Oxidative Stress

Lead Article

Free radicals and tissue injury - B.R. Lucchesi

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Bibliography of One Hundred Key Papers
Free radicals and tissue injury

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Several significant observations have stimulated interest in developing sound approaches toward limiting the extent of irreversible tissue injury associated with an acute ischemic insult or from ischemia followed by reperfusion.

The first observation is the recognition of the number of deaths among patients with ischemic heart disease, accounting for two thirds of the nearly one million

Tissue ischemia of sufficient duration produces irreversible injury and cell death. Associated with the direct ischemic insult, there is an indirect attack on the jeopardized tissue through activation of the complement system. This activation, occurring in response to ischemia, facilitates neutrophil–endothelial cell interactions and neutrophil migration into and across the vascular wall, along with the formation of cytotoxic oxygen metabolites and release of proteolytic enzymes. The neutrophil–dependent actions participate in extending the tissue injury beyond that due to ischemia alone. The invading neutrophils injure the myocardial vasculature and sarcolemma through the generation of oxygen free radicals. Components of the complement system can damage tissue indirectly through formation of neutrophil chemoattractants as well as directly through assembly of the cytolytic “membrane attack complex.” As is the case with any organ, function and cellular viability are dependent upon a blood supply, which, if interrupted for a sufficiently long period, will lead to progressive irreversible cellular changes and necrosis. Thus, arrest of the ischemic process demands that blood flow be restored. It is this self-evident truth which gives rise to a paradox that has aroused the interest of basic scientists and clinicians attempting to comprehend and control the phenomenon of reperfusion injury.

Keywords: ischemic injury; reperfusion injury; respiratory burst; reactive oxygen species; oxygen paradox; selectin; calcium paradox; complement; anaphylatoxin; membrane attack complex; neutrophil; adhesion-promoting receptor.

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SELECTED ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ELAM</td>
<td>endothelial-leukocyte adhesion molecule</td>
</tr>
<tr>
<td>hSOD</td>
<td>human superoxide dismutase</td>
</tr>
<tr>
<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule–1</td>
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<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
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<td>IL-1β</td>
<td>interleukin-1β</td>
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<td>ICAM-110</td>
<td>inducible cell adhesion molecule–110</td>
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<td>LECAM-1</td>
<td>leukocyte–endothelial cell adhesion molecule = 1-selectin</td>
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<tr>
<td>LFA-1</td>
<td>leukocyte function–associated integrin</td>
</tr>
<tr>
<td>LTB4</td>
<td>leukotriene B4</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage-1 antigen</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>PADGEM</td>
<td>platelet-activation–dependent granule external membrane = 1-selectin</td>
</tr>
<tr>
<td>rt-PA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>sCR1</td>
<td>soluble complement receptor 1</td>
</tr>
<tr>
<td>SLeX</td>
<td>sialylated Lewis X antigen</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule–1</td>
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</table>
annual deaths associated with diseases of the cardiovascular system. Ischemic heart disease and, in particular, myocardial infarction, account for one third of all fatalities in the United States each year.

The second observation is that the overall prognosis of patients presenting with acute myocardial infarction correlates directly with the extent of myocardial injury or cardiac reserve. Maximum preservation of cardiac muscle remains an important goal in considering any therapeutic regimen designed to manage the patient with persistent myocardial ischemia.

The third observation in connection with limiting myocardial injury concerns the temporal sequence of events in an ischemic insult, which starts with the onset of ischemia and ends with necrosis of myocardial cells within the region of myocardium at risk. In the absence of coronary artery blood flow, the transformation of ischemic myocardial cells from the reversible to the irreversibly injured state is a gradual process, continuing over a time course of about 6 hours or more and leading to the ultrastructural characteristics of cellular necrosis. The progression of myocyte cell death extends from the subendocardial zone across the ventricular wall to involve progressively more of the transmural thickness of the ischemic region or area at risk. Clinically, the progression of cell death (necrosis) manifests itself in the electrocardiogram as a loss in R-wave voltage and the appearance of a new Q wave. Data obtained in experimental animals show that, in the absence of reperfusion and with little or no collateral blood flow, the earliest evidence of irreversible cell injury in the beating heart is apparent after 45 to 50 minutes and is nearly complete within 6 hours of initiation of the ischemic insult. The pattern of progressive ischemic myocardial injury has been referred to as the “wave front phenomenon.” Efforts to limit the extension of myocardial cell death focus on converting a potentially transmural infarct into one which is subendocardial, or at least nontransmural.

It should be noted that the concept of reperfusion injury has yet to be accepted by all and that there are inconsistencies among experimental results. A number of investigators believe that the reperfusion of an ischemic area only increases the rate of cell death for those cells that were irreversibly injured as a result of the ischemic insult. Conclusive proof of the existence of reperfusion injury would require experimental evidence indicating that cells, viable before reperfusion, are irreversibly injured upon, or soon after, the onset of reperfusion. Direct evidence of the conversion of ischemic cells to an irreversibly injured state upon reperfusion is lacking. However, the preponderance of in vitro and in vivo evidence obtained in a variety of organs and tissues provides a compelling reason for stimulating our interest and inquiry into the phenomenon of reperfusion injury.

For the purpose of this discussion, we will use a strict definition of reperfusion injury that considers the possibility that some or all of the ischemic myocytes that were viable at the time of reperfusion undergo a lethal “explosive” alteration due to the combined influences imposed by complement-mediated loss of membrane integrity, oxidative stress associated with the reintroduction of oxygenated blood, and a time-limited assault mediated by accumulated inflammatory cells. Thus, myocardial cells that were viable at the end of a sufficiently long ischemic interval are subjected to unfavorable conditions related to reperfusion. Thus, it is the act of reperfusion itself, aided by activation of the complement system and the subsequent inflammatory response, that leads to the extension of cell death. This is the basis for the concept of reperfusion injury. Myocardial cell death due to reperfusion injury may be different with respect to its mechanism and separate from the type of cell death that results from persisting myocardial ischemia. Histologically, reperfusion injury manifests itself as “contraction band necrosis,” whereas ischemic cell death presents with the appearance of “coagulative necrosis.”

The detrimental potential of reperfusion injury has received greater attention in recent years due to the use of revascularization procedures and thrombolytic therapy to manage patients with an evolving acute myocardial infarction. While the use of these therapeutic measures is known to reduce mortality in patients undergoing an acute myocardial infarction, it is becoming increasingly evident that the events coincident with the restoration of blood flow are also of importance. It is important to recognize that the process of reperfusion injury is not restricted to the heart, but is a phenomenon observed in many tissues and organs; it represents one of the major obstacles in organ transplantation.

**MYOCARDIAL REPERFUSION**

From the introduction above it is clear that preservation of myocardial tissue within the ischemic zone is dependent upon early reestablishment of blood flow, a concept supported by both experimental and clinical data. Early reperfusion is imperative for salvage of the
remaining viable myocardial cells within the jeopardized region. The question that arises is whether or not reperfusion itself, depending upon the circumstances under which it is done and the timing, has the potential to increase cellular injury beyond that caused by the ischemic insult. That “reperfusion injury” may be a reality is based upon experimental data as well as clinical observations. Despite extensive literature on the subject, not all authorities agree with the concept of “lethal reperfusion.” Opinions on this subject were summarized in a publication² that showed that 56% of authors were in favor of the existence of lethal reperfusion injury, 31% denied its existence or thought there was insufficient evidence to support or refute its existence, and 13% argued that lethal reperfusion injury was an outmoded concept and recommended that “emphasis be placed on attenuating myocyte death during ischemia-reperfusion.” Furthermore, the expression reperfusion injury has been used in many divergent ways that go beyond the original description of irreversible cell injury. The term has been applied to damage involving other tissue components (coronary vasculature), the conduction system, and the extracellular matrix. For example, reperfusion injury may be viewed as lethal or nonlethal. The former refers to the death of tissue that was viable at the moment of reperfusion. On the other hand, nonlethal reperfusion injury might refer to postischemic depression of contractile function that is entirely reversible and referred to as myocardial stunning. The expanded view of what constitutes reperfusion injury has led to the proposal that the potentially deleterious effects of reperfusion should be divided into four separate categories, of which only one involves the death of viable cells due to reperfusion: (i) arrhythmias appearing at the time of reperfusion; for (ii) progressive return of contractile function upon reperfusion (myocardial stunning); (iii) lethal reperfusion-induced injury in which normal or reversibly injured cells, at the end of the ischemic insult, are transformed rapidly into irreversibly injured cells and progress to cell death; and (iv) accelerated appearance of cellular changes in irreversibly injured cells that assume the characteristics of necrotic tissue.

The focus of this article will be on lethal reperfusion injury as it applies to reperfusion of the ischemic myocardium. Reperfusion injury is defined as the conversion of myocytes from a reversibly injured state to that of irreversible injury, an event that can be attributed to reperfusion itself. This type of injury involves the death of myocytes that were not irreversibly injured before reperfusion, and is character-

ized by definite ultrastructural alterations that differ substantially from those that occur as a result of ischemic cell death. It is important to recognize that the phenomenon of lethal reperfusion injury has been observed experimentally and clinically in organs and tissues other than the heart. The conventional view has attributed the injury process to ischemia itself. There is a compelling body of evidence that suggests that a substantial proportion of the injury is associated with the onset of reperfusion and that similar changes have been observed in the intestine, pancreas, liver, skin, skeletal muscle, lung, kidney, spinal cord, and central nervous system.

**MORPHOLOGICAL CHANGES—ISCHEMIC INJURY VS REPERFUSION INJURY**

Reperfusion of the ischemic myocardium results in an apparent acceleration of necrosis, as manifested by cell swelling and the formation of contraction bands, an ultrastructural appearance frequently referred to as “myocardial reperfusion injury.” While essential for the ultimate survival of the myocardial cell, the sudden reintroduction of whole blood reperfusion appears to be detrimental to the previously ischemic myocyte, thus giving rise to a paradoxical situation in which the ideal approach to be employed for the preservation of the ischemic heart is problematic. An understanding of the mechanism(s) of reperfusion injury should encourage efforts to control the circumstances surrounding the time of reperfusion in an effort to minimize those factors contributing to cellular injury upon the reintroduction of nutrient blood flow.

As indicated above, a large body of data pertaining to reperfusion injury has been obtained for the heart, lung, brain, and kidney. The observations strongly favor the position that irreversible tissue injury cannot be attributed entirely to the ischemic insult. A characteristic finding in the reperfused tissue, in contrast to what is observed in the ischemic tissue without reperfusion, is the adhesion of neutrophils to the vascular endothelium and the associated capillary plugging. There is evidence of altered vascular permeability and progressive decline in organ blood flow. The latter, referred to as the “no reflow phenomenon,” places the heart in a suboptimal state with respect to maintaining contractile function. Intramyocardial hemorrhage occurs frequently in acutely reperfused myocardial infarctions, and in most cases is localized in the necrotic zone. Most revealing is the comparison of the morphologic changes observed in myocardial tissue obtained from the ischemic heart without
reperfusion as opposed to cardiac tissue obtained within a brief period of time after the onset of reperfusion. Figure 1 (A and B) illustrates the morphologic changes observed as a result of persistent ischemia of 60 minutes' duration compared to a similar period of ischemia followed by reperfusion with oxygenated blood. The myocardial specimen from the ischemic, but nonreperfused, heart appears essentially normal; that from the heart reperfused after 60 minutes of ischemia shows marked morphologic changes along with the characteristic appearance of hypercontracted myofibrils.

The first subcellular changes to appear as a consequence of uninterrupted ischemia affect the mitochondria, as these organelles are the most sensitive to oxygen...
deprivation. The initial change is a disappearance of the normal dense granules and a clearing of the mitochondrial matrix. The changes become more pronounced with increasing duration of ischemia. Ultimately, the dense matrix granules are no longer observed, the matrix is clear, and the cristae are severely fragmented. The cell nuclei are swollen and exhibit margination of chromatin. Irreversible injury of myocytic cells is best characterized by the presence of large amorphous densities within the mitochondria with clearing of the matrix and loss of the cristae. The nuclei may show severe swelling or shrinkage. The sarcomeres are absent in many parts of the cell and are either in marked relaxation or in a distorted and disorganized pattern. The morphologic changes associated with continuous regional or global myocardial ischemia and characteristic of irreversible myocardial injury occur over a time course of 3 hours. Completion of the progressive morphologic changes can be delayed by the presence of collateral blood flow, cooling of the heart, as well as by inducing cardioplegic arrest.

After 6 hours of regional myocardial ischemia due to coronary artery occlusion, there is extensive transmural necrosis with minimal possibility of tissue salvage by reperfusion. Other observations have led to the assumption that transmural necrosis occurs after as little as 2 hours of ischemia when unmodified reperfusion has been carried out. Beyersdorf et al.3 have reported the interesting observation that necrosis does not occur after 6 hours of coronary occlusion in the absence of reperfusion. Furthermore, muscle salvage by reperfusion is possible after at least 6 hours of regional myocardial ischemia. Emphasis is placed upon the need to control the conditions of reperfusion and the composition of the reperfusate in order to ensure the restoration of myocardial function and prevention of tissue necrosis. These observations suggest that the myocardial injury that occurs with prolonged (>50 min) regional ischemia is not due exclusively to the lack of blood flow to the area at risk, but is associated with the reintroduction of molecular oxygen, plasma proteins, and whole blood cellular components.

The morphologic appearance of tissue obtained from hearts made ischemic and subsequently reperfused differs markedly from myocardium maintained ischemic for a similar interval of time, but subsequently subjected to reperfusion. After 15 minutes of ischemia at 35°C followed by reperfusion for 30 minutes, mitochondria appear normal as do other cellular organelles.

At 120 minutes of reperfusion, all myocardial cell components maintain their normal appearance. When the heart is subjected to a period of 30 minutes of ischemia at 35°C and then reperfused, there is complete reconstitution of the myoccardial cellular components, and normal myocardial cellular structure is maintained throughout the entire observation period terminated at 120 minutes. Thus, ischemia of 15 or 30 minutes’ duration at 35°C is associated with reversible changes in tissue morphology, which are corrected by reperfusion, thereby underscoring the importance of early reperfusion for the salvage of jeopardized myocardium.

Extension of the ischemic period to 60 or 90 minutes followed by reperfusion results in an entirely different morphologic appearance in which there are numerous contraction bands. In contrast to the reperfused tissue samples after shorter periods of ischemia, which all exhibit marked homogeneity of structural alterations, the reperfusion samples after 90 minutes of ischemia show great variability in the mitochondrial alterations with tubular cristae and large amorphous densities and a swollen appearance. Extreme contracture bands and/or distortion and disappearance of sarcomeric units are characteristic of myocardial tissue that has been subjected to reperfusion after an ischemic insult of sufficient duration. The morphologic changes are consistent with those observed in the phenomenon of the “stone heart.” The unresolved question is whether the relatively abrupt and marked changes in tissue morphology that occur between 30 and 60 minutes of ischemia followed by reperfusion are the result of ischemia itself or are related to the period of reperfusion. Does 60 or 90 minutes of myocardial ischemia, in the absence of reperfusion, result in the same extent of irreversible cell injury as that observed if reperfusion is instituted?

In summary, there are recognizable differences in the ultrastructural appearance of tissue subjected to reperfusion after ischemia of sufficient duration as opposed to ischemic tissue that is not reperfused. Ultrastructural changes with reperfusion involve myocyte disintegration with “explosive” cell swelling along with swelling and fragmentation of mitochondria. Most notable is the appearance of contraction band necrosis due to the extensive accumulation of intracellular calcium ions. Irreversible tissue injury due to ischemia, without reperfusion, has the microscopic appearance of pale, relaxed myofibrils with preserved cell structure, commonly referred to as “coagulation necrosis.”
Although many would dispute the concept that reperfusion injury can occur, there is a growing body of data, from studies on the heart as well as on other organs, which suggests that the phenomenon of reperfusion injury is real and can respond to therapeutic measures applied before induction of ischemia or at the time of reperfusion. Up to now, ultimate proof of reperfusion injury has been difficult to obtain, as the only real test of tissue viability during ischemia is to determine if myocyte recovery can be achieved with reperfusion. While there can be no debate regarding the importance and advantages of early myocardial reperfusion, the potentially deleterious events associated with reperfusion must be taken into consideration. This discussion will focus on the current understanding regarding the respective roles of oxygen-derived free radicals and the inflammatory response in reperfusion injury. The two events are not mutually exclusive, but act in concert to extend the degree of tissue damage beyond that resulting from the direct effects of the ischemic insult itself.

**REOXYGENATION INJURY VS REPERFUSION INJURY**

Sustained myocardial ischemia is associated with progressive damage in the affected region (area at risk), so that tissue necrosis is due to the continued lack of oxygen and substrate delivery to the myocardial cells. Even in the presence of severe ischemia, however, the cellular and functional changes associated within the early period (<40 minutes) of the ischemic insult are reversible, thereby giving rise to the concept of a “therapeutic window.” With an appropriately timed intervention, examination several days after reperfusion yields no evidence of functional, biochemical, or morphologic alterations. Extending the duration of ischemia beyond 50 minutes is associated with failure to achieve full functional recovery and development of irreversible injury, which is progressive and directly related to the duration of the ischemic event. Furthermore, extending the period of ischemia (>50 minutes) recruits additional biologic mechanisms that give rise to the distinct, but interrelated, phenomena of “reoxygenation injury” and “reperfusion injury,” which compound the injury resulting from the deprivation of blood flow (ischemia).

Simply defined, myocardial reoxygenation injury is irreversible cellular damage (necrosis) resulting from the reintroduction of molecular oxygen at the time of organ reperfusion. This suggests that the reintroduction of oxygen itself causes injury that would not have occurred, or at least not as rapidly, without reoxygenation. Therefore, reoxygenation injury, as considered in this discussion, relates to the irreversible damage of myocytes that were viable up until the moment of reperfusion.

**THE “OXYGEN PARADOX”—THE DELETERIOUS ACTION OF MOLECULAR OXYGEN**

The reintroduction of oxygen after a period of hypoxia or anoxia in the isolated, buffer-perfused heart is associated with the "oxygen paradox." The “oxygen paradox” is characterized by the development of myocardial contracture upon reoxygenation of the hypoxic or ischemic myocardium, and is accompanied by the release of intracellular enzymes (myocardial creatine kinase) in a pattern consistent with irreversible myocardial cell injury. These events do not occur if the ischemic heart is reperfused with a solution relatively devoid of oxygen (hypoxic reperfusion). This gives the clear impression that the reintroduction of molecular oxygen is associated with an abrupt alteration in the integrity of the myocardial cell and an immediate decline or absolute loss of myocardial contractile function, along with an abrupt increase in resting tension (contracture) and ultrastructural changes in the form of contraction band necrosis.

The cellular morphologic changes observed with reperfusion (reoxygenation) are dependent upon the presence of molecular oxygen. Therefore, it is appropriate to think in terms of “reoxygenation injury” when hearts are studied in vitro under conditions in which a crystalloid perfusion medium is employed. Factors other than oxygen, however, participate in the extension of injury when the ischemic heart is reperfused with whole blood. Therefore, the terms “reoxygenation injury” and “reperfusion injury” are not synonymous. The latter is more inclusive and is the situation most likely to occur under in vivo conditions. With whole blood perfusion, oxygen and its reactive metabolites, plus activation of the complement system together with the cellular elements of the blood (inflammatory response to injury), are involved in the extension of irreversible tissue damage, an extension of tissue injury that is beyond that attributable to the ischemic insult itself. To conclude, ischemia/reperfusion injury is a multifactorial process in which the duration of perfusion deprivation sets the stage for events that will serve to perpetuate the destruction of
tissue during the subsequent period of reperfusion.
An appreciation of the postischemic mechanisms of injury is of paramount importance for developing an appropriate therapeutic approach to the protection of the ischemic-reperfused organ.

OXYGEN-DERIVED FREE RADICALS

Molecular oxygen, although essential for maintaining cell viability, is one of the primary factors involved in reperfusion injury. An early indication that molecular oxygen is involved in the development of reperfusion injury was provided by the observation that reperfusion of the anoxic heart with an oxygenated solution enhanced myocardial injury, while a solution relatively devoid of oxygen (hypoxic reperfusion) did not increase the extent of tissue injury. Since this initial observation, it has become apparent that the introduction of molecular oxygen into the ischemic myocardium results in the formation of oxygen-derived free radicals.

