Free Radicals And Myocardial Injury

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An imbalance between myocardial oxygen and substrate demand and supply leads to ischaemia. For many years, it was believed that reperfusion of ischaemic myocardium led to the recovery of reversibly injured cells and to the death of irreversibly injured tissue. Over the last 15 years evidence has accumulated that this may not be true and that reperfusion may harm as well as benefit ischaemic myocardium — a "double edged sword"[1]. Clearly ischaemic myocardium cannot recover without reperfusion but reperfusion may damage or kill cells that have only suffered reversible injury during the previous period of ischaemia — reperfusion injury. It was Hearse[2] who was largely responsible for the concept of reperfusion injury and since the acceptance of the principle by many workers, much research has been performed in order to understand the mechanisms behind it. One of the most potent stimuli for the work has been the advent of techniques to reperfuse acutely ischaemic myocardium in man: by emergency coronary artery bypass grafting, percutaneous transluminal coronary angioplasty and intracoronary and intravenous thrombolytic therapy. In recent years more and more of the changes of reperfusion injury have been thought to be explainable by the production and effects of oxygen free radical species within the reperfused tissue. An understanding of their production, effects and detoxification is, therefore, important.

1. Free Radical Production

A free radical may be defined as any species that has one or more unpaired electrons. This broad definition includes the hydrogen atom and the oxygen molecule, but these species are not, themselves, important in free radical tissue injury.

The biological half-life of free radicals is only a few microseconds because their reactivity is high; the more reactive the species the smaller is its radius of diffusion. The relatively less reactive species, therefore, may be biologically more hazardous as they are able to initiate damage, or produce secondary radicals, at sites distant from their production. This is especially true of hydrogen peroxide which, although not a free radical, is able to diffuse throughout the cell and cross membranes easily[3] and give rise to the highly toxic hydroxyl radical (OH.) in remote sites.

Singlet oxygen is also not a free radical but it can interact with substances to produce free radicals[4]. The superoxide radical (O_2^{-}.) is formed by the addition of an electron to molecular oxygen. It is formed in almost all aerobic cells and is one of the mechanisms by which phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) act. Superoxide reacts, via several stages, with hydrogen ions to form hydrogen peroxide [1].

[1] $2O_2^{-}. + 2H^{+} . \text{--------} > H_2O_2 + O_2$

Hydrogen peroxide in the presence of a transition metal reacts to form the highly toxic hydroxyl radical [2], as first observed by Fenton in 1894.

[2] $Fe^{++} + H_2O_2 \text{ --------} > Fe^{+++} + OH^{-} + OH.$

Iron is highly bound throughout the body, either chelated to various cellular constituents (eg. citrate) or bound, as iron (+++), to the storage or transport proteins ferritin and transferrin. These proteins are rarely saturated and the amount of free iron is, therefore, kept to an absolute minimum; that which is present is mainly iron (+++) rather than iron (++). Hydroxyl radicals can be generated from hydrogen peroxide and iron (+++), however, if superoxide is also present, via an iron-catalysed Haber-Weiss reaction [3]:

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[3] a) $O_2^{\cdot} + Fe^{+++} \dashrightarrow O_2 + Fe^{++}$

b) $H_2O_2 + Fe^{++} \dashrightarrow Fe^{+++} + OH^- + OH^{\cdot}$
(Fenton reaction)

OVERALL:

$O_2^{\cdot} + H_2O_2 \dashrightarrow O_2 + OH^- + OH^{\cdot}$ (iron catalyst)
(Haber-Weiss reaction)

Copper (+) can also act either as the transition metal for the Fenton reaction or via the iron-catalysed Haber-Weiss reaction by reducing iron (+++) to iron (++). Copper is highly protein-bound (to caeruloplasmin, albumin and amino acids) and very little if any is free. Unlike ferritin, caeruloplasmin does not release copper. To obtain the copper ions, the cell must take up the caeruloplasmin protein (probably by pinocytosis) and degrade the protein. Biologically, copper may therefore be less important than iron in the production of free radicals.

The hydroxyl radical is the most toxic free radical species produced. It is so chemically active that it reacts immediately with whatever biological molecule is in the vicinity, often producing secondary radicals of variable reactivity.

