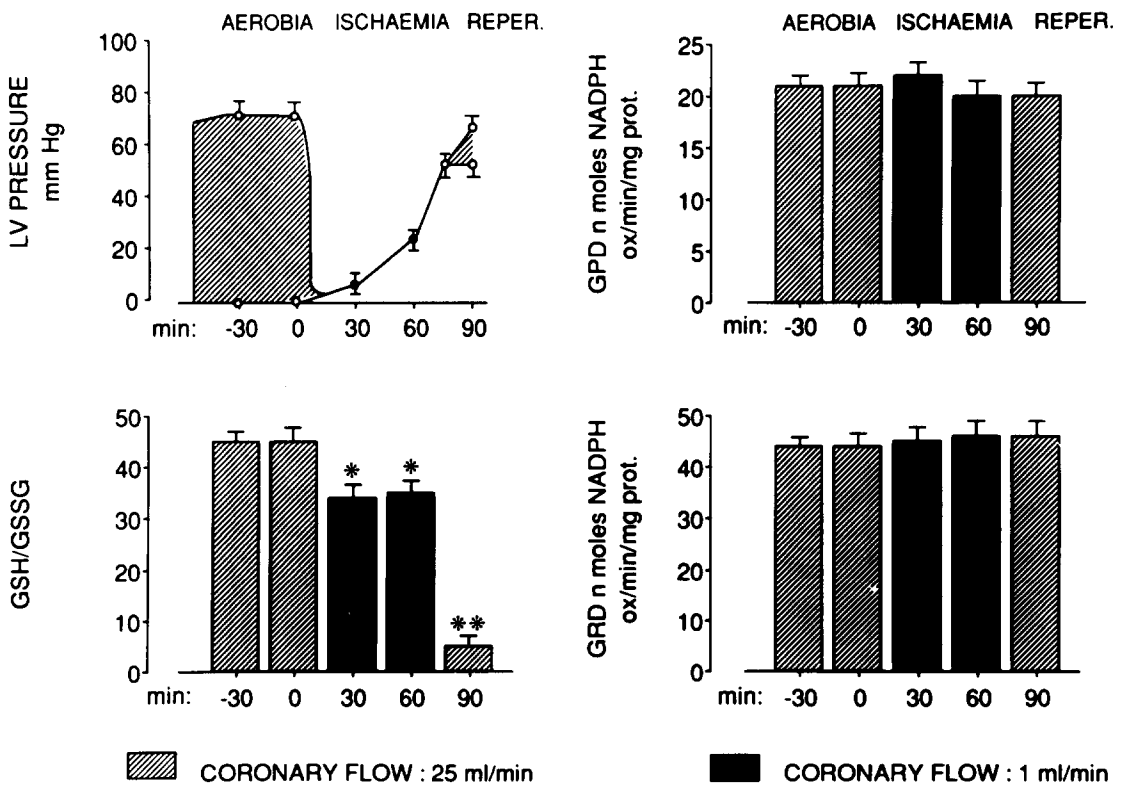

*Fig. 5. Effects of ischemia and reperfusion on tissue GSH/GSSG ratio and on glutathione peroxidase (GPP) and glutathione reductase (GRD) activities of isolated rabbit hearts. All determinations were made in the homogenate. Values are mean ± SE of six experiments. *p < 0.05; ** p< 0.01 with respect to preischemic aerobic values.*