Free radicals are reactive chemical species with an unpaired electron in their outer orbitals. Free radicals derived from oxygen include the superoxide radical (O$_2$•⁻) which can react with hydrogen peroxide to give rise to the hydroxyl anion (OH). The latter is more highly reactive and, since it lacks a charge, penetrates more readily across cell membranes. An additional product of oxygen metabolism is hydrogen peroxide (H$_2$O$_2$). Although hydrogen peroxide is a strong oxidant, it reacts slowly with most organic substrates. However, H$_2$O$_2$ reacts with transition metal ions (Fe$^{3+}$) and their inorganic or organic complexes at rapid rates to generate potential oxidants. In the presence of ferric iron (Fe$^{3+}$), H$_2$O$_2$ leads to the formation of the OH anion, a highly reactive oxidant, which, unlike O$_2$•⁻ or H$_2$O$_2$, is indiscriminately reactive with most biological substrates.

Potential enzymatic sources of oxygen metabolites, xanthine dehydrogenase oxidase of capillary endothelial cells as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase of the polymorphonuclear leukocyte (neutrophil), have been implicated in tissue injury associated with ischemia/reperfusion.

All components within the cell are subject to attack by free radicals. The lipids that constitute the cell membrane, especially those that contain unsaturated double bonds, are susceptible to free radical attack leading to the formation of lipid peroxides, lipid hydroperoxides, and aldehydes. A second important site of free radical attack is the membrane proteins involved in the transport of ions and the maintenance of cellular ionic homeostasis. This is especially true of proteins containing sulfhydryl groups, such as those with methionyl residues and peptides in which the amino acid has a critical role or enzymatic function.

It is suggested that an excessive oxidant stress imposed upon the cell can lead to the disruption of essential components of the cell membrane and associated enzymatic functions.

### Table 1. Formation of oxygen-derived radicals and related metabolites.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equations</th>
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<tbody>
<tr>
<td>REDUCTION OF MOLECULAR OXYGEN TO SUPEROXIDE ANION</td>
<td>O$_2$ + e$^- \rightarrow$ O$_2$•⁻</td>
</tr>
<tr>
<td>DISMUTATION OF SUPEROXIDE ANION AND SUBSEQUENT FORMATION OF HYDROXYL ANION</td>
<td>$2$ O$_2$•⁻ + $2$H$^+$ $\rightarrow$ H$_2$O$_2$ + $O_2$</td>
</tr>
<tr>
<td>H$_2$O$_2$ + O$_2$•⁻ $\rightarrow$ O$_2$ + HO$^-$ + HO$^*$</td>
<td></td>
</tr>
<tr>
<td>HABER-WEISS REACTION</td>
<td>O$_2$•⁻ + H$_2$O$_2$ $\rightarrow$ O$_2$ + HO$^-$ + HO$^*$</td>
</tr>
<tr>
<td>TRANSITION METAL HABER-WEISS CATALYZED REACTION</td>
<td>O$_2$•⁻ + Metal$^{n+}$ $\rightarrow$ Metal$^{n-1}$ + O$_2$</td>
</tr>
<tr>
<td>Metal$^{n-1}$ + H$_2$O$_2$ $\rightarrow$ Metal$^{n+}$ + HO$^-$ + HO$^*$</td>
<td></td>
</tr>
<tr>
<td>Note: Metal$^{n+}$ = Fe$^{3+}$</td>
<td></td>
</tr>
<tr>
<td>XANTHINE OXIDASE REACTION</td>
<td>Hypoxanthine + H$_2$O + 2O$_2$ $\rightarrow$ Xanthine + 2 O$_2$•⁻ + 2H$^+$</td>
</tr>
<tr>
<td>Xanthine + H$_2$O + 2O$_2$ $\rightarrow$ Uric acid + 2 O$_2$•⁻ + 2H$^+$</td>
<td></td>
</tr>
<tr>
<td>NEUTROPHIL MYELOPEROXIDASE REACTION</td>
<td>H$_2$O$_2$ + Neutrophil myeloperoxidase $\rightarrow$ HClO + H$_2$O</td>
</tr>
<tr>
<td>NEUTROPHIL NADPH REACTION</td>
<td>NADPH + 2O$_2$ $\rightarrow$ NADP$^*$ + 2 O$_2$•⁻ + 2H$^+$</td>
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</table>
Free radicals and tissue injury - Lucchesi

gives a summary of the reactions leading to the formation of oxygen-derived free radicals and related metabolites. It is important to recognize that free radical species can be derived from both intracellular as well as extracellular sources. Oxygen free radicals are produced by activated neutrophils that infiltrate the ischemic/reperfused myocardium and constitute an important component of the inflammatory response. The intracellular free radical scavenger superoxide dismutase and the peroxide-degrading enzymes catalase and glutathione peroxidase are able to reduce the contribution of myocardial cell injury attributable to cytosolic reactive oxygen species. These intracellular enzymes, however, may have limited capacity to protect the cell from damage caused by the extracellular free radical attack due to the accumulation of inflammatory cells in response to tissue injury. Thus, exogenous administration of free-radical scavengers such as superoxide dismutase has been explored as a potential therapeutic approach to reducing the injury associated with ischemia/reperfusion.

There is ample evidence, albeit some of it indirect, suggesting that free radicals are formed to a limited degree during myocardial ischemia with a marked increase during the phase of reperfusion. The observations are supported by the use of electron paramagnetic resonance (EPR) spectroscopy and spin-trapping agents to detect free radical formation following perfusion with oxygenated crystalloid perfusion medium or reintroduction of whole blood in the ischemic tissue. Superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the highly reactive hydroxyl radical (OH) are the major free radicals in the pathogenesis of reperfusion injury. In hearts subjected to 30 minutes of ischemia, the appearance of EPR spectroscopy signals indicates the formation of reactive oxygen intermediates upon normoxic reperfusion, with peak signal intensities occurring within 15 seconds. Recombinant human-superoxide dismutase (hSOD) entirely abolishes the burst of oxygen free radical production. The above-mentioned studies demonstrate that superoxide-derived free radicals are generated during postischemic reperfusion and suggest that the beneficial effect of hSOD is due to its specific enzymatic scavenging of superoxide free radicals ($O_2^+$).

**THE “CALCIUM PARADOX”—THE DELETERIOUS EFFECTS OF TISSUE CALCIUM OVERLOAD**

It has been suggested that the “oxygen paradox” and the “calcium paradox” are facets of the same problem. Both are characterized by the development of myocardial contracture, release of intracellular cytosolic enzymes, loss of mechanical activity, and changes in myocyte ultrastructure. The major similarity is a final common pathway leading to intracellular calcium overload and a resultant increase in intracellular calcium.

The “calcium paradox” is characterized by a rapid increase in intracellular free calcium concentration. Whereas no increase in tissue ionized calcium concentration occurs following 60 minutes of global ischemia, there is a ten-fold increase within the first 10 minutes of reperfusion. Several possibilities exist as to the route of calcium entry during the “calcium paradox.” It was widely assumed that calcium was derived from external sources, including entry through voltage-sensitive calcium channels and sodium-calcium exchange mechanisms. However, the use of calcium channel blockers in experimental models of ischemia/reperfusion has shown inconsistent results in protecting the ischemic heart. It is currently debated whether the influx of calcium into the cell is due to ischemia-induced disruption of the membrane or free radical–induced membrane damage. Intracellular sites have been proposed as a potential source of increase in intracellular calcium. It has been hypothesized that the intracellular generation of free radicals after reperfusion may cause leakage of calcium ions from intracellular stores, such as the sarcoplasmic reticulum. In addition, it is conceivable that the cell may lose the ability to extrude calcium, or that the uptake of calcium by the sarcoplasmic reticulum is impaired as a result of the ischemic insult.

A number of biochemical events have been postulated to occur as a result of calcium influx. Foremost is the activation of intracellular enzymes. Activation of phospholipases may lead to the formation of cell-damaging arachidonic acid metabolites and depletion of adenosine triphosphate (ATP) stores. Furthermore, a number of proteases are known to be activated in the presence of calcium and reported to mediate myofibrillar turnover and the degradation of various proteins including cytoskeletal filaments. A number of ultrastructural changes are associated with calcium ion influx. The development of contracture bands and the formation of large amorphous densities within the mitochondria are examples of the most prominent changes in the cellular ultrastructure. The appearance of these ultrastructural markers is considered to be indicative of irreversible injury and cell death.
NEUTROPHILS AND REPERFUSION INJURY

Leukocyte activation, adhesion, and emigration serve a protective role during a well-contained inflammatory response. Under certain pathologic conditions such as endotoxemia, inflammatory bowel disease, and ischemia/reperfusion injury, leukocytes may become injurious and exacerbate—rather than prevent—tissue damage. Experimental studies have investigated the pathophysiologic role of leukocyte accumulation and adhesion during ischemia/reperfusion injury of various organs, in particular the myocardium. Most studies in the heart have investigated whether the consequences of experimental myocardial ischemia/reperfusion could be attenuated by interventions aimed at inhibiting chemotactic leukocyte infiltration in, or adhesion to (or both), microvascular endothelial cells. Although many promising results have been reported, the application of these strategies to experimental or routine clinical management of patients with myocardial infarction has so far not been undertaken.

ADHESION MOLECULES AND CHEMOTACTIC FACTORS IN NEUTROPHIL ACCUMULATION

A sequence of events must occur during and after an ischemic insult for a sufficient number of neutrophils to accumulate in the reperfused tissue and contribute to the extension of the tissue injury above and beyond that attributable to the ischemic process itself. Chemotactic factors, from multiple sources, activate the neutrophils and amplify the local inflammatory response. Chemotactic factors include products derived from activation of the complement cascade, such as C5a and C3a (anaphylatoxins), arachidonic acid metabolites, including leukotriene B4 (LTB4), and tissue cytokines. Concurrent with the formation and release of chemotactic factors there is an upregulation of adhesion-promoting receptors, located on both the granulocytes, especially the neutrophils, and the endothelial cells. Adhesion and subsequent migration of neutrophils into the surrounding tissue is a complex process consisting of a number of steps including: (i) rolling of the neutrophil along the endothelial cell surface; (ii) diapedesis through the endothelium; and (iii) extravascular migration into the tissue. These steps involve distinct groups of adhesion receptors and cell-derived mediators for expression of surface adhesion-promoting receptors and formation of chemotactic factors. The selectins family of cell adhesion molecules consists of three members, each having a unique cellular distribution and mechanism of expression. The selectins mediate the early events associated with neutrophil adhesion. L-selectin is constitutively expressed on neutrophils, monocytes, eosinophils, and some subtypes of B and T cells. P-selectin (also referred to as GMP-140 and platelet activation–dependent granule external membrane [PADGEM] protein) is constitutively present in the α granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon activation of the endothelial cell, P-selectin is mobilized from the Weibel-Palade bodies to the cell surface. L-selectin (Mel-14, leukocyte–endothelial cell adhesion molecule [LECAM-1]), in conjunction with P-selectin, is involved in the “rolling” of the neutrophil along the endothelium. L-selectin is shed from the neutrophil upon activation, coincident with the upregulation of the neutrophil heterodimeric adhesion receptor, CD11b/CD18. The latter interacts with the complement-derived, cell membrane–associated protein IC3b and the endothelial cell–derived intercellular adhesion molecule (ICAM)-1. An additional, endothelial cell–derived molecule that may play a role in the early events of adhesion, is platelet-activating factor (PAF). This biologically active phospholipid is synthesized by the endothelial cell (as well as being stored in the blood platelets) and transported to the cell surface. PAF functions in a dual manner by activating neutrophils and acting directly as an adhesion molecule. PAF, in conjunction with P-selectin, may cause the neutrophil to adhere to the endothelial cell and signal the cell to increase expression of the CD11/CD18 integrins. The combined actions of PAF and P-selectin would provide a means for attaching the neutrophil to the endothelium during the upregulation of the CD11/CD18 complex.

A two-step model has been proposed for neutrophil adhesion in which L- and P-selectin act in concert to facilitate neutrophil recruitment into the microenvironment of the vasculature. Before movement out of the vasculature, a longer-lasting adhesion is mediated via leukocyte β2 integrins (CD11/CD18 complex). The adherent neutrophils may then recruit additional circulating neutrophils and activate these cells via glycoprotein-glycoprotein interactions or leukotrienes.

E-selectin is limited to the endothelial cell and is localized to postcapillary venules where it is expressed in response to inflammatory stimuli (interleukin [IL]-1β, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and lipopolysaccharide). E-selectin has been detected in vivo in inflamed tissues, where it is expressed on the cell surface of cytokine-stimulated endothelial cells and mediates the binding of neutrophils.
The time sequence for expression of E-selectin is dependent upon protein synthesis and requires from 4 to 6 hours for full expression with a return to basal values within 24 hours. The observation that the selectins mediate binding between leukocytes and endothelial cells provided the incentive to search for the respective target cell ligands having a carbohydrate structure. The ligand for E-selectin binding is sialylated Lewis X antigen (SLeX). SLeX is expressed on neutrophils, monocytes, and certain cell lines in the form of glycoproteins and glycolipids. Anti-SLeX antibodies are effective inhibitors of E-selectin binding and are able to prevent the interaction of the neutrophil with the endothelial cell, thereby modulating the inflammatory response to injury.

Before the discovery of the selectins and their recognition as adhesion molecules, attachment of leukocytes to the endothelium was believed to be largely a result of the β2 integrins (LFA-1[leukocyte function–associated integrin], Mo1, and gp150,95). The three members of the β2 integrin family (CD11/CD18 glycoprotein complex) of the leukocyte adhesion promoting receptors, possess a common β subunit (CD18) noncovalently bound to a distinct α subunit (either CD11a, CD11b, or CD11c), to form LFA-1, Mo1 (Mac-1, macrophage-1 antigen), or gp150,95, respectively. Mo1 is stored in intracellular sites and is mobilized to the cell surface in response to appropriate stimuli (eg, C5a). At the surface, the molecule serves as the receptor for complement-derived iC3b opsonized particles and is involved in adhesion, chemotaxis, and spreading of the neutrophil on the endothelial cell. As stated previously, the importance of the Mo1 subunit in mediating neutrophil adherence and reperfusion injury stems from the ability of antibodies directed against Mo1 to decrease myocardial infarct size in experimental models of ischemia/reperfusion injury. In addition to serving as the receptor for iC3b, Mo1 interacts with intercellular adhesion molecule-1 (ICAM-1) located on the endothelial surface. Inactive endothelial cells normally express low levels of ICAM-1. However, stimulation by cytokines such as IL-1 and TNF upregulate expression of ICAM-1 and endothelial-leukocyte adhesion molecule (ELAM), a member of the selectin family. The upregulation of these ligands is maximal within 4 to 6 hours. Antibodies to ICAM-1 not only decrease adhesion of neutrophils, but reduce infarct size in the ischemic/reperfused rabbit heart.

**Figure 2** illustrates the cell adhesion molecules and

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**Figure 2.** Schematic representation of neutrophil adhesion–promoting receptors and their respective ligands. ICAM-1, -2, intercellular adhesion molecule-1, -2; ICAM-110, inducible cell adhesion molecule–110; SLeX, sialylated Lewis X antigen; VCAM-1, vascular cell adhesion molecule–1.
their respective ligands that participate in neutrophil–endothelial cell interaction.

In summary, the sequence of events associated with neutrophil adhesion at a site of tissue injury involves a number of precisely orchestrated cell–cell interactions that are important at different time points. It is likely that the early events (minutes) involved in neutrophil adhesion to the endothelial surface are mediated through a transient interaction between the neutrophil and endothelium mediated via the selectins (L-selectin, P-selectin) and PAF. The β2 integrins Mo1 and LFA-1 are likely responsible for adhesion lasting longer periods of time (minutes to hours) and which depends, in part, upon the activation of the complement system and opsonization of the target site by iC3b, which serves as the ligand for Mo1. During this time, the neutrophil may become firmly attached to the endothelium before movement out of the vasculature into the surrounding tissue.

During chronic episodes of inflammation, the third member of the β2 integrin family, gp150,95, is likely to become the primary mediator of adhesion for the neutrophil and monocyte. Figure 3 summarizes the time-dependent events that are involved in the response to injury and are accelerated in response to reperfusion.

**Figure 3.** Schematic representation of the time-dependent sequence of reactions associated with the development of an inflammatory response to tissue injury as a result of ischemia/reperfusion. ICAM, intercellular cell adhesion molecule; IL, interleukin; MAC, membrane attack complex; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

**MEDIATORS OF NEUTROPHIL-INDUCED CELLULAR INJURY**

Neutrophils are recognized for their capacity to mediate tissue damage in organs subjected to ischemia followed by reperfusion. Ischemia is a common clinical event leading to local and remote injury. Evidence indicates that a significant degree of tissue damage is caused by activated neutrophils that accumulate rapidly in the risk region as a result of tissue reperfusion. If the area of ischemic tissue is extensive, neutrophils also sequester in the lungs, inducing noncardiogenic pulmonary edema, a condition not uncommon in organ transplantation at the time of establishing blood flow. Ischemia/reperfusion injury is initiated by production of reactive oxygen species, which initially appear responsible for the generation of chemotactic activity for neutrophils. Therapeutic options for limiting ischemia/reperfusion injury include inhibition of oxygen radical formation, pharmacological prevention of neutrophil activation and chemotaxis, and also the use of monoclonal antibodies that prevent neutrophil-endothelial adhesion, a prerequisite for a significant component of reperfusion injury in the blood-perfused organ.
Once adherent to the endothelium, activated neutrophils are able to elicit tissue injury through several different mechanisms, including generation of oxygen-derived free radicals and release of cytotoxic lysosomal enzymes. Chemotactic factors, including C5a, PAF, and cytokines, are able to activate neutrophils and promote diapedesis across the vascular bed. Stimulation of the neutrophils by one or more of the chemotactic factors elicits the "respiratory burst," characterized by a sudden increase in oxygen consumption and a release of reactive oxygen intermediates into the surrounding interstitial space, which is relatively devoid of a means for protecting against the damaging influences of reactive oxygen species. Superoxide anion, hypochlorous acid (HOCl), OH, and chloramine (RNHCl-) are oxidants produced by the stimulated neutrophil. In addition, circulating PAF stimulates neutrophils to synthesize H₂O₂, which induces a PAF-dependent adherence of neutrophils to the endothelium. Thus, generation of H₂O₂ by the neutrophil may act as a positive feedback mechanism, allowing the neutrophil to recruit other circulating neutrophils to the injured tissue. Evidence for the role of reactive radicals in reperfusion injury, coupled with the ability of neutrophils to produce free radicals, implicates this mechanism as one way by which neutrophils elicit tissue damage.

Coinciding with the generation of oxygen-derived free radicals by the neutrophil is the release of a number of cytotoxic proteases stored in intracellular granules. Neutrophil-derived cationic proteins and neutral proteases alter vascular permeability and disrupt the basement membrane of the vascular wall. Two metalloproteases, collagenase and gelatinase, when activated by HOCl, degrade collagen and lyse the endothelial cells. Other important lysosomal enzymes released during activation include elastase and heparinase, the latter contributing to the degradation of heparan and heparin sulfate associated with the extracellular matrix or glycocalyx.

It is not entirely clear whether the neutrophils must emigrate from the endothelium into the surrounding tissue in order to elicit injury or if damage to the endothelial cell is sufficient. It is likely that the neutrophil causes deleterious effects in both the vasculature and surrounding tissue. Since neutrophils can form aggregates, small capillaries may become physically "plugged" and represent the underlying mechanism for the "no reflow phenomenon," where areas of the ischemic region are not reperfused adequately. Neutrophils also may affect larger vessels such as arterioles and precapillary vessels. Release of vasoconstricting agents from the activated neutrophils are thought to decrease vessel diameter, resulting in decreased perfusion of the surrounding tissue. The decrease in perfusion may be exacerbated by release from the neutrophil of factors such as PAF, which activate circulating platelets. Accumulation of platelets in the reperfused area would result in both an increase in vascular plugging and a release of platelet-derived factors acting upon the vasculature.