Free radicals are produced in the heart at several sites and by several mechanisms:

Leukocytes — the major bacteriocidal mechanism of leukocytes is via free radical production. Seventy per cent of the oxygen consumed by activated granulocytes produces superoxide[8]. This has generated interest in defining the role of the leukocyte in reperfusion injury. Whilst some knowledge has been gained into the behaviour of leukocytes in regional ischaemia and reperfusion, their role in open-heart surgery and cardiac transplantation remains to be defined.

Xanthine-oxidase —Granger et al[6] were the first to propose that xanthine oxidase may be an important source of free radical production during ischaemia and reperfusion. The theory proposes that 2 important events occur during ischaemia that cause a burst of free radical production on reperfusion.

Firstly, in ischaemia, xanthine dehydrogenase is converted to xanthine oxidase. In normal tissue, about 90% of the enzyme is in the dehydrogenase form[7,8]. Ischaemia causes the conversion of the dehydrogenase form to xanthine oxidase by proteolytic cleavage[7,9] or sulphydryl oxidation[7]. Both forms of the enzyme convert xanthine to uric acid; xanthine dehydrogenase by reduction of nicotinamide adenine dinucleotide (NAD^+) via reaction [4] and xanthine oxidase by using molecular oxygen instead of NAD^+, via reaction [5], producing superoxide.

[4] xanthine + H_2O + NAD^+ $\dashrightarrow$ uric acid + NADH + H^+ (xanthine dehydrogenase)

[5] xanthine + H_2O + $2O_2$ $\dashrightarrow$ uric acid + $2O_2^{\cdot}$ + $2H^+$ (xanthine oxidase)

Thirty minutes of ischaemia results in an increase in xanthine oxidase activity from the baseline 6-10% up to 27-33% of the total activity [8,10], most of the conversion occurs in the first 5 minutes of ischaemia[8].

The second change that occurs during ischaemia is the degradation of high energy compounds which increases the amount of xanthine present in the tissues. The stage is set, therefore, for a burst of free radical production upon reperfusion, when molecular oxygen is reintroduced.

The flaw in this theory is that there appears to exist a species difference with regard to the enzyme. Downey et al[10] have shown that whilst xanthine oxidase accumulates in the ischaemic rat and dog myocrdium, it does not appear to be present in the human and rabbit heart. This may partly explain the variations seen in the ability of xanthine oxidase blocking drugs (allopurinol and oxypurinol) to protect the heart against reperfusion injury[11-13]. The situation is clouded because, although some groups concur with the findings of Downey et al[14,15] others have found xanthine oxidase-like activity present in human myocardium[16]. At present, it appears that xanthine oxidase is not present, in appreciable activities, in the human heart, although a form of the enzyme, which is neither oxidase nor dehydrogenase, may be present[10].

Mitochondria — Superoxide is produced under physiological conditions in mitochondria via NADH

dehydrogenase[17], and this accounts to 1% of the electron flow. Rates of mitochondrial respiration increase markedly on reperfusion so that greater quantities of superoxide production are to be expected. The exact role of mitochondria in free radical production, however, is unknown.

Catecholamine metabolism—Catecholamines are released in response to ischaemia and their degradation, by monoamine oxidase, may produce hydroxyl radicals[18]. The significance of this pathway in reperfusion injury is unknown.

2. Effects of Free Radicals

Because of their high reactivity, free radicals react with virtually all cellular components[19]. The most important reactions are with the sulphydryl groups of unsaturated fatty acids and amino acid residues. This has many effects, including alteration in membrane permeability, loss of enzyme activity and cleavage of DNA[13]. The most important of these is alteration in membrane permeability by lipid peroxidation which, as well as altering the structure of the lipid bilayer, may inactivate or alter membrane transport systems by non-enzymatic autocatalytic reactions[19].

A free radical (R.) reacts with a fatty acid (LH) (which may be esterified as in a phospholipid) to produce an alkyl radical of the fatty acid (L.) — reaction [6]. The alkyl radical is then stabilised by resonating to a conjugated diene. Oxygen is taken up to produce a lipid peroxy radical (LOO.) — reaction [7]. This peroxy radical is able to extract hydrogen from another fatty acid [8] forming a lipid hydroperoxide (LOOH) and another alkyl radical [L.]. Thus an initial triggering event can be amplified, in the presence of oxygen, leading to a wave of damage:

[6] $LH + R. \text{------} > L. + RH$

[7] $L. + O_2 \text{------} > LOO.$

[8] $LOO. + LH \text{----} > LOOH + L.$

In practice, several terminating reactions exist to overcome the propagation. Two alkyl radicals can combine [9] as can two peroxy radicals [10] or combinations can occur [11].