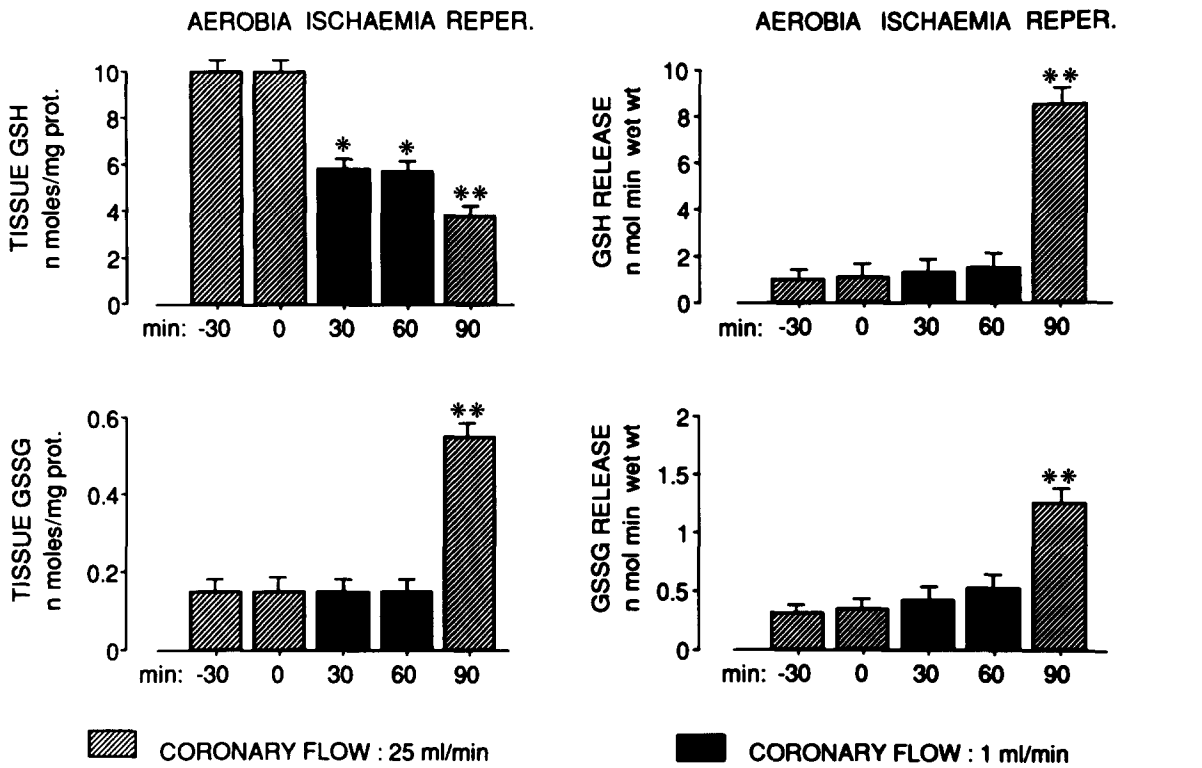

*Fig. 6. Effects of ischemia and reperfusion on tissue content and release of reduced (GSH) and oxidized (GSSG) glutathione of isolated and perfused rabbit hearts. Values are mean ± SE of six experiments. *$p < 0.05$; **$p < 0.01$ with respect to preischemic aerobic values.*

quently, the second line of defense against oxygen toxicity, GPD, is likely to be highly stimulated. We found a severe alteration of the glutathione status, indicating that this was the case and that myocardial oxidative damage had occurred and that it had probably been counteracted at that level.

Studies on animals show that the oxidative stress on reperfusion is correlated with the duration of the ischemic period [16]. Reperfusion after a short period of ischemia (30 to 60 minutes) does not result in oxidative stress, probably because the defense mechanisms are still able to protect the myocardial cells against the burst of oxygen free radicals generated by readmission of oxygen. Reperfusion after more prolonged period of ischemia, when the defense mechanisms are likely to be reduced, results in further damage, with no recovery in function.

Occurrence of Oxidative Stress After Ischemia and Reperfusion in Man

Clinical investigations are hampered by the impossibility of directly measuring the ephemeral free radicals and by the difficulties in following the molecular changes occurring during early phases of reperfusion. In addition, in the clinical setting it is almost impossible to standardize the onset, severity, and duration of ischemia and reperfusion.

We attempted to resolve this problem by measuring the arterial and coronary sinus difference of GSH and GSSG of coronary artery disease (CAD) patients subjected to different periods of global ischemia followed by reperfusion during coronary artery bypass grafting. Because of the high rate of glutathione autooxidation and its disappearance in the blood, we determined plasma levels of GSH and GSSG using a method modified by us [68] in which the blood is treated immediately after collection with thiol, stabilizing agents.

In Figures 7 and 8 are reported the arterial-coronary sinus differences for GSH (Figure 7) and GSSG (Figure 8) of CAD patients subjected to aortic cross-clamping in which the mean clamping period was as long as 25 ± 3 minutes (group 1, 12 patients) and 55 ± 5 minutes (group 2, 10 patients), followed in both groups by 30 minutes of reperfusion. In all patients, before clamping there was a small positive arteriovenous difference for GSH and GSSG. During the following 25 (group 1) or 55 (group 2) minutes of global ischemia, it was not possible to sample from the coronary sinus because of the abolition of coronary flow.