A critical aspect of reperfusion injury is the infiltration of polymorphonuclear leukocytes into the reperfused region. While the primary role of the neutrophil is to protect the host from infectious agents, the infiltration and subsequent activation of the neutrophils may prove detrimental to the surrounding, but still viable, myocardial tissue. The involvement of neutrophils in the control of local infection or repair of injured tissue requires that the cells leave the vascular compartment and emigrate to the affected tissue site. Accumulation of neutrophils in the interstitial space contributes to the inflammatory response. It is now recognized that leukocyte migration includes: (i) initial rolling of the leukocyte along the vessel wall, (ii) followed by firm adhesion to the wall, and (iii) finally, emigration through the endothelial cell layer in a process known as diapedesis. Neutrophil rolling is mediated by the interaction of a member of the selectin family of proteins with a specific carbohydrate ligand. As discussed previously, the process of neutrophil adhesion is mediated by activation of a member of the β₂ (CD11/CD18) integrin family of proteins, which then binds to a counterligand on the surface of the endothelial cell (iC3b and ICAM-1). Pathophysiologic accumulation of neutrophils has been determined from animal models to play an important role in ischemia/reperfusion injury.

Activated neutrophils coming into contact with endothelial cells stimulate the conversion of xanthine dehydrogenase to xanthine oxidase. This conversion is rapid, occurring within 5 to 10 minutes, and is dependent upon the presence of the complement component C₅a. Thus, the peptide chemotactic mediator can interact directly with endothelial cells to initiate the production of free radical species, and thus amplify the response to injury. The sequence of events involving the conversion of xanthine dehydrogenase to xanthine oxidase is depicted in Figure 4.

During the period of flow deprivation (ischemia), emigration of the inflammatory cells from the vascular bed into the interstitial space progresses slowly over
in the course of 8 to 12 hours, ultimately culminating in the formation of a region of myocardial infarction. The infarcted region is characterized histologically by tissue necrosis with the presence of numerous inflammatory cells comprised primarily of polymorphonuclear neutrophils. On the other hand, myocardial tissue subjected to reperfusion after a suitable period of ischemia (>50 minutes), undergoes a rapid infiltration of neutrophils into the previously ischemic zone. The emigration of the inflammatory cells begins within minutes of the onset of reperfusion and increases progressively for up to 90 to 120 minutes. Evidence for the involvement of neutrophils in reperfusion injury was obtained from earlier studies that showed that the nonsteroidal anti-inflammatory agent ibuprofen could protect the myocardium in experimental models of myocardial infarction.\textsuperscript{14,15} Romson et al\textsuperscript{14} noted that the reduction in infarct size seen in dogs treated with ibuprofen was associated with a reduction in leukocyte infiltration into the area at risk. The use of nonsteroidal anti-inflammatory agents to reduce myocardial infarct size has demonstrated a dichotomy between ibuprofen, which reduces myocardial infarct size, and indomethacin and aspirin, which do not, despite the fact that the three nonsteroidal anti-inflammatory agents inhibit cyclooxygenase.\textsuperscript{15} These results suggest that ibuprofen acts in a manner distinct from that of either aspirin or indomethacin. In vitro studies with granulocytes exposed to zymosan-activated plasma (activation of the complement system) demonstrated a similar dichotomy between ibuprofen and aspirin. Ibuprofen inhibited granulocyte aggregation, superoxide production, lysosomal enzyme release, and granulocyte-mediated endothelial cytotoxicity, while aspirin was without effect. It is proposed that ibuprofen's beneficial effect in experimental myocardial ischemia is related to its ability to inhibit activated granulocytes and thus to diminish myocardial cell death in experimental myocardial infarction.

Further evidence for the role of the neutrophil in eliciting myocardial injury came from studies showing that neutrophil depletion before the induction of ischemia decreased infarct size in a canine model of myocardial reperfusion injury.\textsuperscript{17,18} Furthermore, Simpson et al\textsuperscript{18} administered a monoclonal antibody directed against the CD11b/CD18 adhesion-promoting molecule (Mo1) to an open-chest dog 45 minutes into a 90-minute period of myocardial ischemia. After 6 hours of reperfusion, it was noted that the administration of the monoclonal antibody decreased infarct size by 46% when compared to controls. The ability of the Mo1 CD11b/CD18 antibody to reduce infarct size suggests the possibility of a therapeutic approach to limiting tissue damage associated with ischemia/reperfusion, and illustrates the role of adhesion molecules in modulating the neutrophil-mediated component of reperfusion injury.

**THE COMPLEMENT SYSTEM IN REPERFUSION INJURY**

The complement system (Figure 5) is composed of two separate pathways, the classic one and the alternative one. The classic pathway provides the “specific” adaptive response involving immune complex formation, while the alternative pathway is responsible for “nonspecific,” innate immunity. Although triggered by different mechanisms, both pathways converge at the level of C3, ultimately forming the anaphylatoxins (eg, C3a, C4a, and C5a) and the membrane attack complex (MAC). The latter consists of the distal complement
components C5b-9. Both the anaphylatoxins and the MAC play an important role in mediating the pathogenesis of ischemia/reperfusion injury.

Activation of the complement system in the setting of myocardial ischemic injury was proposed over 25 years ago and represents an integral mechanism through which the ischemic tissue undergoes cellular injury and ultimately necrosis. A subsequent study provided evidence demonstrating the consumption of classic complement components by heart subcellular membranes in vitro and in patients after acute myocardial infarction. Nonimmune activation of the first component of complement (C1) by the heart mitochondrial inner membrane has been demonstrated, and the findings are consistent with the fact that cardiolipin, the only strong activator of C1 among phospholipids, is present in large amounts in the heart mitochondrial inner membrane. The proteins of the mitochondrial inner membrane were found to activate C1 only weakly, in contrast with the phospholipid fraction which induces strong C1 activation. Furthermore, the digestion of mitochondrial inner membranes with proteolytic enzymes did not affect C1 activation. A subsequent study demonstrated the accumulation of C1q, an early component of the classic pathway, in a canine model of myocardial ischemia and reperfusion injury. An interesting observation was that ischemia-induced complement activation, as evidenced by the accumulation of complement components, was not apparent immediately and required approximately 45 minutes. This would suggest that ischemic insults of limited duration (45 minutes), in contrast to longer intervals of ischemia, may not elicit injury associated with the complement cascade. Recognition of the time-dependent mechanism for activation of the complement cascade places emphasis on the ischemic episode as the initiator of the detrimental consequences arising during the period of reperfusion. Ischemic insults of short durations, not associated with activation of the complement system, would not engender the abrupt
changes in tissue viability observed with ischemic insults of longer duration (>50 min).

Complement proteins in most infarcted myocardial fibers and associated blood vessels coincided with sequestration of polymorphonuclear leukocytes. However, neither accumulation of complement proteins nor of leukocytes was observed in myocardial tissue that was not subjected to an ischemic insult.

Accumulation of the MAC is not limited to the membrane of target cells, but it may be found in the cytoplasmic regions. Thus, complement components can diffuse into the interstitial space, where the MAC is assembled. The resulting accumulation of C5b-9 on the cell membranes could contribute to functional disturbances (signal transduction) and irreversible damage (altered intracellular electrolyte and water balance) of cells during ischemia and reperfusion. Other studies have shown a significant increase in accumulation of C3bBb, and the degradation products of the anaphylatoxins in the plasma of patients after acute myocardial infarction, indicating that products of complement activation, in addition to the MAC, play an important role in ischemia/reperfusion injury.

ACTIVATION OF COMPLEMENT IN THE SETTING OF REPERFUSION INJURY

The mechanism(s) by which complement is activated upon reperfusion is (are) not clear. Since complement appears to be activated within a short time span after reperfusion, one should focus on the initial events known to occur following reperfusion of the ischemic myocardium. The fifth component of complement, C5, can be converted to an active form, C5b, by oxygen-derived free radicals, which rapidly appear in the extracellular compartment following reperfusion. The nonenzymatic conversion of C5 to a functionally active C5b-like form by hydroxyl radicals results in the formation of the complete, lytic MAC. Products of neutrophil activation, including superoxide anion, hydrogen peroxide, hydroxyl radical, peroxide-like radicals, and myeloperoxidase have also been implicated in promoting complement activation. Exposure of basement membranes and subcellular organelles that may appear after an ischemic event has been shown to activate complement. Early investigations demonstrated that isolated myocardial membranes bind C1, thus leading to activation of the entire cascade. Furthermore, cytoplasmic constituents, including the mitochondria, are able to elicit activation of the complement system.

Damage to the cellular membrane may provide an indirect mechanism by which free radicals initiate activation of complement. Damage to the membrane may take the form of denaturation of integral proteins and/or altered membrane integrity. Denaturation of protective membrane proteins impairs the ability of the cell to ward off injury via activation of the complement system. The expression of various membrane regulators of complement activation in normal and infarcted human tissue has been analyzed in an effort to address the question of why the strictly controlled complement system reacts against autologous tissue subjected to ischemia and reperfusion. For example, in the heart, protectin (CD59) is strongly expressed by normal myocardium. Infarcted cells, however, exhibit a substantial decrease in the expression of this regulator of MAC formation. The expression of CD59, but not of C8-binding protein, was diminished in the lesions. Results show that C8-binding protein, vitronectin, and C4b-binding protein do not prevent complement attack against the infarcted myocardium, but accumulate together with the MAC. Ischemia-induced transformation of complement-resistant viable cells into activators of complement may be due to the acquired loss of resistance to the MAC by shedding of CD59 and other complement-protective proteins.

Plasmin-dependent fibrinolytic agents used for thrombolysis are known to activate complement in vitro and may contribute to its activation in vivo. The extent of complement activation was studied in patients suffering an acute myocardial infarction, treated or not with streptokinase. Streptokinase treatment caused abrupt activation of the complement system, whereas no significant complement activation was detected in plasma of myocardial infarct patients not treated with fibrinolytic agents. In like fashion, plasmin generation subsequent to the administration of recombinant tissue plasminogen activator (rt-PA) has been associated with activation of complement. Reports in the literature indicate that complement activation does occur following the administration of rt-PA, but that it is definitely independent of reperfusion. Similar results have been obtained when streptokinase was used as the thrombolytic agent, thereby providing evidence that plasmin can initiate activation of the complement cascade. A direct comparison between the effects of streptokinase and rt-PA on complement activation during thrombolytic therapy demonstrated that complement activation occurred following administration of either compound. However, the influence of streptokinase on the generation of complement activation products, including the anaphylatoxins, was more pronounced than that of rt-PA.


ACTIONS OF THE ANAPHYLATOXINS

Production of the anaphylatoxins C3a and C5a occurs in both the classic and alternative pathways, while C4a is produced via the classic pathway only. Both C3a and C5a are derived from the actions of convertases on the N-terminal ends of the α-chain of their respective precursors. The proteolytic generation of the anaphylatoxins C3a, C4a, and C5a during the early phases of complement activation is associated with the inflammatory reaction during the evolution of a myocardial infarction. The anaphylatoxins induce localized vasoactive effects in a variety of tissues, including those damaged during the process of ischemia and reperfusion. The anaphylatoxins mediate alterations in vascular permeability, induce smooth muscle cell contraction, and release histamine from mast cells and basophils. The spasmogenic properties of C3a and C4a are regulated by plasma carboxypeptidase N, which removes the C-terminal arginine from the anaphylatoxins that is essential for activity. The lack of a C-terminal arginine on C5a, however, does not reduce its chemotactic action or the facilitation of the inflammatory response. On a molar basis, C5a is ten times more active than C3a. There are, however, fewer C5a molecules produced during complement activation, thus the net effectiveness of C5a may be less than that of C3a.

Human umbilical vein endothelial cells (HUVECs) stimulated with purified C5a exhibit an increased P-selectin expression, demonstrating that C5a has a role in both the recruitment and adhesion of neutrophils. C5a alone does not cause significant injury in the reperfused myocardium, but, in the presence of neutrophils, it is associated with marked injury, suggesting that complement-derived products are required for neutrophil activation and subsequent contractile dysfunction. This conclusion is in agreement with other observations showing a reduction in infarct size in a pig model of myocardial infarction with the administration of a monoclonal antibody against C5a. The fact that ibuprofen, but not aspirin or indomethacin, could reduce experimental infarct size is of interest in view of the ability of ibuprofen to prevent C5a-induced activation of the neutrophil. These observations support the concept of an important role for the alternative complement pathway and C5a in the propagation of myocardial damage during reperfusion. C5a is also likely to mediate the early events of neutrophil recruitment. In a rabbit model of ischemia and reperfusion injury, greater concentrations of C5a were present during the early stages of reperfusion, whereas it was not detectable during the ischemic period. The increase in C5a generation correlated with increases in neutrophil accumulation within the ischemic zone during reperfusion. As C5a concentrations declined, interleukin-8 (IL-8) concentrations in myocardial tissue increased, demonstrating a biphasic generation of neutrophil chemotactic factors.

ROLE OF THE MEMBRANE ATTACK COMPLEX

Previous studies demonstrated that the MAC has a functional role in mediating the pathogenesis of ischemia/reperfusion injury. The presence of complement-derived products in human tissues after an ischemic episode, plus the ability of cobra venom factor and the complement inhibitor soluble complement receptor-1 (sCR1) to decrease tissue injury in experimental models is evidence of the detrimental role of complement and the MAC. The importance of reperfusion in mediating complement activation and subsequent accumulation of the MAC is exemplified in a study delineating the temporal characteristics of MAC accumulation in the nonreperfused and reperfused myocardium. In the reperfused myocardium, MAC accumulation occurs rapidly (30 min) as compared to the 5 to 6 hours required for MAC accumulation in hearts that did not undergo reperfusion. The data suggest that reperfusion is a prerequisite for rapid activation of the complement cascade and subsequent MAC accumulation. The detrimental role of the MAC in augmenting myocardial injury after ischemia/reperfusion is further substantiated by use of experimental animals deficient in the complement protein C6. There is a decrease in myocardial infarct size and in the no-reflow phenomenon in C6-deficient rabbits as compared to C6-sufficient rabbits. Neutrophil accumulation within the area at risk of C6-deficient rabbits was decreased when compared to C6-sufficient animals. This observation demonstrates a link between MAC accumulation and subsequent recruitment and accumulation of leukocytes within the ischemic region. The ability of the MAC to modulate the expression of proinflammatory chemokines in vitro suggests that, in addition to its direct lytic effects, the MAC may participate in mediating the recruitment of neutrophils in the setting of ischemia/reperfusion injury.

The deleterious effects of MAC formation on cellular membranes have been attributed primarily to the direct, lytic effects of the complex upon nucleated cells. Nucleated cells, through cell-associated complement
inhibitors, can limit the degree of local complement activation and subsequent MAC formation. Thus, the partial formation of the terminal complex or the complete formation of the MAC, while not directly lysing target cells, may act to stimulate the cell, thereby modulating the proinflammatory response noted during the reperfusion period. The MAC promotes the inflammatory response by inducing the expression of proinflammatory mediators. Stimulation of granulocytes or mesangial cells with nonlytic MAC concentrations promotes formation of reactive oxygen and arachidonic acid metabolites (eg, prostaglandins, leukotrienes) and modulates the expression of cellular adhesion molecules, eg, P-selectin, which is mobilized rapidly from its intracellular storage granules. The MAC is able to enhance the recruitment of leukocytes by increasing the expression of cytokines from both effector cells of the immune system and nonimmune cells. Proinflammatory cytokines, including TNF-α and IL-1β, are secreted by mesangial cells and monocytes after MAC accumulation. The recruitment of inflammatory cells (neutrophils and monocytes) may be increased by the formation of sublytic MAC concentrations on endothelial cells as a result of the local induction of chemotactic cytokines. Both the neutrophil chemotactic cytokine IL-8 and the monocyte chemokine monocyte chemoattractant protein-1 (MCP-1) are secreted in response to sublytic MAC formation in vitro.

The mechanisms by which MAC formation promotes increased expression of proinflammatory mediators has yet to be ascertained, although initial in vitro studies have provided some insight. The complex has been linked to a number of second-messenger signaling pathways. Indirectly, the membrane-associated, pore-forming protein complex provides a mechanism for transmembrane ion fluxes, including calcium, which, once within the cytoplasm, may promote activation of calcium-dependent phospholipases, increased ATPase activity, and the uncoupling of oxidative phosphorylation in the mitochondria. The development of contraction bands and the formation of large amorphous densities within the mitochondria are examples of changes in cellular ultrastructure that are, in part, mediated by excess tissue calcium. The appearance of the ultrastructural markers is indicative of irreversible injury and cell death.

The MAC also may elicit activation of signal transduction mechanisms in a receptor-independent manner. For example, MAC formation is associated with increased intracellular concentrations of sn-1,2-diacylglycerol (DAG) and activation of a variety of protein kinases, including protein kinase C and protein kinase A. An increase in protein kinase C and cAMP activity in Ehrlich cells is noted after exposure to the “sublytic” MAC. The interaction of the MAC with multiple signal transduction pathways suggest a possible link between MAC formation and the subsequent upregulation of proinflammatory mediators. It is noteworthy that formation of the entire MAC (C5b-9) is not required to achieve second messenger activation, since the nonlytic membrane-associated complexes C5b-7 and C5b-8 can impart a signal to the target cell in the absence of pore formation. It is known that MAC accumulation modulates the activity of transcription factors such as nuclear factor-κB (NF-κB), which controls the expression of proinflammatory cytokines and adhesion molecules. These observations strengthen the hypothesis that the terminal complement complexes C5b-7, C5b-8, and C5b-9 are able to generate nonlethal, and perhaps reversible, cell signals independent of those arising from lethal pore formation. It is apparent that MAC deposition, via its ability to alter the activation state of nucleated cells, may serve an important role in amplifying the inflammatory response to injury.

Myocardial ischemia/reperfusion injury is accompanied by an inflammatory response contributing to reversible and irreversible changes in tissue viability and organ function. Endothelial and leukocyte responses are involved in tissue injury, and are orchestrated primarily by the complement cascade. Anaphylatoxins and assembly of the membrane attack complex contribute directly and indirectly to further tissue damage. Tissue salvage can be achieved by depletion of complement components, underscoring the contributory role of the complement cascade in ischemia/reperfusion injury. The complexity of the complement cascade provides numerous sites as potential targets for therapeutic interventions designed to modulate the complement response to injury. The latter is exemplified by the ability of a soluble form of complement receptor 1, sCR1, to decrease infarct size in in vivo models of ischemia/reperfusion injury as well as preventing myocyte and vascular injury and organ dysfunction by stopping assembly of the membrane attack complex. Effective inhibitors of complement are not limited to newly developed compounds or solubilized forms of endogenous regulators of complement activation. Therapeutic agents in common use such as heparin and related nonanticoagulant glycosaminoglycans are known to inhibit complement activation in vitro as well as in vivo and may prove useful as cytoprotective agents. It thus appears logical to use experimental models representative of clinical
settings in which complement activation is associated with tissue injury from the standpoint of organ function and cell viability. Furthermore, there is a need to identify pharmacologic interventions able to inhibit the human complement system before recommending such agents for human clinical investigation. The data obtained with a specific pharmacologic intervention can then be correlated using in vivo animal models of myocardial ischemia/reperfusion injury. There has been renewed emphasis on complement in recent years with the result that new pharmacologic interventions are in the early stages of development and awaiting the opportunity to enter the stage of clinical trials. Better knowledge of the disease states associated with inappropriate activation of the complement system is being sought in the hope that a pharmacologic approach to therapy may become a reality.

**ISCHEMIC PRECONDITIONING**

Brief periods of myocardial ischemia followed by reperfusion provide endogenous protection from a subsequent ischemic insult. This paradoxical finding gave rise to the concept now known as “ischemic preconditioning.” Despite extensive investigation during the past 10 years, the process by which such protection is conferred remains to be elucidated.