[9] $L. + L. \text{----} > L - L$

[10] $LOO. + LOO. \text{-----} > LOOL + O_2$

[11] $LOO. + L. \text{-----} > LOOL$

There also exist defence mechanisms which can terminate the propagation reactions (eg, glutathione peroxidase). Although lipid hydroperoxides are fairly stable entities, transition metals can catalyse their decomposition to produce more radicals [12] [13] and so continue the damage[3].

[12] $LOOH + Fe^{+++} \text{-----} Fe^{++} + LOO. + H^+$

[13] $LOOH + Fe^{++} \text{-----} Fe^{+++} + LO. + OH^-$

Lipid peroxidation changes the ratio of polyunsaturated fatty acids to other fatty acids and the overall effect is to decrease membrane fluidity[4]. Cross-linking reactions may further damage the structure and function of the membrane.

3. Host Defences Against Free Radical Injury

The body possesses 3 principal enzymes capable of detoxifying free radicals. McCord and Fridovich[20] were the first to describe a copper-containing enzyme with the ability to cause the dismutation of superoxide and they named it superoxide dismutase (SOD). The protein had been isolated over 30 years earlier[21] and it was then thought to be a copper-storage protein. It was later found to contain zinc also, although this is only involved in the structure rather than the function of the enzyme. Two other types of SOD have been isolated: one containing manganese and the other iron. The enzyme is present in most organisms; aerobic organisms containing more than aerotolerant organisms, with none detectable in obligatory anaerobes[22]. SOD is essential for the natural defence against oxygen toxicity, which is mediated by free radicals[23]. It is located almost totally intracellularly[9] and exists in two forms in the heart: the copper zinc-containing enzyme in the cytoplasm and the manganese-containing protein in mitochondria. (Iron-containing SOD is found predominantly in prokaryotypes). The enzyme catalyses the dismutation of superoxide to hydrogen peroxide and oxygen [14] at a very fast rate.

[14] $2O^{2-}. + 2H^{+}$ ------> $H_2O_2 + O_2$

Whilst hydrogen peroxide is potentially toxic, via a Fenton or an iron-catalysed Haber-Weiss reaction, it is naturally decomposed by the tissue-bound haemoprotein catalase to yield oxygen and water [15].

[15] $2H_2O_2$ ------> $2H_2O + O_2$

A further enzyme, glutathione peroxidase, which is present in both the cytosol and mitochondria, catalyses the degradation of organic peroxides and hydrogen peroxide. The enzyme contains selenium and converts the lipid peroxides (LOOH) to alcohols (LOH) and water at the expense of reduced glutathione (GSH) which becomes oxidised (GSSG) [16]:

[16a] 2 GSH+ H_2O_2 ------> GSSG + $2H_2O$

[16b] 2 GSH+ LOOH ------> GSSG + LOH + H_2O

During ischaemia the concentrations of SOD, catalase, reduced glutathione and glutathione peroxidase fall[24], so that the protection afforded by these agents, on reperfusion, is reduced. The levels of SOD and catalase in the heart are much lower than other tissue (eg, SOD 25% and catalase <1% of the activity in the liver) which may make the heart particularly sensitive to free radical damage[25].

There also exist several non-enzymatic free radical scavengers in the heart, including: β-carotene, vitamin A, vitamin C and vitamin E, which ameliorate free radical damage. Their role, however, is secondary to the principal scavengers[9].

4. Free Radicals and Reperfusion

Whilst much is known about free radicals in general, direct evidence for their involvement in reperfusion injury is still lacking. This is because it is not possible to measure directly the various oxygen species produced due to their short half-lives. There is, however, a large volume of evidence that free radicals are produced and have toxic effects when ischaemic myocardium is reperfused. This evidence is based both on measurements of an increase in the products of free radical damage after reperfusion[26] and on the limitation of damage afforded by the administration of free radical scavengers[27].

Older assays of free radical damage (eg, thiobarbituric acid reactivity) may be inaccurate in measuring the absolute amount of lipid peroxidation[13] but as markers of free radical production and damage they are valid[26]. These methods have been used to show a gradual rise in lipid peroxidation during regional ischaemia, with a dramatic increase occurring on reperfusion[28]. Other techniques for measuring free radical production have been introduced more recently. Electron spin resonance has been used to confirm the presence of free radicals during ischaemia with a large rise occurring on reperfusion[29,30].