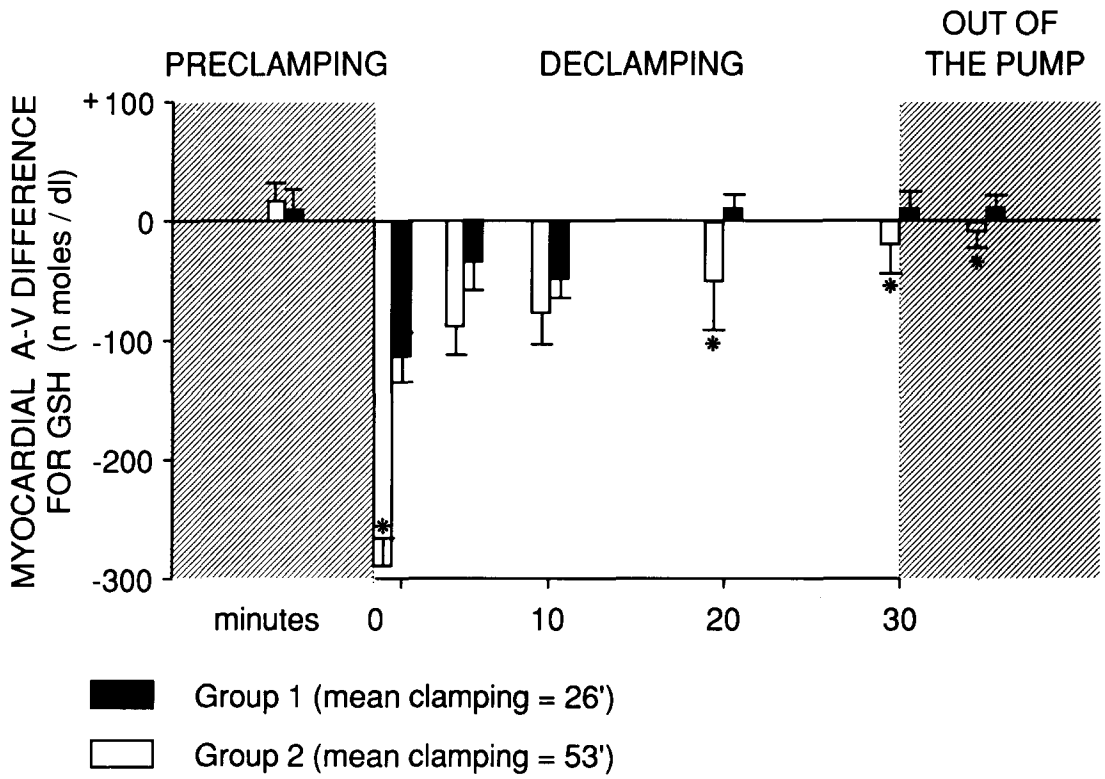

*Fig. 7. Myocardial arterio-coronary sinus difference for reduced glutathione (GSH) of CAD patients subjected to open-heart surgery. *p < 0.05; **p < 0.01 with respect to group 1.*