Preconditioning was defined originally as a rapid, adaptive response to a brief ischemic insult which slowed the rate of cell death during a subsequent, prolonged period of ischemia. The definition has been expanded to include other adverse consequences of ischemic insults, such as cardiac rhythm disorders and altered cardiac contractility—end points that may not represent the same phenomenon as originally described. Reduction in myocardial infarct size was found to be induced by brief (5-minute) intermittent periods (6-12 cycles) of regional myocardial ischemia and reperfusion (10 minutes), which were then followed by a longer (60-minute) ischemic insult that should have resulted in a significant loss of myocardial tissue. When compared to control hearts not subjected to ischemic preconditioning, the hearts of preconditioned animals exhibited a marked reduction in infarct size.

A complete discussion of ischemic preconditioning is beyond the scope of this article. However, the subject is of paramount importance. Clinical use of preconditioning could lead to improved patient outcomes not only with respect to myocardial preservation, but in other organs subjected to periods of flow deprivation. In addition to coronary artery bypass grafting and percutaneous transluminal coronary angioplasty procedures, both stable and unstable angina, as well as cardioplegia during cardiac transplantation, may provide examples of situations in which the human myocardium is preconditioned. Better understanding of the mechanisms involved is needed. A major goal would be to develop pharmacological and therapeutic interventions that could mimic ischemic preconditioning through activation of the essential biochemical events that enhance the tissue’s ability to withstand a sustained, albeit limited, ischemic insult. At present, adenosine and related analogs in addition to agents that open the ATP-dependent potassium channel have provided positive results in animal studies.

Development of an effective pharmacologic intervention could find important clinical applications when used in conjunction with procedures currently employed during surgery as well as in the treatment of acute cardiac ischemic syndromes. The interested reader is encouraged to consult the more recent literature on this fascinating and potentially important subject.

**ISSUES FOR FURTHER CONSIDERATION**

The increasing use of revascularization procedures in the management of patients with occlusive arterial disease, as well as the growing demand for organ transplantation, require a more thorough understanding of how deprivation of blood flow to an organ alters long-term tissue viability and function. The need to reperfuse the ischemic tissue within a reasonable time frame is clear. The damaging consequences of organ reperfusion have been observed experimentally as well as clinically in many vascular beds. There is a definite recognition that the reintroduction of blood flow brings about an abrupt change in the functional state of the reperfused tissue. However, many unresolved issues remain pertaining to ischemic injury and the potential for extension of tissue damage as a result of reperfusion.

Three particularly pertinent aspects will be addressed in this issue of Dialogues in the following section: Giuseppe Ambrosio will answer the question "How important is oxidative stress in ischemia, reperfusion, and heart failure?"; a point with far-ranging implications is looked into by Malcom Mitchinson: "Are free radicals a major factor in atheroma?"; this of course leads directly to the question of potential therapeutic applications, explored by Michael Hess and Rakesh Kukreja: "What are the prospects of antioxidants as a new therapeutic modality?"
REFERENCES

1. Reimer K, Jennings R.
The “wavefront phenomenon” of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow.

2. Przyklenk K (Guest editor).
Lethal myocardial “reperfusion injury”: The Opinions of Good Men.

Studies on prolonged acute regional ischemia. I. Evidence for preserved cellular viability after 6 hours of coronary occlusion.

4. Hearse DJ, Humphrey SM, Nayler WG, Slade A, Border D.
Ultrastructural damage associated with reoxygenation of the anoxic myocardium.
J Mol Cell Cardiol. 1975;7:315-324.

Measurement and characterization of postischemic free radical generation in the isolated perfused heart.

Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity.
J Mol Cell Cardiol. 1985;17:937-945.

Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase.

Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury.

9. Hearse DJ, Humphrey SM, Bullock GR.
The oxygen paradox and the calcium paradox: two facets of the same problem?

10. Mullane K.
Neutrophil and endothelial changes in reperfusion injury.

11. Zimmerman GA, McIntyre TM, Mehra M, Prescott SM.

12. Lucchesi BR.
Modulation of leukocyte-mediated myocardial reperfusion injury.

13. Springer TA.
Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm.

The effect of ibuprofen on accumulation of indium-111-labeled platelets and leukocytes in experimental myocardial infarction.

Ibuprofen inhibits granulocyte responses to inflammatory mediators. A proposed mechanism for reduction of experimental myocardial infarct size.
Inflammation. 1984;8:33-44.

Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog.

Protective effects of N-2-mercaptopropionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals.

Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function.

The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats.

20. Pinckard RN, Olson MS, Giclas PC, Terry R, Boyer JT, O’Rourke RA.
Consumption of classical complement components by heart subcellular membranes in vitro and in patients after acute myocardial infarction.


Oxidative Stress

Expert Answers to Three Key Questions

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How important is oxidative stress in ischemia, reperfusion, and heart failure?

Giuseppe Ambrosio, MD, PhD; Isabella Tritto, MD

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Cellular metabolism is normally accompanied by generation of small amounts of oxidants. Under certain pathophysiological conditions, the amount of oxidants formed is greatly increased, with consequent oxidative stress, and it is now widely appreciated that this phenomenon may have important consequences. Experimental studies have shown that generation of large amounts of oxygen radicals can occur upon postischemic reflow, inducing a specific form of myocardial damage, that adds on ischemic injury; evidence is accumulating that this phenomenon may also occur in man. Oxidative stress also accompanies experimentally induced heart failure. Its pathogenetic role and possible occurrence in man are hot research topics.

Over the past decade, it has been clearly shown that reperfusion, after a period of ischemia, is associated with formation of large amounts of oxygen radicals,4 and that this phenomenon is accompanied by specific biochemical abnormalities, such as release of oxidized glutathione5 and formation of conjugated dienes,6 which are the “signature” of oxidative attack. Beside its biological consequences, the very demonstration of this phenomenon has already had a major impact on our understanding of the consequences of myocardial ischemia, in that it has challenged the simplistic notion that myocytes having survived an ischemic insult merely go “back-to-business-as-usual” upon restoration of blood flow. Instead, it has become evident from these and other observations (eg, occurrence of specific contractile and electrophysiological abnormalities7-12) that reperfused myocardium shows a number of peculiar characteristics. Thus, while there is no doubt that timely reperfusion is the most effective means to halt the progression of ischemic cell injury, it is now also clear that the early postischemic state represents a pathophysiological condition characterized by specific peculiarities.

**IMPORTANCE IN ISCHEMIA-REPERFUSION**

**Conceptual importance**

Different aspects should be considered when it comes to gauging the importance of oxidative stress in the pathophysiology of myocardial ischemia-reperfusion. The first major aspect is a conceptual one. That free radicals and other oxygen metabolites may oxidize a number of biological molecules in the test tube has been known for quite some time. However, evidence that a similar phenomenon could also occur in vivo is much more recent.

Over the past decade, it has been clearly shown that reperfusion, after a period of ischemia, is associated with formation of large amounts of oxygen radicals,4 and that this phenomenon is accompanied by specific biochemical abnormalities, such as release of oxidized glutathione5 and formation of conjugated dienes,6 which are the “signature” of oxidative attack. Beside its biological consequences, the very demonstration of this phenomenon has already had a major impact on our understanding of the consequences of myocardial ischemia, in that it has challenged the simplistic notion that myocytes having survived an ischemic insult merely go “back-to-business-as-usual” upon restoration of blood flow. Instead, it has become evident from these and other observations (eg, occurrence of specific contractile and electrophysiological abnormalities7-12) that reperfused myocardium shows a number of peculiar characteristics. Thus, while there is no doubt that timely reperfusion is the most effective means to halt the progression of ischemic cell injury, it is now also clear that the early postischemic state represents a pathophysiological condition characterized by specific peculiarities.

**Biological importance**

The second aspect to consider is the biological importance of oxidant stress, as evidenced in the experimental laboratory. Much information

**Keywords:** oxidative stress; reperfusion injury; neutrophil; myocardial ischemia; oxygen radical scavenger; trimetazidine; heart failure; nitric oxide.

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Oxidative stress in ischemia, reperfusion, and heart failure - Ambrosio & Tritto

has been obtained in a number of studies in which the outcome of hearts reperfused under “standard” conditions was compared to that of hearts that, in addition to reperfusion, were treated with various antioxidants. Other studies investigated the effects of various antineutrophil interventions, since production of oxygen radicals is a major mechanism of toxicity by activated neutrophils. By this approach, it has been possible to dissect out the relative component of myocardial injury due to oxygen deprivation during ischemia from possible alterations brought about by oxidative stress upon reflow. It has thus been shown that oxidative stress can exert major consequences on various aspects of cardiac function, and that hearts receiving interventions aimed at preventing oxidant toxicity scored better on various indices of cell injury (as outlined below) as compared to untreated hearts receiving reperfusion alone.

Reduction of reperfusion arrhythmias. It is well known that postischemic reperfusion may be associated with the sudden onset of life-threatening arrhythmias (i.e., ventricular tachycardia and/or ventricular fibrillation) upon reflow. This phenomenon can be induced by oxygen radicals, and it is significantly reduced by free radical scavengers in animal models. By this approach, it has been possible to dissect out the relative component of myocardial injury due to oxygen deprivation during ischemia from possible alterations brought about by oxidative stress upon reflow.

Improvement of contractile recovery. Many studies using different experimental models have sought whether antioxidant therapy would improve recovery of contractile function after ischemia in reperfused hearts. There is general agreement that this is certainly the case for “stunned” hearts, i.e., hearts that have been subjected to periods of coronary artery occlusion of relatively short duration (5-15 min) and in which recovery of function with reperfusion is slow in spite of the absence of irreversible tissue injury. This model reproduces the situation of transient ischemic episodes in patients with unstable angina or vasospasm. In this setting, administration of scavengers has been associated with greater and/or more rapid recovery of regional contractile function during reperfusion. Similar results have also been observed in isolated hearts subjected to global ischemia, both under normothermic and hypothermic conditions that simulate cardiac surgery. Importantly, in some of these studies, treatment was begun only at the moment of reflow, and, therefore, any beneficial effect observed could not possibly be due to reduction of ischemic damage. These results demonstrate that, although ischemic injury undoubtedly affects cardiac function, at least part of the contractile impairment of stunned hearts can be a specific consequence of oxidative stress upon reflow (Figure 1).

In other studies, the effects of scavenger treatment were assessed on the recovery of function of hearts.

Figure 1. Possible components of postischemic contractile dysfunction. Transient coronary artery occlusion induces a marked depression of regional contractility (indexed as wall thickening), which recovers slowly over several hours. This myocardial “stunning” may arise from the additive effects of ischemia-induced injury (light shading), and of a second deleterious component that can be specifically reduced through the use of an antioxidant intervention given at reflow, and therefore might denote oxidant-mediated injury (dark shading). Adapted from Hearse DJ: Stunning: a radical re-view (Cardiovasc Drugs Ther. 1991;5:853-876).
subjected to longer periods of ischemia (60-120 min), to mimic the clinical scenario of patients with acute myocardial infarction in whom salvage of a portion of the ischemic myocardium is achieved by thrombolysis. Also in this case, a beneficial effect on functional recovery has been documented, although there are some negative reports.

Reduction of vascular injury.
While endothelial cells can tolerate ischemia relatively well, reperfusion brings about marked alterations, as evidenced by decreased responsiveness to endothelium-dependent vasodilators, and occurrence of ultrastructural changes. These alterations can be considerably reduced through administration of oxygen radical scavengers or antineutrophil interventions.

Reduction of cell death. This issue has stirred considerable interest and controversy. Many studies have reported that the extent of necrosis caused by coronary artery occlusion can be further reduced if reperfusion is complemented by administration of scavengers for review, 24-26). This finding would imply that a component of reperfusion may have caused death of myocytes that would otherwise have survived a transient ischemic insult. However, a similarly impressive list can be made of studies in which oxygen radical scavengers failed to protect from possible cell death linked to reperfusion (for review, 26 and 27).

The reasons for this apparent discrepancy are unclear. It is possible that the conditions under which oxygen radicals may promote myocyte necrosis are different from what is established for other manifestations of oxidant toxicity, such as arrhythmias or stunning. Similarly, it is also conceivable that the mechanisms of oxygen radical generation in hearts subjected to prolonged ischemia (which might lead to necrosis) may differ from those activated by shorter ischemic episodes. In this respect, it is important to recognize that, in contrast to the conflicting results obtained with use of oxygen radical scavengers, interventions aimed at preventing neutrophil infiltration or activation have almost invariably been associated with significant and persistent reduction of the extent of myocardial infarction in reperfused hearts.

Clinical importance.
In spite of the vast knowledge accumulated in experimental studies, the importance of oxidative stress in patients during postischemic reperfusion is still a matter of current research (Table I). One reason is the inherent difficulty of investigating this issue in the clinical setting. There is evidence that under certain conditions human myocardium can undergo oxidative stress, as indexed by the release of oxidized glutathione and lipid peroxides in the coronary sinus of patients subjected to cardiac arrest and reperfusion during cardiac surgery. However, direct demonstration of oxygen radical generation in patients is still lacking. This is because oxygen radicals are extremely short-lived species and their detection requires use of sophisticated techniques that currently do not lend themselves to use in humans.

Additional information has been obtained through studies in which antioxidant therapy has been administered to patients who underwent reperfusion after a period of ischemia. Again, much data have been collected during cardiac surgery. Here, administration of various antioxidants has been associated with reduced ultrastructural damage, and better functional outcome. As for patients with acute myocardial infarction who underwent recanalization, the two available studies convincingly show that administration of the oxygen radical scavenger superoxide dismutase can markedly decrease the incidence of reperfusion arrhythmias.

Preliminary data also indicate that adenosine, a drug that interferes with neutrophil activation, can reduce the extent of necrosis in patients with acute myocardial infarction receiving thrombolytic therapy. Improved regional wall motion in these patients has also been reported with administration of trimetazidine; interestingly, we have recently shown that trimetazidine can reduce neutrophil-mediated injury and oxygen free radical generation in an experimental model of acute myocardial ischemia/reperfusion.

### Table 1. Evidence for oxidative stress during postischemic reperfusion. Comparison of clinical and experimental data.

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<thead>
<tr>
<th></th>
<th>Experimental Data</th>
<th>Clinical Evidence</th>
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<tr>
<td>Oxygen radical formation</td>
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<td>?</td>
</tr>
<tr>
<td>Membrane lipid peroxidation</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Glutathione oxidation</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophil activation</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Arrhythmias</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Contractile impairment</td>
<td>+++</td>
<td>?</td>
</tr>
<tr>
<td>Vascular alterations</td>
<td>+++</td>
<td>?</td>
</tr>
<tr>
<td>Myocyte cell death (necrosis and/or apoptosis)</td>
<td>+ –</td>
<td>?</td>
</tr>
</tbody>
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Dialogues in Cardiovascular Medicine - Vol 3 · No. 1 · 1998
**IMPORTANCE IN HEART FAILURE**

**Biological importance**

Evidence is accumulating that oxidative stress may also play a role in the pathophysiology of heart failure. While myocardial hypertrophy is accompanied by increased antioxidant defense, progression towards heart failure is accompanied by reduced antioxidant defenses and increased oxidative stress. Several studies have shown that heart failure is accompanied by a decrease in myocardial content of superoxide dismutase and glutathione peroxidase, a reduction in redox state, and accumulation of oxidized glutathione, and by a concomitant increase in lipid peroxidation. These findings have been reported in various experimental models of heart failure, regardless of etiology (aortic banding, post-myocardial infarction, idiopathic cardiomyopathy, pacing-induced heart failure) and animal species (rat, guinea pig, Syrian hamster, rabbits). Oxidative stress during heart failure can be not only the consequence of reduced antioxidant defenses, but it can also be secondary to increased generation of oxidant species. In this regard, increased oxygen radical generation has been demonstrated in mitochondria obtained from failing hearts. Nitric oxide metabolism is another potential source of oxidant species. Heart failure is accompanied by increased nitric oxide production, with a positive correlation between plasma nitrate concentrations and severity of heart failure. This increase might be secondary to increased basal nitric oxide release by constitutive endothelial nitric oxide synthase, or to activation of the inducible form of this enzyme by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), which are produced in greater amounts in heart failure. Since nitric oxide synthase also generates oxygen radicals, the increase in its activity might result in oxidative stress. In addition, enhanced release of proinflammatory cytokines might result in neutrophil activation, with attendant production of oxygen radicals.

A peculiar form of heart failure that is considered secondary to oxidative stress is adriamycin-mediated contractile impairment. Exposure to adriamycin is accompanied by indices of oxidative injury to myocytes, and several studies have shown that adriamycin cardiotoxicity can be prevented by oxygen radical scavengers or antioxidants.

**Clinical importance**

The recognition of a potential role of oxidative stress in the pathogenesis of heart failure has drawn attention to its potential as a therapeutic target. High plasma levels of malondialdehyde, an end-product of lipid peroxidation, have been detected in patients with heart failure. The occurrence of oxidative stress in patients with heart failure is also suggested by the detection of elevated breath pentane excretion, a noninvasive index of lipid peroxidation.

Recent studies have documented the beneficial effects of carvedilol administration on morbidity and mortality in patients with chronic heart failure. These effects have been related to its β-blocker activity, but recent evidence suggests that carvedilol also has antioxidant properties that might contribute to its beneficial effects.

The possibility of preventing adriamycin toxicity has raised considerable attention, since the possible occurrence of heart failure severely limits its clinical use. In addition to several experimental studies, clinical data are also available showing that ICRF-187, an iron chelator with antioxidant properties, can prevent development of myocardial contractile impairment in patients receiving adriamycin.

In conclusion, the bulk of evidence clearly indicates that reperfusion of ischemic hearts is accompanied by generation of oxygen radicals, at concentrations which can induce marked biochemical, metabolic, and functional abnormalities, in various animal species and possibly in man. These alterations lead to a condition of oxidative stress that can be significantly reduced through appropriate control of the modalities of reperfusion, and therefore they can be considered a specific consequence of reperfusion, in addition to injury caused by ischemia itself. Oxidative stress may also accompany heart failure, although in this case its pathogenetic role is still unclear. Much research is currently aimed at establishing whether prevention of oxidative stress in the clinical setting would translate into better metabolism and function of myocytes, and whether antioxidant drugs may represent a novel adjunct to our therapeutic armamentarium.
REFERENCES


Oxidative stress in ischemia, reperfusion, and heart failure - Ambrosio & Tritto

25. Opie LH. 
Reperfusion injury and its pharmacologic modifications. 

26. Engler R, Gilpin E. 
Can superoxide dismutase alter myocardial infarct size? 

27. Reimer KA, Murry CE, Richard VJ. 
The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and the controversy? 

Occurrence of oxidative stress during reperfusion of the human heart. 
_Circulation_. 1990;81:201-211.

Assessment of myocardial oxidative stress in patients after myocardial revascularization. 

Reduction of reperfusion injury with mannitol cardioplegia. 

31. Johnson WD, Kayser KL, Brenowitz JB, Saedi SF. 
A randomized controlled trial of allopurinol in coronary bypass surgery. 

Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. 

33. Murohara Y, Yui Y, Hattori R, Kawasaki C. 
Effects of superoxide dismutase on reperfusion arrhythmias and left ventricular function in patients undergoing thrombolysis for anterior wall acute myocardial infarction. 

34. Mahaffey KW, Puma JA, Barbagelata A, et al. 
Does adenosine in conjunction with thrombolysis reduce infarct size? Results from the controlled, randomized AMISTAD trial. 
_Circulation_. 1997;96(suppl I):1206.

Free radicals, thrombolytic therapy and myocardial infarction: results of the EMIP-FF angiographic substudy. 
_Circulation_. 1997;96(suppl I):1330.

Trimeprazin prevents neutrophil-mediated myocardial injury in postischemic rat hearts. 

37. Dhall A, Hilly MF, Singhal PK. 
Role of oxidative stress in transition of hypertrophy to heart failure. 

38. Oskarsson HJ, Marinelli C, Vandenberg B, Coppey L, Lund D. 
Oxidative stress plays a role in the pathophysiology of tachycardia-induced cardiomyopathy. 