The administration of free radical scavengers has produced mixed results, although the majority of papers do show a beneficial effect with their use. The question — why do some studies show a benefit from the addition of free radical scavengers, whilst others do not? — must be addressed. The answer is far from clear. In the reports using free radical scavengers many different protocols have been used and they differ in several ways:

— method of coronary occlusion

— duration of ischaemia

— duration of reperfusion

— dosage and method of administration of scavengers

— species

— criteria used for assessment of recovery

These factors may all be important when trying to assess the overall benefit of free radical scavengers. The method of coronary occlusion (when used) varies from total[8,28] to subtotal occlusion[31,32] altering residual flow to tissue. A large collateral flow may also affect the results. The type of ischaemia imposed in the studies is either regional or global, the type being chosen to mimic a clinical setting of ischaemia. The

duration of ischaemia varies considerably, often again to simulate a clinical situation. The duration and manner of reperfusion is important in assessing the role of free radical-induced injury. Most studies with short reperfusion periods show a benefit from the administration of free radical scavengers. Those studies with a longer duration of reperfusion often show no protective benefit, perhaps indicating that free radical scavengers delay rather than prevent myocardial cell necrosis[33,34]. The results of Chi et al[31] are particularly interesting because they used a long-acting form of SOD (conjugated with polyethylene glycol which has a half-life over 30 hours). Reperfusion for 24 hours showed a persistent improvement in myocardial function. It may be that, whilst there is an initial burst of free radical production with reperfusion, free radical production and damage continues well into the period of reperfusion. Certainly all the studies showing no benefit from the administration of free radical scavengers did not administer the drug throughout the reperfusion period. The dosage and method of administration of SOD varies in almost every report. The total dosage used ranges from 1,000 U/kg to 19,000 U/kg. The delivery of the scavenger varies from pre-ischaemic administration continued through to the end of reperfusion to administration just prior to reperfusion only. The situation is further complicated as some workers administered the drug as a bolus whilst others have used continuous infusion techniques. Riva et al[35] have performed dose-response curves for SOD and shown the optimal antiarrhythmic dose to be 27,000 U/kg. If one assumes that this dose is optimal for the suppression of other forms of free radical damage, then most studies have used too low a dosage. This does not, however, explain the differences between the results.

Free radical production peaks within 30 seconds of reflow and then rapidly returns to normal. It has been proposed that for SOD to work, it must be present at the onset of reperfusion[33] and must continue for at least 10-15 minutes. This duration of infusion would be expected to be sufficient to protect against the burst of free radical production associated with reperfusion. One reason that those studies assessing myocardial damage, after a significant period of reperfusion, showed no benefit from SOD may be that delayed free radical release occurred from invading neutrophils. Neutrophil depletion has been shown to reduce infarct size[36] and the beneficial effects shown by Chi et al[31] may relate to sustained anti-free radical therapy preventing neutrophil-derived free radical damage.

SOD is a protein with a molecular weight of 32,000 daltons and, therefore, it would not be expected to enter cells readily. Some may be taken up by endothelial cells and its mechanism of action may be to maintain vascular integrity, rather than act intracellularly. If this is true, it would, therefore, be expected to show protection only with ischaemia which is severe enough to cause endothelial damage — this is clearly not so.

Several other anti-free radical agents have been used to assess the role of free radicals in reperfusion injury. Allopurinol has been investigated extensively[10] and as with SOD and catalase, variable protection has been seen. Oxypurinol, the active metabolite of allopurinol, has also been studied[37]. Glutathione depletion[25] or enhancement with N-acetyl cysteine[24] has been investigated, again with varying results. Other agents have been used also, in an attempt to define the role of free radicals and myocardial damage.

It appears that oxygen-derived free radicals are almost certainly involved in the injury occurring to ischaemic myocardium upon reperfusion. Where the most significant damage occurs (endothelium, cell membrane etc.) remains to be defined and whether the pattern of injury differs in different settings (eg, regional and global ischaemia/reperfusion) is also unclear. Free radical scavengers appear to have a part to play in preventing or ameliorating the damage of reperfusion but the dosage of the agents, the method of administration and the timing of delivery remain to be defined before they can be adopted clinically.

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