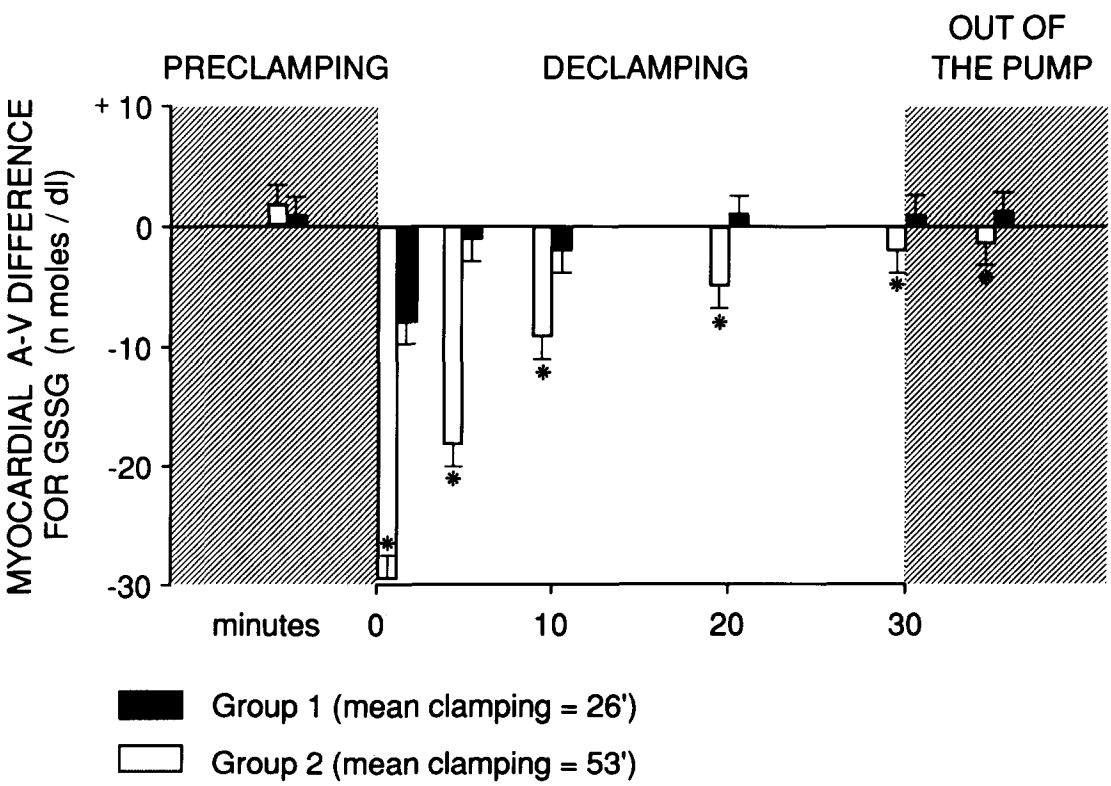

*Fig. 8. Myocardial arterio-coronary sinus difference for oxidized glutathione (GSSG) of CAD patients subjected to open-heart surgery. *p < 0.05; **p < 0.01, with respect to group 1.*

During reperfusion, after the short period of ischemia (group 1), there was a small and transient release of GSH (Figure 7) and GSSG (Figure 8) into the coronary sinus, reaching a peak 3 minutes after declamping; the GSH and GSSG concentrations in the coronary sinus then started to decline and fell below the arterial values. In contrast, reperfusion of patients of group 2 after a more prolonged period of ischemia resulted in more pronounced and sustained release of GSH and GSSG from the myocardium. GSSG production was still continuing after 30 minutes of declamping, the arteriocoronary sinus difference remaining negative, even after the patients were disconnected from the pump (Figure 8).

The results obtained in the two groups of patients show that, as in animal studies, reperfusion results in an oxidative stress, depending on the duration of the ischemic period. When the period of clamping was reduced to 25 minutes, reperfusion resulted in a small and transient rise of GSH and GSSG in the coronary sinus. It is probable that this represented a washout process. Reperfusion reinstated after 55 minutes of ischemia led to a significant release of GSH and GSSG, which was still on at the end of the procedure. This is similar to the effects of reperfusion after prolonged ischemia in the isolated heart and presumably implies oxidative stress. It is interesting to note that in patients of group 2, there was a direct correlation between the duration of the clamping period and the release of GSSG into the coronary sinus and an inverse correlation between the degree of GSSG release in the coronary sinus and the recovery of hemodynamic function after surgery [71]. Thus these cases indicate that reperfusion of CAD patients might induce oxidative damage after a prolonged period of ischemia, and oxygen free radicals may be involved in reperfusion damage.

Conclusion

Without doubt, reperfusion is the most effective way to treat the ischemic myocardium. Later reperfusion may cause, or at least accelerate, myocardial damage, the extent of which can be modified, although the underlying mechanism is still not understood and it is likely that it has multifactorial origin. Myocardial production of oxygen free radicals above the neutralizing capacity of the myocytes may be an important cause of the deleterious effects of reperfusion. This possibility, however, is by no means proven.

There is evidence that prolonged ischemia reduces the naturally occurring defense mechanisms of the heart against oxygen free radicals. Although each method had limitations, when several different methods have been compared, there is also evidence, albeit preliminary, that the formation of these toxic species is enhanced after ischemia, and particularly so after reperfusion. It seems that the presence of blood is not a prerequisite for oxygen free radical formation and that the mitochondria are likely to be an important site of production. Experimental work does indicate that the oxygen free radical-mediated component of the reperfusion damage can be circumvented, providing optimism for pharmacologic treatment. However, several controversies exist regarding the meaning of studies in which agents known to interfere with oxygen free radicals have provided protection, and conclusions derived from such studies should be considered with caution.

Almost no data are available on the role of oxygen free radicals in reperfusion injury in humans. This, in our opinion, is the goal to achieve in the very near future, before antioxidants are blindly and irrationally combined with reperfusion therapy in the treatment of acute myocardial infarction in humans.

Acknowledgments

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