39. Singh N, Dhall A, Seneviratne C, Singhal PK. 
Oxidative stress and heart failure. 

40. Dhall A, Singhal PK. 
Antioxidant changes in hypertrophied and failing guinea-pig hearts. 

41. Hilly MF, Singhal PK. 
Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. 

42. Khaper N, Singhal PK. 
Effects of afterload-reducing drugs on pathogenesis of antioxidant changes and congestive heart failure in rats. 

43. Kobayashi A, Yamashita T, Kaneko M, Nishiyama T, Hayashi H, Yamazaki N. 
Effects of verapamil on experimental cardiomyopathy in the bio 14.6 Syrian hamster. 

44. Belch JJF, Burgus AB, Scott N, Chopra M. 
Oxygen free radicals and congestive heart failure. 

45. Guarneri C, Muscarci C, Caldarera CM, Stefanelli C, Pretolani E. 
The effect of treatment with coenzyme Q10 on the mitochondrial function and superoxide radical formation in cardiac muscle hypertrophied by mild stenosis. 

46. Winlaw DS, Smythe GA, Keogh AM, Schyven CS, Spratt PM, Macdonald PS. 
Increased nitric oxide production in heart failure. 

47. Winlaw DS, Smythe GA, Keogh AM, Schyven CS, Spratt PM, Macdonald PS. 
Nitric oxide production and heart failure. 

48. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. 
Negative inotropic effect of cytokines on the heart mediated by nitric oxide. 

49. Moncada S. 
The l-arginine-nitric oxide pathway. 

50. Drexler H, Hayoz D, Munzel T. 
Endothelial function in chronic congestive heart failure. 

51. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. 
Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. 
52. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL.
Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury.

53. Hansen PR.
Role of neutrophils in myocardial ischemia and reperfusion.

54. Luo X, Evrovsky Y, Cole D, Trines J, Benson LN, Lehotay DC.
Doxorubicin-induced acute changes in cytotoxic aldehydes, antioxidant status and cardiac function in the rat.
*Biochim Biophys Acta.* 1997;1360:45-52.

55. Doroshow JH, Locker GY, Ifrim I, Myers CE.
Prevention of doxorubicin cardiac toxicity in the mouse by N-acetylcysteine.

56. Siveski-Iliskovic N, Hill M, Chow DA, Singal PK.
Prolactol protects against adriamycin cardiomyopathy without interfering with its antitumor effect.

57. McMurray J, McLay J, Chopra M, Bridges A, Belch JIF.
Evidence for enhanced free radical activity in chronic congestive heart failure secondary to coronary artery disease.

58. Sobotka PA, Brottman MD, Weitz Z, Birnbaum AJ, Skosey JL, Zarling EJ.
Elevated breath pentane in heart failure reduced by free radical scavenger.

Beneficial effects of intravenous and oral carvedilol treatment in acute myocardial infarction. A placebo-controlled, randomized trial.

60. Packer M, Bristow MR, Cohn JN, et al.

61. Feuerstein GZ, Ruffolo RR Jr.
Carvedilol, a novel vasodilating beta-blocker with the potential for cardiovascular organ protection.

62. Aruoma OL.
Scavenging of hypochlorous acid by carvedilol and ebselen in vitro.

Protective effect of the bispiperazinedione ICRF-187 against doxorubicin-induced cardiac toxicity in women with advanced breast cancer.
Are free radicals a major factor in atheroma?

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The most persuasive prima facie case for involvement of a particular factor in atherogenesis arises when epidemiological and experimental data agree. This is true for only a few of the many risk factors that have been proposed. Undoubtedly, the best known is high plasma levels of low-density lipoprotein (LDL) cholesterol. However, plasma cholesterol levels nearer the average are not reliable predictors of an individual's risk; other factors must be involved. There is increasing evidence, both experimental and human, that oxidative processes may be the missing link.

THE LIPID OXIDATION HYPOTHESIS

The hypothesis states that low-density lipoprotein (LDL) and its constituent lipids, such as cholesterol, cause focal thickening of the artery wall only when they have been oxidized. It dates in its present form from the early 1980s, but many observers had raised the possibility earlier. Oxidized lipids had repeatedly been found on chemical analysis of human lesions.1 One series of experiments even suggested that experimental atherosclerosis in rabbits was due not to the cholesterol added to the diet, but to the toxic oxidation products of stored cholesterol.2 The presence in human lesions of the insoluble pigment ceroid, a product of lipid/protein oxidation, suggested that oxidative activity also occurred in the lesion itself.3 The localization of ceroid in the characteristic foam cells of atherosclerosis suggested that the foam cells might be macrophages whose microbicidal free radicals oxidized lipids, leading to ceroid formation and eventually foam cell death with creation of the lipid core. It also suggested that antioxidants such as vitamin E might hinder the process.3

A crucial finding was the discovery by Goldstein and Brown4 that macrophages, unlike most cells, take up LDL by scavenger receptors only after it has been chemically altered, or modified, in one of several ways. These scavenger receptors are not downregulated by the cell's increasing cholesterol content. Therefore, macrophages can become bloated with lipid, to resemble foam cells. This discovery was quickly followed by the findings that endothelial cells were able to carry out such LDL modification in vitro and, soon after, that the type of modification, by these and other cell types, was in fact oxidation.5 Human foam cells were soon identified as macrophages by immunocytochemistry.6

Experiments since then have revealed a wide variety of biological effects of oxidized LDL (oxLDL) and its components in vitro that could be relevant to atherogenesis in vivo.7 Among these effects are that oxLDL is chemotactic for monocytes and possibly hinders further macrophage migration, and that oxLDL is avidly taken up by macrophages, which oxidize it further. The oxidation products are toxic for endothelial and smooth muscle cells. They are also toxic for the macrophages themselves, which may be at least partly responsible for the development and enlargement of the lipid core (the "necrotic base" or "atheroma" of the advanced plaque).

The precise sites and mechanisms of the proposed LDL oxidation in vivo are still uncertain, but the oxidation is probably not only free radical–mediated but also enzymatic. There is now evidence that macrophage foam cells in the lesion are an important site of oxidation and that the process is accompanied by increased release of macrophage cytokines that might be responsible for the oxidation.
for smooth muscle cell migration and secretion, and for increased endothelial cell activity. The macrophages may also release proteases that degrade and weaken the fibrous cap. More detailed accounts of these and other related findings can be found in recent reviews.7,8

BASIC CHEMISTRY OF LDL OXIDATION

The lipid composition of LDL is of course variable and partly dependent on the diet. In the context of LDL oxidation, there are two relevant dietary variables. One is the content of lipid-soluble antioxidants, such as α-tocopherol, that are carried in the lipoproteins. The other is the balance of dietary fatty acids, the importance of which is well documented, but generally underestimated.

Oxidation of LDL in vitro, caused, for instance, by cells or by transition metals, is always accompanied by the appearance of cholesterol oxidation products and the depletion of unsaturated fatty acids with two or more double bonds, notably linoleic and arachidonic acids. Oleate, with only one double bond, is unaffected.9 Correspondingly, pure cholesterol esters of polysaturated fatty acids, such as cholesteryl linoleate and arachidonate, are rapidly oxidized in vitro with evidence of cholesterol oxidation, whereas cholesterol alone and cholesteryl oleate are not oxidized.10 The findings suggest that cholesterol oxidation is triggered by oxidation of the polyunsaturated fatty acid (Table I). Similar events might occur in vivo. Firstly, human lesions contain cholesterol oxidation products, and macrophage-rich lesions show depletion of linoleate and arachidonate, but not oleate.11 Secondly, the ceroid pigment found in lesion foam cells also accumulates in macrophages in vitro exposed to cholesteryl linoleate and arachidonate, but not oleate.12 (Table I). Thirdly, decreased susceptibility of LDL to oxidation can be conferred by artificial enrichment with oleate, and is also seen in LDL from humans whose diet has been supplemented with oleate.13 Oxidation of both LDL and its oxidizable components is inhibited in vitro by antioxidants, including α-tocopherol.9,10 Antioxidants appear to be more effective in preventing lipid and LDL oxidation than in mitigating the effects of that oxidation once it has occurred.

The findings suggest that most dietary polysaturated fatty acids enhance the oxidizability of LDL, but increased dietary oleate and antioxidants both have an opposite effect. The lipid-soluble antioxidants are most effective because they are carried in the lipoprotein particle, but in vivo water-soluble antioxidants such as vitamin C may also contribute.

The lipid oxidation appears to trigger changes in apo B, the protein moiety of LDL, leading to recognition of the altered apo B of oxLDL by macrophage scavenger receptors. Antibodies raised against various forms of oxLDL react with human lesions, often in a pattern similar to the distribution of ceroid.14 Many patients with advanced atherosclerosis have plasma antibodies that cross-react with both ceroid and oxidized LDL.15

TOXICITY OF OXIDIZED LDL

Chisolm and colleagues showed not only that cell-mediated LDL modification in vitro was due to oxidation,5 but also that the oxLDL produced was toxic to various cell types in vitro, including smooth muscle cells and endothelial cells.16 The possible contributions of these effects to atherogenesis are of great interest, but still not completely explained.

<table>
<thead>
<tr>
<th>FATTY ACID DEPLETION</th>
<th>CHOLESTEROL OXIDATION</th>
<th>CEROID FORMATION</th>
<th>TOXICITY</th>
<th>INHIBITION OF EFFECTS BY ANTIOXIDANTS</th>
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<tr>
<td>Cholesterol (alone)</td>
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<td>–</td>
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<tr>
<td>Cholesteryl oleate (18:1)</td>
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<td>–</td>
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<tr>
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Table 1. Results of incubation of cholesterol and some of its esters with human macrophages in culture. Number of carbon atoms: double bonds shown for each fatty acid. Lipids taken up by cells in the form of emulsions with bovine serum albumin. See text for references. In different systems, cholesterol alone can be toxic.
However, one cell type in the lesion that dies in considerable numbers, and which is at least partly responsible for the development and progression of the lipid core, is the macrophage foam cell. Toxicity of oxLDL for macrophages has been demonstrated in vitro, and occurs at least partly by the controlled form of cell death known as apoptosis. Again, both cholesterol esters and triglycerides containing polyunsaturated fatty acids are toxic, although oleates are not (Table I). An exception to this general rule may be linolenic acid, an observation that awaits explanation. Importantly, human macrophages in vitro can themselves oxidize native LDL sufficiently to cause self-inflicted toxicity.

A number of studies have shown that apoptosis occurs in human atherosclerosis, including among the deeper macrophage foam cells near the lipid core. The cause of apoptosis in vivo is, however, unknown. If oxLDL were responsible for the lipid core, it would suggest that it is only in industrialized populations that this component of oxidative injury is manifest. This is because nonindustrialized human populations develop fatty streaks, but not advanced lesions; hence their relative immunity from the complications (Figure 1).

**ANTIOXIDANTS AND Atherosclerosis in vivo**

The in vitro evidence to support the oxidation hypothesis has accumulated inexorably. Experimental findings to refute it are conspicuous by their absence. However, all of the in vitro studies use highly artificial systems over a very short time scale. Moreover, the experimental variables such as cell culture conditions require adjustments to produce results, many of which might be unrealistic, such as adding pro-oxidant transition metals. The laboratory findings have, however, at least provided the stimulus for studies in vivo.

**Experimental atherosclerosis**

The most dramatic demonstration of the role of oxidation in experimental atherosclerosis was perhaps the atherogenic effect of cholesterol oxidation products in the diet, already mentioned. Since then, experiments have centered on the effects of inclusion of antioxidants in the diet of hyperlipidemic animals. Inhibition of diet-induced atherogenesis in animals has been reported for several antioxidants, including probucol and vitamin E, although not all experiments have shown such positive results.

The antioxidant drug probucol dramatically alleviates the spontaneous atherosclerosis seen in Watanabe (heritable hyperlipidemic) rabbits. Although the drug appears to inhibit the development of the lipid core. This latter observation is important because it supports the idea that oxidation might cause the foam cell death seen in human lesions (Figure 1).

The impact of these studies in Watanabe rabbits has been insufficiently recognized. It lies in the fact that, as far as we know, the only abnormality leading to atherosclerosis in these animals is the very high plasma LDL cholesterol level. The rabbits are receiving a normal rabbit diet, not by any means high in fat, nor deficient in naturally occurring antioxidants. However, even these extremely high plasma LDL and cholesterol levels are much less effective in producing atherosclerosis in these animals if they receive antioxidant supplements. The implication for human atherosclerosis is that normal antioxidant levels might be insufficient to protect abnormally large amounts of oxidizable lipid against oxidation. Thus, humans would develop more severe atherosclerosis if the dietary intake of oxidizable lipids were greater than the capacity of their normal antioxidant levels. The analo-
gies are even more direct, of course, with human familial hypercholesterolemia.

One defect in the data from animal experimentation is that the effects of antioxidants on oxidizability of plasma LDL have not always tallied with their observed effects on arterial lesions. The findings are in general supportive, but not totally conclusive.

**Epidemiological studies**

This is not the place to conduct another detailed review of all the epidemiological data concerning the protective effect of antioxidants against the clinical complications of atherosclerosis. In summary, the data suggest at best a weak protective effect for vitamin C and β-carotene; only α-tocopherol, the most active component of vitamin E, has consistently shown an inverse relationship between intake and incidence of ischemic heart disease. Most of the studies have suggested that only the high plasma levels achieved by active supplementation are effective and that dietary manipulation alone is of dubious value. However, at least one report suggested that high dietary intake was protective.

These studies do not prove that the effect of α-tocopherol is entirely attributable to its antioxidant activity. However, taken in conjunction with the experimental data, this seems the most likely mode of action. Epidemiology doesn’t explain either what events during atherogenesis are being affected. Studies of human lesions removed from patients receiving α-tocopherol supplementation will be necessary to investigate this question.

**Intervention studies**

Any intervention study, to be practical, must assess the effects of antioxidants in subjects who already have atherosclerosis, since the disease develops in adolescence and young adulthood. However, it is sufficient to test the ability of the intervention to slow the subsequent development of the disease, especially to diminish the incidence of clinical events. This rationale assumes that oxidative processes are still actively involved in the later stages of plaque development that lead to complications, especially plaque rupture and thrombosis at the macrophage-rich shoulders of the advanced plaque (Figure 1).

The very few human trial reports up to now have failed to show any convincing benefit from β-carotene, the only trial of probucol had negative results, possibly because of adverse effects on high-density lipoprotein (HDL) levels. A large Finnish trial, using only 50 mg vitamin E, produced no convincing effect.

The only trial reported so far that shows a positive effect is the Cambridge Heart AntiOxidant Study (CHAOS). This was a secondary prevention trial in patients with angiographic evidence of coronary stenosis and clinical complications, such as angina. α-Tocopherol supplements in high doses (400 or 800 IU daily) produced a dramatic (75%) decrease in incidence of myocardial infarction in the α-tocopherol group. There was apparently no effect on deaths, in the period studied.

**CURRENT PERCEPTIONS OF THE OXIDATION HYPOTHESIS**

The only disease that has been investigated sufficiently to provide more than a tentative causative link with radical-mediated injury is atherosclerosis; the findings now merit serious consideration. The only relevant official advice is to increase the daily intake of fruit and vegetables. Sad, it appears that, at least in the short term, this may have little effect on plasma levels of lipid-soluble antioxidants.

Up to now, the findings suggest that surprisingly high doses of lipid-soluble antioxidants are necessary to bring about the slowing of atherosclerosis. Thus, 400 IU of α-tocopherol daily are required to decrease the oxidizability of LDL. The only clinical trial (CHAOS) with a dramatic beneficial effect used 400 or 800 IU of α-tocopherol. But both these studies were relatively short term. Lower doses given to symptomless subjects over a longer period might be equally effective.

If the oxidation hypothesis were true, it would require that for some reason the daily requirements for lipid-soluble antioxidants in many developed countries are significantly higher than normal. What could be the reasons for this? Exposure to industrial emissions, combustion of fossil fuels, and active and passive smoking would no doubt contribute, by greatly increasing respiratory exposure to free radicals.

Perhaps one more clue lies in the relative immunity enjoyed by the fortunate inhabitants of the Mediterranean rim, with their diet rich not only in antioxidants but also in oleate, provided by the ready availability of olives. The diet of the more Northern European countries contains not only less α-tocopherol, but nowadays also considerably increased intake of polyunsaturated fatty acids, especially in cooking oils. Indeed, this increase has been powerfully encouraged by the advice to replace “saturated” with “unsaturated” fats in the diet. This stemmed, of course, from the attempts to achieve a healthier plasma lipid profile, with lower LDL/HDL ratio and lower cholesterol levels. It has presumably had the additional effect of making LDL more oxidizable,
and perhaps even charged with the lipid oxidation products of the cooking process. A meta-analysis has shown that replacing dietary saturates with monounsaturates such as oleate has a similar beneficial effect on plasma lipids to replacement with polyunsaturates. Perhaps this would be a better solution.

A reasonable criticism of the antioxidant hypothesis of atherosclerosis would be that the oxidative processes do occur in the lesion, but are secondary to other processes and not themselves involved in disease progression. Oxygen radical production and secondary tissue damage almost certainly occur in other inflammatory diseases. However, if it were a secondary, unimportant event, it would not explain the epidemiological association of low antioxidant status with incidence of complications, still less the beneficial effect of vitamin E in preventing myocardial infarction.

CONCLUSIONS

The classic pieces of evidence necessary to establish that a particular infective disease is caused by a particular micro-organism were enunciated by Robert Koch. Koch’s postulates are:

1. The organism must be isolated from all cases of the disease, from the affected tissues.
2. The organism must be isolated in pure form in the laboratory, through several subcultures.
3. The disease must be reproducible by introducing the same organism into a susceptible experimental animal.

These tests have arguably been fulfilled, in a manner of speaking, for the oxidation hypothesis of atherosclerosis. If lipid oxidation products are the suspected cause of the disease, they are found in the lesions of all patients, are easily purified, and can cause a very similar disease by being introduced into the diet of rabbits. This last point is undoubtedly the weakest, it has only been shown by one group and probably does not reflect exactly how the disease happens in man, if the oxidation usually occurs in the artery rather than being of dietary origin.

The lipid oxidation hypothesis states that LDL cholesterol causes atherosclerosis only when it is oxidized. The strongest support so far comes from two findings. Firstly, that even the dramatic hypercholesterolemia seen in Watanabe rabbits cannot cause extensive advanced disease in the presence of antioxidant supplements; and secondly that, even in patients with coronary atherosclerosis that is already advanced, high-dose α-tocopherol supplements can improve the clinical outlook dramatically.

The answer to the question posed in the title, in the face of all this evidence, is nevertheless still only a somewhat tentative yes. The final answer should be provided by clinical trials now in progress.

REFERENCES

Toxicity of polyunsaturated fatty acid esters: the anomalous behaviour of cholesteryl linoleate.

Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development.
*Biochim Biophys Acta.* 1995;1256:141-150.

12. Ball KY, Carpenter K LH, Enright JH, Hartley SL, Mitchinson MJ.
Ceroid accumulation by murine peritoneal macrophages exposed to artificial lipoproteins.

Feasibility of using an olate-rich diet to reduce the susceptibility of low-density lipoprotein to oxidative modification in humans.

Gene expression in macrophage-rich human atherosclerotic lesions. 13-Lipoxygenase and acetyl low-density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts.

15. Parbums DV, Brown DL, Mitchinson MJ.
Serum antibodies to oxidised LDL and ceroid in chronic periorbitis.

16. Cathcart MK, Morel DW, Chisolm G.
Monocytes and neutrophils oxidise low-density lipoprotein making it cytotoxic.

17. Ball KY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ.
Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma.
*Atherosclerosis.* 1995;114:45-54.

Apoptosis in human monocyte-macrophages exposed to oxidised low-density lipoprotein.

Oxidation of low-density lipoprotein by human monocyte-macrophages results in toxicity to the oxidising culture.

20. Hegyi L, Skepper JN, Cary NR, Mitchinson MJ.
Foam cell apoptosis and the development of the lipid core of human atherosclerosis.

Distribution of coronary and aortic atherosclerosis by geographic location, race and sex.

22. Daugherty A, Zweifel BS, Schonfeld G.
Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits.

23. Verlangieri A, Bush MJ.
Effects of d-alpha-tocopherol supplementation on experimentally induced primate atherosclerosis.

24. Carew TE, Schwenke DC, Steinberg D.
Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit LDL degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the WHHL rabbit.
*Proc Natl Acad Sci USA.* 1987;84:7725-7729.

25. Braesen JH, Beisiegel U, Niendorf A.
Probucol inhibits not only the progression of atherosclerotic disease, but causes a different composition of atherosclerotic lesions in WHHL rabbits.

Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women.

The effect of probucol on femoral atherosclerosis: the Probucol Quantitative Regression Swedish Trial (PQKST).


A randomised controlled trial of vitamin E in patients with coronary disease: the Cambridge Heart Antioxidant Study (CHAOS).

30. Zino S, Skeaff M, Williams S, Mann J.
Randomised controlled trial of effect of fruit and vegetable consumption on plasma concentrations of lipids and antioxidants.

31. Jialal I, Fuller C, Huet BA.
The effect of alpha-tocopherol supplementation on LDL oxidation.

32. Gardner CD, Kraemer HC.
Monounsaturated versus polyunsaturated dietary fat and serum lipids. A meta-analysis.

33. Staprans I, Pan XM, Rapp JH, Feingold KR.
Oxidized cholesterol in the diet accelerates the development of aortic atherosclerosis in cholesterol-fed rabbits.
What are the prospects of antioxidants as a new therapeutic modality?

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Antioxidants have entered the era of therapeutic application. Potent antioxidant drugs such as probucol, carvedilol, and trimetazidine have been added to the cardiovascular armamentarium. Human epidemiological data suggest that the incidence of cardiovascular diseases is lowered by dietary supplementation with antioxidants such as vitamins C or E, particularly in combination with lipid-lowering drugs. However, epidemiological studies are not sufficient to establish causal relationships. Available results from several large-scale randomized trials with antioxidants do not yet allow the risk-to-benefit ratios for antioxidant supplements to be fully assessed. Nevertheless, the risk of many common and highly disabling human diseases may be reduced by increasing the intake of natural flavonoids and carotenoids, of minerals such as selenium, and of nutrients such as coenzyme Q10.

Keywords: vitamin E, atherosclerosis; heart failure; ischemia; free radical; antioxidant.

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OXIDATIVE STRESS AND ANTIOXIDANTS

In order to survive in the “unfriendly” environment created by oxygen radicals, living organisms generate a variety of water- and lipid-soluble antioxidants. A biological antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays the oxidation of that “substrate.” Antioxidants may work by preventing the generation of oxidizing species, by scavenging free radicals, or by reducing a free radical—thereby inactivating it without the antioxidant itself becoming a highly reactive free radical in the process. This process of inactivation of free radicals is known as “chain breaking” and is particularly important in lipid structures that contain easily oxidizable, unsaturated fatty acids and through which chains of peroxidation may develop after each free radical “hit.” A series of endogenously synthesized antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, intercept and inactivate these reactive oxygen species.

The battle against oxidative stress is part of aerobic life. Problems arise when the amount of oxidative stress overwhelms the capacity of antioxidants. This may be due to excessive free radical generation by a toxic agent or pathological process, or to a lack of antioxidant power, ie, lack of defense. The increased oxidative stress has been linked to a wide variety of degenerative processes and diseases, such as cancer, atherosclerosis, stroke, ischemia/reperfusion injury, heart failure, chronic inflammatory diseases (rheumatoid arthritis, lupus erythematosus), inflammatory processes (eg, wound healing), photo-oxidative stresses to the eye (cataracts), familial amyotrophic lateral sclerosis, glutathione-linked adolescent seizures, Parkinson’s disease, Alzheimer’s dementia and a wide variety of age-related disorders, and the aging process. It is difficult to demonstrate cause and effect relationships between these diseases and antioxidant status because oxidant damage is subtle and difficult to measure, and the associated diseases develop over years.

OXIDATIVE STRESS AND CARDIOVASCULAR DISEASE (CVD)

Prolonged ischemia such as that following myocardial infarction or occurring during long-term coronary bypass procedures causes serious damage to the myocardium. Early reperfusion is an absolute prerequisite for the survival of ischemic tissue. However, reperfusing ischemic myocardium carries with it a component of injury known as “reperfusion injury.” There is currently abundant data indicating that free radicals and their metabolites derived from
molecular oxygen contribute to myocardial dysfunction during the syndrome of ischemia and reperfusion1 (Figure 1). Congestive heart failure (CHF) after myocardial infarction (MI) is a common clinical syndrome with poor prognosis. In the guinea-pig model of CHF following MI, improved myocardial redox state with vitamin E therapy, coupled with the modulation of the development of heart failure, was observed, suggesting a pathophysiological role for oxidative stress. It has been shown that patients with ischemic heart disease have higher plasma concentrations of lipid peroxides. Increase in exhaled pentane in patients with chronic heart failure as well as coronary artery disease (CAD) suggests increased lipid peroxidation. Breath pentane was significantly lowered in chronic heart failure patients maintained on free radical scavengers. Inhibition of peroxidation by vitamin E might favorably influence the balance between peroxidative damage and the repair mechanisms of the body.

Oxidation of low-density lipoprotein (LDL) plays an important role in atherosclerosis. Atherogenesis is the accumulation of lipid (mainly cholesterol), smooth muscle cells, foam cells, collagen, calcium, and necrotic debris in the intima of the arterial wall. Atherosclerosis develops as a consequence of oxidized LDL entering the subendothelial space of the arterial wall and becoming trapped. Oxidized LDL is chemotactic for macrophages and smooth muscle cells, encouraging cell migration to this budding sclerotic site, causing proliferation of smooth muscle (Figure 2). Oxidized LDL inhibits macrophage migration away from the site and is cytotoxic. In addition, in situ macrophage action results in further oxidation of LDL, as activated...
Macrophages produce free radicals during their oxidative burst. The earliest manifestation of atherogenesis is the fatty streak, which may regress or stabilize or progress to complicated fibrous or lipid-rich atheromatous plaques (atheroma).

**ANTIOXIDANTS IN CORONARY ARTERY DISEASE**

The heart may be the most susceptible of all organs to premature aging and free radical oxidative stress. This may be the result of acute ischemia/reperfusion injury, endothelial damage of hyperhomocysteinemia, as well as chronic oxidative damage secondary to lipid peroxidation. Although highly responsive, and therefore vulnerable to the effects of oxidative stress, the heart is also receptive to the benefits of targeted phytonutrients, antioxidants, and nutritional supplements. The biological membranes of myocytes, besides being potential sites of lipid peroxidation, also provide hydrophobic binding sites for drugs and natural antioxidants (vitamin E and ß-carotene).

Antioxidants, either natural or synthetic, can interrupt peroxidation by a number of different mechanisms and prevent the production of oxygen free radicals. Membrane-associated α-tocopherol (vitamin E) is the major lipid-soluble antioxidant present in human blood and cardiac muscle. It is the antioxidant that has been known longest in biological systems, and is present in relatively high concentrations in both the cell and mitochondrial membranes. As an antioxidant, it inhibits lipid peroxidation as a chain-breaking agent by intercepting phospholipid polyunsaturated fatty acid radical intermediates. The membrane α-tocopherol tone is a critical protective mechanism of myocardial phospholipid against oxidative injury, and acts as a determinant of the course of heart-membrane peroxidative damage.

Antioxidants can slow the progression of experimental atherosclerosis in several animal species. They also have an inhibitory effect on smooth muscle cell growth both in vitro and in vivo. These findings suggest a possible effect of antioxidants on restenosis after percutaneous transluminal coronary angioplasty (PTCA). Nine studies have investigated the effects of probucol, ß-hydroxytoluene, and vitamin E antioxidants on atherosclerosis in rabbits and monkeys. Seven studies, most of which used the antioxidant drug probucol, yielded positive results. Clinical trials in patients receiving multivitamins, vitamin E, or probucol, and undergoing PTCA, suggested a trend towards reduction in restenosis. The preventive effect appeared to be independent of the lipid-lowering effects.

The α-Tocopherol, ß-Carotene Cancer Prevention Study was designed to test for protection against lung cancer, but data on heart disease were also recorded. No evidence of benefit was found with either vitamin E or ß-carotene supplementation. However, the subjects were heavy long-term smokers and the dose of vitamin E used in the study was probably low. In another recent randomized trial, it was reported that the proportion of major coronary events in men with a previous MI who smoked was not decreased with either α-tocopherol or ß-carotene supplements. In fact, the risk of fatal CAD increased in the groups that received either ß-carotene or the combination of α-tocopherol and ß-carotene. Two separate studies provide evidence for an association between a high intake of vitamin E and a reduced risk of CAD in both men and women.

Ascorbic acid (vitamin C) provides in vivo antioxidant protection primarily by acting as an aqueous phase peroxyl and oxygen radical scavenger. It is concentrated in tissues and fluids with a high potential for radical generation, such as the eye, brain, liver, lung, heart, semen, and leukocytes. The oxidized or “spent” form of the vitamin (dehydroascorbic acid) is readily converted back to the reduced form by reduced glutathione, reduced nicotinamide adenine dinucleotide phosphate (NADPH), or both. Evidence suggests that antioxidant protection from vitamin C may reduce the risk or slow development of certain diseases, including CAD, cancer, and age-related eye and neurodegenerative diseases. Epidemiological studies show that higher ascorbate intakes are associated with reduced risk of developing cancer—particularly cancer of the stomach, esophagus, or oral cavity. Furthermore, a strong correlation between fruit and vegetable intake and reduced risk of disease have been noted by epidemiological studies. However, randomized controlled intervention trials in human subjects are needed to provide convincing evidence that increased intakes of vitamin C or other micronutrients would by themselves or in combination reduce the incidence of disease.

Carotenes are pigments found in yellow and green vegetables such as carrots, spinach, and sweet potatoes. Some carotenoids, in particular ß-carotene, are precursors of vitamin A. Many of the carotenoids, including ß-carotene, act as chain-
Breaking antioxidants or quenchers of singlet oxygen (the excited form of molecular oxygen), and may function as part of the antioxidant defense system. Epidemiological studies suggest that people who eat diets rich in carotene-containing foods have lower rates of heart disease and cancer than the general population. However, these studies failed to separate the effects of carotenes from the effects of other substances such as fiber, vitamin C, folic acid, and other phytochemicals. Although these studies provide interesting correlations between high carotene consumption and improved health status, they do not provide firm evidence that carotene consumption is beneficial.

Flavonoids have a wide variety of polyphenolic structures, are about the molecular weight of cholesterol, and are ubiquitously distributed in plant foods. As such, they are small lipid-soluble compounds like α-tocopherol and β-carotene. Flavonols such as quercetin and kaempferol are predominantly found in onions, kale, broccoli, apples, cherries, berries, tea, and red wine. They have a staggering list of actions, including anti-inflammatory, antiallergic, antiinhibitors, venotonic, antimicrobial, and antineoplastic activities. Some are antithyroidal, and certain flavonoids can be mutagenic. On a molecular level, flavonoids can modify the actions of a host of enzymes, including those involved in cell respiration and replication, drug metabolism, and immune function. Several flavonoid compounds have been shown to have antioxidant properties in vitro, inhibiting the oxidation of LDLs and reducing thrombotic tendencies by inhibiting platelet aggregation. Because oxidation of LDLs is thought to be a necessary precursor of atherosclerosis, flavonoids that reduce this oxidation process may reduce the risk for coronary heart disease. Epidemiological reports suggest a role for the flavonoids and quercetin in the prevention of CAD. A small cohort study in elderly men demonstrated a significant inverse association between intake of those flavonoids most commonly consumed in the Netherlands and coronary mortality. An ecological study based on middle-aged men from 16 different cohorts showed a similar inverse association between flavonoid intake and coronary mortality. The case for a specific cardioprotective role of quercetin comes from the data showing that apples and onions—important sources of quercetin in the Finnish diet—were foods most strongly related to mortality risk. A recent study using a 26-year follow-up period suggested that low flavonoid intakes result in higher risks of CAD.

The risk of many diseases, including CAD, is related to fat in the diet. Fat is energy dense, but low in nutrients found in plant materials and phytochemicals. A high-fat diet is unhealthy in part because it lacks plant material. This probably explains the so-called “French Paradox.” The French consume a diet that is both high in fat and high in plant flavonoids (wine). As a result, the antioxidant components of wine might attenuate the oxidative stress provided from their high-fat diet. Despite these positive epidemiological findings, it should be noted that some flavonoids have biochemical effects that could translate into toxicities in humans, particularly if taken in large doses.

Trimetazidine (TMZ) has been described as a new anti-ischemic agent thought to have a direct effect on the ischemic myocardium by a metabolic mechanism. A number of experimental studies have demonstrated that TMZ exerts direct anti-ischemic effects by limiting intracellular calcium accumulation and acidosis, reducing infiltration of neutrophils in the ischemic myocardium, and scavenging oxygen-derived free radicals during reperfusion. This agent appears clinically promising in the treatment of reperfusion injury.

The coenzyme Q10 (or ubiquinone) is a natural antioxidant that plays a key role in oxidative phosphorylation. It has been found that myocardial tissue levels of coenzyme Q10 were lower in patients with more advanced heart failure compared with milder stages of the disease. Furthermore, coenzyme Q10 was found to have a positive effect on morbidity and on the quality of life in heart failure patients.

There are large numbers of other antioxidant compounds that have proven to be beneficial in animal studies and could find potential clinical use. Carvedilol is a novel, multiple-action cardiovascular drug that is currently approved in many countries for the treatment of hypertension and congestive heart failure, for which increased survival has clearly been demonstrated. The reduction in blood pressure produced by carvedilol results from β-receptor blockade and vasodilation. Carvedilol and several of its metabolites are potent antioxidants, and this activity may account in part for the cardioprotective effects observed in animal models of acute myocardial ischemia. In theory, it could also serve to protect the myocardium of patients with hypertension, CAD, and congestive heart failure, in which oxidative stress is now recognized to occur. The antioxidant effects of carvedilol may both inhibit the direct cytotoxic actions of oxygen radicals and prevent oxygen-radical–induced activation of transcription factors and genes.
Prospects of antioxidants as a new therapeutic modality - Hess & Kukreja

associated with the remodeling process. Carvedilol inhibits gene expression of the intercellular adhesion molecule-1, which is responsible for the infiltration of neutrophils during ischemia, leading to exacerbation of reperfusion injury. It also inhibits the oxidation of LDL in vitro, preventing the formation of this cytotoxic and atherogenic form of LDL.

A number of drugs containing sulfhydryl groups have antioxidant properties (reviewed in reference 23). Captopril is an SH-containing antioxidant and an angiotensin-converting enzyme inhibitor receiving attention for its anti-inflammatory action, and the attenuation of reperfusion-induced depression of myocardial function. Captopril inhibits cellular lipid peroxidation in cultured endothelial and smooth muscle cells when exposed to the O$_2^•^*$ and •OH generating systems. $N$(2-mercapto-propionyl)glycine (MPG) is a free radical scavenger that has been used clinically in Europe and Japan in the treatment of several disorders linked to abnormal free radical production. It can be administered orally and can theoretically scavenge both intracellular and extracellular oxygen free radicals because of its ability to cross cell membranes. It is a synthetic thiol compound that scavenges the O$_2^•^*$ and possibly the •OH radical. In addition, the drug is able to substitute as a sulfhydryl donor for glutathione, an important intracellular antioxidant that is significantly depleted by hypoxia and reperfusion. Glutathione functions as the hydrogen donor for glutathione peroxidase, a protective enzyme that eliminates H$_2$O$_2$ and inactivates lipid peroxide. MPG may, therefore, exert its protective effect both through the direct elimination of oxygen free radicals and through prevention of lipid peroxidation.

The protective effect of thiol-containing compounds may be attributed not only to their antioxidant properties, but also in part to the inhibition of the complement cascade. Complement-mediated injury of myocardial tissue has been documented in experimental models. Cellular components released from ischemic myocardial tissue have been shown to activate the complement system. Components of the systems, such as C3, C4, C5, and the membrane attack complex (MAC) have been identified in experimentally infarcted tissue as well as in human ischemic and infarcted myocardium. In decomplemented animals, reduced neutrophil infiltration into infarcted myocardium has been demonstrated after experimental ischemia/reperfusion. Reduced loss of myocardial creatine kinase and limitation of infarct size after coronary artery occlusion has been demonstrated in decomplemented animals. Both captopril and MPG have been shown to provide protection against complement-mediated increases in end-diastolic pressure and coronary artery perfusion pressure in a concentration-dependent manner. The mechanism of action of penicillamine is thought to inactivate the fourth component of complement, leading to inhibition of the complement cascade. The structure and adverse reactions of captopril are similar to those of penicillamine, suggesting that these two agents may act in similar fashion.

L-Propionyl carnitine has also been shown to provide protection against ischemia-reperfusion damage and also act as an antioxidant and atherogenic form of LDL.

The protective effect of thiol-containing compounds may be attributed not only to their antioxidant properties, but also in part to the inhibition of the complement cascade. Complement-mediated injury of myocardial tissue has been documented in experimental models. Cellular components released from ischemic myocardial tissue have been shown to activate the complement system. Components of the systems, such as C3, C4, C5, and the membrane attack complex (MAC) have been identified in experimentally infarcted tissue as well as in human ischemic and infarcted myocardium. In decomplemented animals, reduced neutrophil infiltration into infarcted myocardium has been demonstrated after experimental ischemia/reperfusion. Reduced loss of myocardial creatine kinase and limitation of infarct size after coronary artery occlusion has been demonstrated in decomplemented animals. Both captopril and MPG have been shown to provide protection against complement-mediated increases in end-diastolic pressure and coronary artery perfusion pressure in a concentration-dependent manner. The mechanism of action of penicillamine is thought to inactivate the fourth component of complement, leading to inhibition of the complement cascade. The structure and adverse reactions of captopril are similar to those of penicillamine, suggesting that these two agents may act in similar fashion.

L-Propionyl carnitine has also been shown to provide protection against ischemia-reperfusion damage and also act as an antioxidant agent, protecting myocardial cells from oxidative damage. Ferrari et al$^{24}$ have shown that L-propionyl carnitine was more effective than L-carnitine plus propionic acid in improving recovery of cardiac mechanical function after ischemia and reperfusion. The exact mechanism of protection by L-propionyl carnitine is not known, although three hypotheses have been proposed. First, L-propionyl carnitine can serve as energy source for heart muscle cells by being enzymatically converted to propionyl-CoA and subsequently utilized in the Krebs cycle. Another mechanism proposed by Ferrari et al$^{24}$ is the membrane-stabilizing effect of L-propionyl carnitine, but experimental results have not supported this hypothesis. On the other hand, they found that both L- and D-propionyl carnitine chelate iron, thus preventing the formation *OH radical by Fenton’s reaction. Although both isomers exhibited identical antiradical activity, the protective effect of L-propionyl carnitine was remarkably superior during ischemia/reperfusion injury.

EGb 761 is a stringently standardized extract from Ginkgo biloba leaves, which mainly contain flavonoid substances and terpenoids. EGb 761 is commonly used to treat peripheral arterial disease and organic brain syndromes. EGb has superoxide dismutase (SOD)-like activity and *OH radical–scavenging ability, and also has the property of inactivating oxoferryl radical species, which is a more efficient oxidative agent than the classic *OH radical. The properties of this antioxidant are thought to provide many beneficial effects against biological free radical injuries. EGb 761 also preserves the total ascorbate content and, primarily, maintains the reduced form of ascorbate in the myocardium following ischemia/reperfusion.

Deferoxamine is a naturally occurring iron chelator derived from Streptomyces pilosus. It is a low-molecular-weight protein and is currently used clinically for treatment of iron-overload states. Several features of deferoxamine
make it advantageous for studying the effects of iron chelation in the prevention of reperfusion injury. Several lines of evidence support the ability of deferexamine to enter cells, which would allow it to also chelate intracellular iron. Deferoxamine and other chelators have been reported to limit creatine kinase release, preserve myocardial phosphocreatine content, lower vascular resistance, and preserve myocardial structure and/or enhance recovery of left ventricular developed pressure. Deferoxamine may also exert a biphasic antioxidant/pro-oxidant effect, ie, it may paradoxically amplify oxidative damage in the presence of reducing agents (such as DMTU). Dimethylthiourea (DMTU) is a scavenger of •OH radical, \(H_2O_2\), and hypochlorous acid, the powerful oxidant generated from activated neutrophils. DMTU is also highly diffusible across lipid membranes, allowing it to act intracellularly as well. DMTU may require lower tissue levels for a protective effect in large part because it scavenges \(H_2O_2\). Other approaches to blocking formation of oxygen free radicals are via inhibiting the enzymes used in their production by the alternate pathways employed during ischemia/reperfusion, namely, by using the xanthine oxidase inhibitor, allopurinol, a structural analog of hypoxanthine. This compound competitively inhibits xanthine oxidase-catalyzed urate production with its subsequent formation of oxypurinol. Oxypurinol itself is a noncompetitive inhibitor, which forms a stable complex with xanthine oxidase, thus preventing further enzymatic activity. While both agents inhibit urate production, in the presence of molecular oxygen, the oxidation of allopurinol to oxypurinol generates \(O_2^•\) radicals during its association with the enzyme. Oxypurinol may therefore be seen as the “active” form of allopurinol. It remains unclear, however, whether exogenously administered oxypurinol offers any true therapeutic advantage over allopurinol. Although allopurinol may also be a •OH radical scavenger at high concentrations in cell-free systems in vitro, it has been found that this highly inefficient scavenging capability almost certainly does not explain reports of classic experiments in biological systems. Uric acid has also been shown to interact directly with reactive oxygen species such as •OH radicals. Urate also protects human plasma ascorbate from oxidation. Thus, it appears that this metabolic waste product has antioxidant properties.

**CONCLUSIONS**

Antioxidants have now entered the era of therapeutic application. Drugs such as probucol, carvedilol, and TMZ, with potent antioxidant properties, have been added to our cardiovascular armamentarium. There is a large body of human epidemiological evidence suggesting that incidence of these diseases is lowered in populations having a high level of antioxidants, such as vitamin E, ascorbate, \(\beta\)-carotene, or flavonoids in their diet, or who are taking dietary supplements of these antioxidants. Dietary supplements of vitamin E significantly lower the risk of disease, in particular CVD. Clinical use of this concept has been successful with vitamin E in combination with lipid-lowering drugs and improvement in coronary atherosclerosis. This concept of adjunct therapy with antioxidants for better results is gaining recognition, so that antioxidants should become a key player in future treatments. Vitamin C intake is also inversely related to CVD and total mortality in men. However, causal relations can never be established by epidemiological studies. Results from several large-scale randomized trials of antioxidant supplements are now available, but inconsistent, since they do not prove or disprove the value of antioxidant vitamins, nor incriminate them as harmful. They do, however, raise the possibility that some of the benefits from observational epidemiology may have been overestimated, particularly in view of the greater health consciousness (as reflected by decreased smoking and increased exercise) of subjects increasing vitamin or dietary supplement consumption. At this point, randomized trial data are not yet sufficient to fully assess the risk-to-benefit ratios for antioxidant supplements. Nevertheless, data suggesting the benefits of antioxidants outweigh the information questioning their beneficial role in the prevention of diseases. By enhancing the intake of phytoneutrients, such as natural flavonoids and carotenoids found in fresh fruits, fortification of foods by antioxidants, or dietary supplements, it may be possible to reduce the risk of a large number of common, highly disabling human diseases. In addition, minerals like selenium and nutrients such as coenzyme Q10 will minimize free radical risk and optimize a favorable outcome from the ubiquitous presence of oxidative stress in the cardiovascular system. Measures to minimize accumulation of heavy metals (eg, iron and copper), which are able to initiate adverse free radical reactions, will also help to mitigate oxidative stress. It may thus be possible to prevent the risk for CVD and improve the quality of life, particularly in elderly patients.
References

1. Kukreja RC, Janin Y.
Reperfusion injury: basic concepts and protection strategies.

Antioxidants and atherosclerotic heart disease.

3. Steinberg D.
Antioxidants and atherosclerosis: a current assessment.


Effectiveness of an antioxidant in preventing restenosis after percutaneous transluminal coronary angioplasty: the Probucol Angioplasty Restenosis Trial.

Vitamin E supplementation, plasma lipids and incidence of restenosis after percutaneous transluminal coronary angioplasty (PTCA).

7. Alpha-Tocopherol, Beta-Carotene, Cancer Prevention Group.

Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction.

Vitamin E consumption and the risk of coronary disease in men.

10. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC.
Vitamin E consumption and the risk of coronary disease in women.

11. Singh RB, Niaz MA, Rastogi SS, Rastogi S.
Usefulness of antioxidant vitamins in suspected acute myocardial infarction (The Indian Experiment of Infarct Survival–3).

12. Enstrom JE, Kanim LE, Klein MA.
Vitamin C consumption and mortality among a sample of the United States population.

13. Block G.
Vitamin C status and cancer: epidemiological evidence of reduced risk.

14. Gaziano JM, Hennekens CH.
The role of beta-carotene in prevention of cardiovascular disease.

15. Hertog MG, Feskens EJM, Hollman PCH, Kartam MB, Kromhout D.
Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study.

Flavonoid intake and long-term risk of coronary heart disease and cancer in Seven Countries Study.

Flavonoid intake and coronary mortality in Finland: a cohort study.

18. Detry JM.
Clinical features of an anti-anginal drug in angina pectoris.
Eur Heart J. 1993;14(suppl G):2-5.

19. Mortensen SA.
Perspectives on therapy of cardiovascular diseases with coenzyme Q10 (ubiquinone).

20. Kukreja RC, Hess ML.
Free Radicals, Cardiovascular Dysfunction and Protection Strategies.


22. Ruffolo RR Jr, Feuerstein GJ.
Cardiovasc Drugs Ther. 1997;11(suppl 1):247-256.

23. Kukreja RC, Nayeem MA, Qian Y, Hess ML.
Adaptation Biology Medicine.

24. Ferrari R, Ceconi C, Curello S, Pasini E, Visioli O.
Protective effect of propionyl-L-carnitine against ischaemia and reperfusion damage.

25. Anderson TJ, Meredith IT, Yeung AC, Frei B, Selwyn AP, Ganz P.
The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion.
Oxidative Stress

Summaries of Ten Seminal Papers

1. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release
   D.J. Hearse and others. J Mol Cell Cardiol. 1973

2. The “wavefront phenomenon” of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow

3. The effect of ibuprofen on accumulation of indium-111–labeled platelets and leukocytes in experimental myocardial infarction
   J.L. Romson and others. Circulation. 1982

4. Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity
   R. Ferrari and others. J Mol Cell Cardiol. 1985

5. Granulocytes as active participants in acute myocardial ischemia and infarction

6. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury

7. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial “stunning” is a manifestation of reperfusion injury
   R. Bolli and others. Circ Res. 1989

8. The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats

9. Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function
   P.J. Simpson and others. Circulation. 1990

10. Early accumulation of the terminal complement-complex in the ischaemic myocardium after reperfusion
    D. Mathey and others. Eur Heart J. 1994

Summaries prepared by Michael J. Shattock, PhD
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Events selected by Dr P.B. Garlick - Division of Radiological Sciences - Guy’s Hospital - London SE1 9RT - UK
Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release

D.J. Hearse, S.M. Humphrey, E.B. Chain

*J Mol Cell Cardiol.*, 1973;5:395-407

In the early 1970s, the attention of cardiologists was firmly focused on the deleterious effects of ischemia and oxygen deprivation. Many centers around the world were then investigating the mechanisms of ischemic injury. The concept that, in the absence of reperfusion, infarct size and ischemic damage could be limited by pharmacological intervention was slowly starting to evolve. This understandable fixation on the ischemic process and its manipulation distracted attention from reperfusion.

In 1972, however, Hearse et al reported a series of studies, which were to focus attention on the possible damaging effects of reoxygenation and reperfusion, and were the catalyst for many others investigating “reperfusion injury.”

Hearse et al investigated the effects of reoxygenation on hypoxic tissue injury and enzyme leakage, believing that “...enzyme release could be reduced or even halted by reoxygenation.” They were, however, clearly surprised by their results. In their introduction, they stated that “In the course of this study the unexpected observation was made that reoxygenation during glucose-free (hypoxic) perfusion exacerbated enzyme release.” Although they did not coin the term “oxygen paradox” until later, that sentence marks the birth of this concept—ie, reoxygenation, while undoubtedly essential for the long-term survival of the tissue, can paradoxically itself cause massive tissue injury.

To investigate reoxygenation, Hearse et al used the isolated perfused rat heart preparation. To simplify the profiles of enzyme leakage, they arrested the hearts during hypoxia and reoxygenation by raising extracellular potassium, and measured the time-course of leakage of a number of enzymes and total protein loss during a range of hypoxia/reoxygenation protocols. During hypoxia, they showed that enzymes typically leak from the heart in two phases. Firstly, there is a small “blip” of enzyme release after about 40 to 50 minutes, but this small release (just 2% to 5% of the total release) is a prelude to a much larger and more sustained release. In this second phase, enzymes are progressively lost from the hypoxic heart as the cell membranes become increasingly damaged. This phase of enzyme release peaks at around 3 to 4 hours and represents the loss of the major portion of soluble intracellular proteins as cells literally fall apart and injury becomes severe and irreversible.

These two distinct phases of hypoxic tissue injury are important as between phase 1 (≈50 min) and the peak of phase 2 (≈200 min) lies the transition from reversible to irreversible injury, during which reoxygenation has its most dramatic effects. Reoxygenation induced massive enzyme loss, which peaked after about 2 min reoxygenation at levels 100 to 200 times the immediately preceding hypoxic level. It was concluded that this massive release of myocardial enzymes reflected “…sudden and major ultrastructural damage.” Whichever myocardial enzyme was measured (or even total protein), the profile of release was largely similar, implying that the release process is not some selective change in membrane permeability, but rather a massive nonspecific disruption of cellular architecture, loss of membrane integrity, and cell lysis. A further important observation was made. The integrated total enzyme loss from hypoxic hearts was estimated and compared to the amount lost from reoxygenated hearts. Hearse et al found that, although reoxygenation accelerates enzyme release, the total amount of enzyme release was similar to that seen with sustained hypoxia.

Thus started the still unresolved controversy: does reoxygenation induce new cell death per se or does it simply accelerate the demise of cells already destined to die?

One of the most prescient conclusions of this study was that the readmission of molecular oxygen and the associated oxidant stress may induce these effects. The authors had questioned “some of the basic assumptions concerning the advantages of reoxygenation or reperfusion.” The much-debated concept of reoxygenation/reperfusion injury was thus born, with its subsequent implications for stunning, arrhythmias, contractile dysfunction, and necrosis.

The Sydney Opera House is opened in Australia; President Allende of Chile is assassinated by right-wing opponents; and Pablo Picasso, arguably the greatest artist of the 20th century, dies, aged 91
The “wavefront phenomenon” of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow

K.A. Reimer, R.B. Jennings
Lab Invest. 1979;40:633-644

By 1979, the idea that it might be possible to salvage the acutely ischemic myocardium by pharmacological intervention had received much attention. The general, but unsubstantiated view, was that cell death started somewhere towards the center of the subendocardium of an ischemic territory and then gradually expanded concentrically, moving both laterally and transmurally. The concept that there were lateral “border zones” of jeopardized tissue (in which blood flow may be intermediate between the severely ischemic center of an evolving infarct and the adjacent normal tissue) was the basis for the idea that this tissue could provide the target for “infarct-limiting” drugs. However, in a series of experiments in the dog heart, Reimer and Jennings carefully and unequivocally debunked this idea.

Mongrel dogs were anesthetized and the circumflex coronary artery was occluded for 40 min, 3 h, or 6 h, and the ischemic territory then reperfused for 4 days. In a fourth group of animals, coronary occlusion was maintained without reperfusion for 4 days. Hearts were then excised and sliced into rings of tissue. The occluded territory was delineated with dyes, the extent and geometry of the necrosis estimated histologically, and the residual flow in the ischemic territory measured using radioactive microspheres introduced into the heart earlier in the protocol.

After short periods of ischemia (40 min), necrosis was largely confined to a thin subendocardial zone, but, crucially, extended laterally to the limits of the occluded zone, i.e., even after this short period of ischemia, there was no evidence of a lateral border zone of salvageable tissue. As ischemia progressed, the area of necrosis expanded towards the endocardium, such that by 4 days, 79% of the occluded territory had infarcted, with the small surviving rim of tissue being confined to the epicardial surface. This transmural (rather than lateral) wavefront of necrosis was shown to be a primary determinant of the amount of tissue salvageable by reperfusion. At 40 min, the wavefront had not progressed very far and thus reperfusion limited the size of the infarct to about 64% of the total area at risk of infarction. By 3 hours, however, little or no tissue could be salvaged by reperfusion, with infarcts being largely similar in size to those seen after a permanent occlusion.

Reimer and Jennings then investigated the relationship between the transmural evolution of infarction and what is now accepted to be its principal determinant—collateral blood flow. They showed that, in the ischemic territory, there was always a gradient of collateral blood flow from subendocardium to subepicardium. Subendocardial flow in the ischemic territory was invariably very low (about 3% of normal), while that of the subepicardium could be as high as 40% (average 17%). This transmural gradient of flow was closely correlated with both the rate of development and the eventual extent of cell death. Since there is no lateral border zone, the size of an infarct at any moment in time is determined by the size of the occluded zone and the extent to which the wavefront of cell death has spread transmurally towards the epicardial surface. The rate of spread of this wavefront is essentially determined by the gradient of transmural collateral blood flow.

Although this study did not directly investigate reperfusion injury, the understanding of the temporal and spatial pattern of an evolving infarct it provided was essential for future studies. Reimer and Jennings demonstrated that, if the myocardium at risk was reperfused within 3 hours, tissue could be salvaged. The fraction of salvageable tissue could, however, change as a consequence of the reperfusion conditions. In this study, the extent of cell death at the end of a submaximal period of ischemia (i.e., <3 h) without reperfusion was not assessed. Thus, it was impossible to determine how much of the necrosis in ischemic/reperfused hearts was due to the preceding ischemia or the process of reperfusion itself. While this study did not, therefore, answer this particular question, it did provide the all-important framework for future studies by characterizing the nature of the evolving infarct.
The effect of ibuprofen on accumulation of indium-111–labeled platelets and leukocytes in experimental myocardial infarction

J.L. Romson, B.G. Hook, V.H. Rigot, M.A. Schork, D.P. Swanson, B.R. Lucchesi

Circulation. 1982;66:1002-1011

In the late 70s and early 80s the quest for the magic "anti-infarct" drug was in full swing. Perhaps one of the more promising candidates was ibuprofen. It had been shown to either limit or delay the evolution of an infarct, without however, affecting what were considered to be the two principal determinants of myocardial injury, namely, oxygen consumption and residual blood flow. This study therefore set out to investigate two other possible mechanisms underlying the protective effects of ibuprofen: (i) its ability to suppress platelet aggregation, and (ii) its anti-inflammatory properties, which may limit tissue damage by suppressing leukocyte infiltration into the injured myocardium.

Platelets or leukocytes were labeled with \(^{111}\)In-oxine in mongrel dogs subjected to 40 min total occlusion of the left circumflex (LCx) coronary artery, followed, in most studies, by 24 h of reperfusion. Three different experimental protocols were used. (i) \(^{111}\)In-labeled platelets or leukocytes infused before occlusion and reperfusion (with or without ibuprofen treatment); (ii) labeled platelets infused 72 h after occlusion and reperfusion; or (iii) labeled platelets infused before a 40-min occlusion, but with no reperfusion. The authors reasoned that platelet and leukocyte infiltration would be profoundly affected by hemorrhagic infarction. They therefore partially restricted LCx flow throughout the 20 min prior to total occlusion and for the duration of reperfusion. This was insufficient to reduce resting blood flow, but could limit reactive hyperemia on reperfusion, thus limiting hemorrhagic infarction. On postmortem, myocardial tissue was stained to identify both the region at risk and the infarcted tissue, and the heart was sliced into 1-cm rings. The region at risk was further subdivided into blocks of tissue containing different, but quantifiable amounts of infarcted tissue vs jeopardized noninfarcted "normal" tissue. The infiltration of either platelets or leukocytes into the region at risk was then assessed by counting the radioactivity in these different blocks of tissue.

Ibuprofen was shown to substantially limit infarct size in hearts made ischemic for 40 min and reperfused for 24 h. In control hearts, early during the time course of reperfusion, platelets accumulated in the injured myocardium—a process unaffected by ibuprofen. However, in the absence of reperfusion, or when the labeled platelets were administered later, accumulation was markedly reduced. Thus, the authors concluded that while reperfusion was essential for substantial platelet accumulation—which occurs only during the early phase of reperfusion—it is unlikely to be a critical factor in the infarct-limiting effect of ibuprofen.

Regarding leukocyte infiltration, ibuprofen both reduced infarct size and substantially limited leukocyte infiltration into the injured myocardium. However, this correlation between leukocyte accumulation and infarct size did not prove that these two phenomena were causally related. The chicken and egg question remained: was there less tissue injury because there was less leukocyte infiltration, or was there less leukocyte infiltration because there was less injury? Although no definitive answer was provided, evidence was found that leukocyte infiltration may actually be initiating injury rather than responding to it. Romson et al showed that ibuprofen could limit leukocyte infiltration in all ischemic regions of the myocardium at risk of infarction whatever the ischemic severity, suggesting that the ability of ibuprofen to limit leukocyte infiltration is not dependent on the severity of ischemia. To put it another way, ibuprofen limits leukocyte infiltration even in those small regions of the ibuprofen-treated hearts containing the most severely ischemic necrotic tissue. Thus, it appeared that ibuprofen was not simply limiting the severity of ischemia, and hence leukocyte infiltration, but actually preventing leukocyte-induced myocardial injury during reperfusion. Such observations led the authors to conclude that "The influx of leukocytes into regions of myocardial necrosis in numbers far greater than necessary for the repair and resolution of injured myocardium may exacerbate the ischemic insult."

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1982

Lindy Chamberlain is jailed in Melbourne, after the Dingo Baby trial; Hugh Hudson’s “Chariots of Fire” wins the Best Picture Oscar; and the board game “Trivial Pursuit” is launched
Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity

R. Ferrari, C. Ceconi, S. Curello, C. Guarnieri, C.M. Caldarera, A. Albertini, O. Visioli

J Mol Cell Cardiol. 1985;17:937-945

By 1985, it had been repeatedly demonstrated that the readmission of oxygen, rather than the readmission of flow, might contribute to reperfusion injury. The recognition of the role of molecular oxygen focused attention on oxidant stress and free radicals as possible mediators of injury. However, it would be another 2 years before the burst of oxygen-centered radical production that is now known to accompany reperfusion was directly measured using electron spin resonance.

Ferrari and colleagues, however, recognized both the role of oxidant stress and the potential importance of the cell’s antioxidant defenses. They hypothesized that ischemia and reperfusion might lower the myocardial cell’s antioxidant defenses, thus rendering the cell more vulnerable to oxidative injury.

To test this hypothesis, they used the isolated Langendorff-perfused rabbit heart preparation subjected to 90 min low-flow (4%) ischemia and 30 min reperfusion. At various times during this protocol, they measured tissue high-energy phosphate (ATP and PCr) content, a variety of antioxidant enzymes, and tissue oxidized (GSSG) and reduced (GSH) glutathione.

In one series of experiments, the hearts were paced throughout at a rate of 180 bts/min, while in a second series, hearts were allowed to beat spontaneously. In the unpaced hearts, although ATP and PCr fell during ischemia, contractile function recovered well on reperfusion (to 70% of its preischemic value), and there was little evidence of cell necrosis assessed as creatine phosphokinase (CPK) release on reperfusion. In these hearts, GSH content fell during ischemia and remained depressed during reperfusion. However, superoxide dismutase (SOD) and other antioxidant enzymes remained unaffected.

This contrasted with hearts paced throughout the protocol. In these hearts, the pacing protocol clearly increased the severity of the ischemic insult. These paced hearts went into contracture during ischemia, and, on reperfusion, ATP, PCr, and contractile function all failed to recover significantly. In addition, Ferrari and colleagues observed that these paced hearts showed release of CPK and GSH into the coronary effluent. Since GSH is not thought to cross the intact cell membrane, the logical inference was that this loss of soluble cytosolic enzymes was likely to reflect ultrastructural damage and irreversible injury.

The appearance of GSH in the coronary effluent was accompanied by depletion of tissue GSH and SOD and accumulation of GSSG. While it is possible that the general loss of soluble proteins occurred through lysis of cell membranes, the loss of these key antioxidants may also increase the vulnerability of reversibly injured cells to oxidant stress. This increased vulnerability was reflected in the paced hearts in an accumulation of GSSG in hearts at the end of the reperfusion period.

The authors concluded that the readmission of oxygen in hearts in which SOD levels are depleted by the preceding ischemia overwhelms the cell’s depleted defenses and oxidizes glutathione to form GSSG. Since GSH is also important in protecting protein thiols from terminal oxidation, GSH may also have been sacrificially depleted by the reversible process of protein S-glutathiolation. This was supported by an observed decrease in total cellular protein thiol groups.

Thus, the importance of this paper is that it demonstrated that ischemia can deplete the myocardial cell’s antioxidant defenses, increasing the heart’s vulnerability to the oxidant stress of subsequent reperfusion.

1985

Mikhail Gorbachev becomes the new leader of the Soviet Union; 17-year-old Boris Becker becomes the youngest Men’s Singles champion at Wimbledon; and Ruth Lawrence, aged 13, gains a first class degree in mathematics at Oxford University
Granulocytes as active participants in acute myocardial ischemia and infarction

G.W. Schmid-Schonbein, R.L. Engler

Am J Cardiovasc Pathol. 1987;1:15-30

While it may seem unusual to choose a review article as a seminal paper in this field, this one is really a must for anyone wishing an introduction to reperfusion injury.

In this article, Schmid-Schonbein and Engler take the reader on a whistle-stop tour of the microvasculature and its interactions with granulocytes.

Initially, this tour starts off with the authors providing a review of the basics of the deformability of circulating cells so as to introduce the reader gently to the biophysics of plasticity, friction, and adhesion.

Schmid-Schonbein and Engler point out that large granulocytes such as neutrophils are typically around 8 µm in diameter—almost twice the diameter of a capillary. Thus, granulocytes are squeezed, rather than swept, through the capillary bed even under the best of conditions, and changes in adhesion, deformability, or perfusion pressure can rapidly lead to capillary plugging. During ischemia, the drop in perfusion pressure alone may be sufficient to result in capillary plugging.

In addition, the release of numerous substances which can activate granulocytes (such as platelet-activating factor [PAF], arachidonic acid and its metabolites, cytokines, etc) may exacerbate plugging.

Whatever the exact mechanism, the consequence is that during low-flow ischemia, as much as 60% of the capillaries may be plugged with granulocytes.

Plugging of the larger vessels does not seem to occur. Nonetheless, on reperfusion, this microvascular plugging can significantly compromise reflow to the previously ischemic zone. This “no-reflow” phenomenon, first described by Kloner and colleagues in 1974, can occur after relatively short periods of ischemia and has a profound impact both on the extent and microvascular redistribution of flow on reperfusion. Thus, capillary plugging is an important determinant of the ability of reperfusion to salvage reversibly injured tissue.

In addition to the essentially passive role of granulocytes in the “no-reflow” phenomenon, Schmid-Schonbein and Engler also review the evidence that granulocytes may play a much more active and direct role in mediating ischemia/reperfusion injury. In ischemia and reperfusion, granulocytes may release large quantities of oxygen-derived free radicals and may also undergo degranulation and release of lytic enzymes.

Pharmacological studies in which granulocytes are either manipulated or eliminated are reviewed. The ability of such interventions leads the authors to conclude:

“The classic hypothesis that the granulocytes appear in the ischemic or postischemic myocardium as a response to the associated inflammatory process may have to be revised. Instead, the granulocytes may actually be the key factor causing the inflammation.”

Colonel Oliver North testifies before the US Congress in the Iran-Contra hearings; the Minnesota Twins win the Baseball World Series on November 2nd; and work begins on the English Channel Tunnel (the Chunnel)
Measurement of superoxide–derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury

J.L. Zweier


By 1987, many pharmacological studies had implicated free radicals in the genesis of reperfusion injury. However, since radicals exist for only the tiniest fraction of a second, their detection and quantification in intact beating hearts was, to say the least, challenging! In 1987, Jay Zweier et al in Baltimore and Pamela Garlick et al in London used electron paramagnetic resonance spectroscopy techniques to detect radical production in isolated hearts during early reperfusion. This paper, published in 1988, extends Jay Zweier's work and demonstrates the way in which radical production in the early seconds of reperfusion may be correlated with functional recovery.

Adult rabbit hearts were perfused in the Langendorff mode, and left ventricular pressure was measured with an intraventricular balloon. 5,5-Dimethyl-1-pyrroline-\(n\)-oxide (DMPO, a spin-trapping agent which reacts with the short-lived radicals to form a longer-lasting radical adduct) was infused into the coronary circulation via a side-arm in the aortic cannula. During aerobic perfusion, no radical adducts could be detected in the coronary venous drainage.

However, when hearts were subjected to 30 min global ischemia and reperfusion, there was a rapid appearance of radical adducts in the coronary effluent in the early seconds after the restoration of flow. Reperfusate samples were collected in 10-second aliquots and, after 10 to 20 seconds, the spin-trap signals reached a maximum before gradually declining over the next 2 to 3 minutes.

The nature of the spectroscopic signals indicated that superoxide and hydroxyl radicals were the most likely initiators of this generation of spin-trap adducts. In order to test this, Zweier perfused hearts with recombinant superoxide dismutase (SOD), which was crucially administered at the time of reperfusion and not prior to ischemia. In the SOD-treated group, radical adduct formation on reperfusion was reduced by over 80% and, importantly, this radical scavenging was associated with an increase in functional recovery. SOD treatment improved both the recovery of left-ventricular developed pressure (from 46%±6% to 67%±5%) and diastolic pressure (from 47±5 mm Hg to 29±4 mm Hg). Having demonstrated that the superoxide radical may be responsible for much of the oxidant stress on reperfusion, Zweier then reasoned that the detection of signals reflecting hydroxyl radical formation may occur as a consequence of iron-catalyzed Fenton chemistry. In experiments where SOD treatment was combined with an iron-chelating agent (Chelex), the radical signal on reperfusion was totally eliminated and functional recovery was again inversely correlated with the fall in oxidant stress.

This paper demonstrated the influence of radical production during the early seconds of reperfusion on the recovery of contractile function. Many studies both before and since have failed to distinguish between the therapeutic benefits of an agent added during ischemia and reperfusion, and one added during reperfusion alone.

In this study, Zweier showed that radical scavengers administered at the time of reperfusion could both scavenge these radicals and improve functional recovery. This observation has led to many subsequent studies both from Jay Zweier and others, including the classic experiments of Roberto Bolli showing the role of free radicals during the early seconds of reperfusion as mediators of myocardial stunning.
Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial “stunning” is a manifestation of reperfusion injury

R. Bolli, M.O. Jeroudi, B.S. Patel, O.I. Aruoma, B. Halliwell, E.K. Lai, P.B. McCay

Circ Res. 1989;65:607-622

While recognizing the wealth of evidence suggesting that free radicals may play a role in ischemia/reperfusion injury, Bolli et al identified four remaining key questions: (i) does this critical free radical–mediated damage occur during ischemia, reperfusion, or both? (ii) if the burst of radical production occurs during reperfusion, what is its time course? (iii) are antioxidants given at the time of reperfusion protective, and is this related to radical scavenging? and (iv) if reperfusion injury is mediated by free radicals, which species are involved? In order to answer these questions, Bolli et al used the anesthetized open-chest dog model subjected to 15 min ischemia and 2 h reperfusion. N-(2-mercapto-propionyl)-glycine (MPG, a membrane permeable antioxidant) was administrated to the animals at various times and four groups of animals were studied:

- **Group I**: MPG infusion was started 15 min before coronary occlusion and ended 2 h after reperfusion.
- **Group II**: MPG infusion was started 1 min before coronary occlusion and ended 2 h after reperfusion.
- **Group III**: MPG infusion was started 1 min after coronary occlusion and ended 2 h after reperfusion.
- **Group IV**: (control group) received equivalent volumes of vehicle.

Throughout the experiment, systolic wall thickening was assessed in all dogs using ultrasonic crystals sewed to the epicardial surface. Free radical production was measured using electron paramagnetic resonance spectroscopy and the spin-trapping agent α-phenyl N-tert-butyl nitrone (PBN) and, at the end of the protocol, hearts were stained with triphenyltetrazolium chloride (TTC) to demonstrate the lack of myocardial necrosis following this short period of reversible ischemia. In all groups, during ischemia, regional wall motion fell rapidly until, by the end of the 15-min ischemic period, the ischemic zone was akinetic and showed systolic wall thinning (with a systolic loss of 75% of control thickness). During reperfusion, contractility remained depressed in the control hearts (Group V) and in the hearts given MPG 1 minute after the start of reperfusion (Group III). However, when MPG was given before the onset of reperfusion (Groups I and II), on reperfusion, contractile function was rapidly restored, such that by 4 hours of reperfusion systolic wall thickening had recovered to about 50% of its control value.

In a separate, but similar group of dogs, Bolli et al went on to measure free radical production during early reperfusion using electron paramagnetic resonance. MPG given 1 minute before reperfusion virtually abolished free radical production, while MPG given 1 minute after reperfusion was almost ineffective at attenuating the early peak in radical production. The striking difference between the effect of MPG given 1 minute before reperfusion and that given 1 minute after reperfusion is, perhaps, the crucial observation in this paper. This observation implies that there is a narrow and critical time window during the first minute of reperfusion where radical production may initiate whatever processes underlie myocardial stunning—MPG administered 1 minute into reperfusion is too late and ineffective. In a series of in vitro experiments, Bolli et al went on to demonstrate that it is the hydroxyl radical that is the most likely radical species responsible for these effects.

In summary, Bolli et al answered the four key questions posed in the introduction of their article, concluding that: (i) free radical–mediated damage occurs primarily during reperfusion, (ii) the time course of the radical burst is very rapid: antioxidants must be present in the first crucial minute of reperfusion if they are to effectively protect against myocardial stunning, (iii) the efficacy of antioxidants given at the time of reperfusion is directly related to their ability to scavenge radicals, and (iv) the hydroxyl radical is the most likely species responsible for these effects.

Romanian leader Nicolae Ceausescu and his wife are executed by firing squad; David Dinkins is elected as New York City’s first black mayor; and Kazuo Ishiguro wins the Booker Prize for “The Remains of the Day”

1989
The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats

J.H. Hill, P.A. Ward


**Phlogiston** (n.) the mythical element originally proposed to be contained in all combustible materials and metals in the phlogistic theory of combustion (a theory largely disproved by Lavoisier shortly before losing his head in the French revolution). [f. Gk *phlox, phlogos* flame].

If, like me, you feel the need to reach for a dictionary at the sight of the word “phlogistic,” fear not—it means “inflammatory” (in its most basic sense of the word), or, more literally, “of phlogiston.” This paper therefore describes the way in which the inflammatory process following acute myocardial infarction is exacerbated by the direct activation of the complement system.

At the time this paper was written, the classic sequential activation pathway of the complement cascade had been well characterized. This paper, however, demonstrates the direct activation of a component of this system bypassing the normal sequential pathway.

Hill and Ward demonstrated that heart tissue contains a proteolytic enzyme that can break down complement C3 to form cleavage products that are potently chemotactic. This enzyme is normally confined within heart cells and is released only when the membrane is disrupted either experimentally by homogenization or by tissue injury such as occurs during myocardial infarction. This proteolytic enzyme released in infarcting hearts cleaves C3 to form smaller chemotactic fragments, which then attract leukocytes into the damaged tissue.

The studies of Hill and Ward involved two basic approaches: (i) they established the presence of this enzyme in the normal myocardium and its ability to cleave C3, and (ii) they demonstrated the role of this enzymatic pathway in chemoattraction and in leukocyte infiltration in infarcting tissue.

In their first studies, they showed that rat heart homogenates incubated either with sera or human C3 produced a potent chemotactic factor. However, the tissue homogenate alone or C3 in the absence of the homogenate did not show any chemotactic activity. The enzyme present in the myocardium responsible for the interaction with C3 was soluble (it appeared in the supernatant of crude homogenates) and, when incubated with radiolabeled C3, produced chemotactic labeled proteolytic fragments.

In the second series of studies, Hill and Ward demonstrated that these chemotactic proteolytic breakdown products appear in the infarcting myocardium, peaking after 3 to 4 hours’ ischemia. The activity of this chemotactic fraction could be inhibited by coincubation with an antibody to C3. In addition, rats pretreated 1.5 days before infarction with a C3 inactivator extracted from cobra venom showed no proteolytic chemotactic products in the infarcting myocardium. Control rats showed massive neutrophil infiltration into the infarcting region 24 to 48 hours after infarction. In contrast, in the C3-depleted rats, very few neutrophils accumulated in the infarcting tissue. Finally, the authors concluded that, although C3 depletion reduced neutrophil infiltration, they could detect no differences in the ultimate extent of myocardial damage, or in the process of scar formation.

In summary, Hill and Ward showed that the myocardium contains enzymes capable of proteolytically cleaving C3 to active chemoattractant fragments. This reaction appears to be set in motion in infarcting tissue (perhaps by the release of this enzyme [or enzymes] from damaged cells), resulting in massive neutrophil infiltration. While this direct activation of the complement cascade may play an important role in the inflammatory process, these studies, in the absence of reperfusion, could not demonstrate a relationship between this process and the eventual extent of cellular injury.
Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function


This study, published in 1990, came at a time when there was abundant evidence that neutrophil infiltration and activation in the reperfused myocardium can exacerbate ischemia/reperfusion injury. Simpson and colleagues had demonstrated 2 years previously that an antibody to the neutrophil adhesion complex CD11b/CD18 (Mo1) can reduce infarct size measured in dogs after 6 hours of reperfusion.

In this study, they extended their observations by asking two further questions: (i) can a F(ab’)2 fragment of the Mo1 antibody protect against longer periods of reperfusion (ie, after 72 hours)? and (ii) is the protection afforded by anti-Mo1 F(ab’)2 mediated by inhibiting neutrophil adhesion or by some other mechanism?

Simpson et al subjected dogs to 90 min of ischemia (ligation of the left circumflex coronary artery) followed by 72 hours of reperfusion. Anti-Mo1 F(ab’)2 antibodies were given as bolus infusions 45 minutes after ischemia and at 12, 24, 36, and 48 hours (control dogs received inactivated antibodies).

The principal finding of this study was that anti-Mo1 F(ab’)2 administration substantially reduced infarct size even when measured after 72 hours of reperfusion. Infarct size was 21.6%±2.8% of the area at risk in the Mo1 antibody protect against longer periods of reperfusion (ie, after 72 hours)? and (ii) is the protection afforded by anti-Mo1 F(ab’)2 mediated by inhibiting neutrophil adhesion or by some other mechanism?

Simpson et al subjected dogs to 90 min of ischemia (ligation of the left circumflex coronary artery) followed by 72 hours of reperfusion. Anti-Mo1 F(ab’)2 antibodies were given as bolus infusions 45 minutes after ischemia and at 12, 24, 36, and 48 hours (control dogs received inactivated antibodies).

The principal finding of this study was that anti-Mo1 F(ab’)2 treatment with the antibody reducing PMA-stimulated neutrophil aggregation to 36.3%±3.6% of control.

The authors concluded that the profound ability of antibodies to the Mo1 antigen on the neutrophil mediates its protective effect on ischemia/reperfusion injury by preventing neutrophil aggregation and hence preventing plugging of the microvasculature. The lack of effect on tissue neutrophil infiltration measured at 72 hours of reperfusion may, as the authors suggest, indicate that the assessment at 72 hours of reperfusion may miss the crucial early moments of neutrophil aggregation (see review of Schmid-Schonbein and Engler’s paper), and effects early in reperfusion may have resolved by the end of the reperfusion period.

Finally, the authors conclude that the profound protective effects mediated by anti-Mo1 antibodies administered before or during reperfusion may prove useful in limiting irreversible myocardial injury following thrombolysis or angioplasty.

1990
Liberian president Samuel Doe is killed by rebels; Mexican novelist Octavio Paz wins the Nobel Prize for literature; and US industrialist Armand Hammer dies aged 92 years
Early accumulation of the terminal complement-complex in the ischaemic myocardium after reperfusion


**Eur Heart J.** 1994;15:418-423

In this study, Mathey and coworkers used two independent methods to assess the deposition of complement C5b-9 in the ischemic myocardium. Firstly, they used immunohistochemistry to visualize tissue localization of C5b-9 using a dual antibody technique in frozen tissue sections from rabbit hearts subjected to a range of ischemia and ischemia/reperfusion protocols. Secondly, they took tissue biopsies from ischemic and normal myocardial segments and measured C5b-9 content using an enzyme-linked immunosorbent assay (ELISA) assay.

Both techniques essentially showed the same results.

- During ischemia (in the absence of reperfusion), C5b-9 first appears in the ischemic tissue after about 1.5 hours and then gradually accumulates such that by 5 hours there is significant C5b-9 accumulation. By 12 hours of ischemia, tissue C5b-9 was essentially maximal, and intense staining was seen in the immunohistochemical sections.

- In the absence of reperfusion, since the major accumulation of C5b-9 occurs over a time scale longer than that required to induce a maximal infarct, the authors conclude that it is unlikely that C5b-9 influences the progression of ischemic injury. However, this is not the case in tissue that is subjected to ischemia followed by early reperfusion.

- Reperfusion accelerated C5b-9 accumulation such that maximal accumulation was seen after 3 to 4 hours’ reperfusion (vs 12 hours in the absence of reperfusion). In hearts that were reperfused, as little as 15 to 30 minutes of ischemia was sufficient to induce significant C5b-9 infiltration.

Interestingly, in a separate series of hearts subjected to 3 hours of ischemia followed by reperfusion, 30 minutes of reperfusion was sufficient to induce maximal C5b-9 infiltration, with longer periods of reperfusion inducing no further complement infiltration. Since C5b-9 and C5a can evoke degranulation of neutrophils, production of superoxide anions, increased neutrophil adhesion, and cellular calcium overload, the authors concluded that the early infiltration of C5b-9 into the reperfused myocardium may play a role in the pathogenesis of reperfusion injury.

The authors finally concluded that if the complement system does contribute to reperfusion injury, the temporary inactivation of this system may be a valuable adjunctive therapy during elective reperfusion following cardiac surgery, thrombolysis, or angioplasty.

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The Baltic ferry “Estonia” sinks with the loss of more than 900 lives; Red Rum, three times winner of the Grand National, retires, aged 29; and the Norwegians say “nei” to the European Union in a referendum.
## Oxidative Stress

**Bibliography of One Hundred Key Papers**

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<tr>
<th>Author(s)</th>
<th>Title</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Birnbaum Y, Hale SL, Kloner RA.</td>
<td>Differences in reperfusion length following 30 minutes of ischemia in the rabbit influence infarct size, as measured by triphenyltetrazolium chloride staining.</td>
<td><em>J Mol Cell Cardiol.</em> 1997;29:657-666.</td>
</tr>
</tbody>
</table>
Bibliography of One Hundred Key Papers


Frangogiannis NG, Youker KA, Entman ML. The role of the neutrophil in myocardial ischemia and reperfusion. *EXS.* 1996;76:263-284.


Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. *J Mol Cell Cardiol.* 1973;5:395-407.
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title of the Study</th>
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<tr>
<th>Author(s)</th>
<th>Title</th>
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<tr>
<td>Mullane KM, Westlin W, Kraemer R.</td>
<td>Activated neutrophils release mediators that may contribute to myocardial injury and dysfunction associated with ischemia and reperfusion.</td>
</tr>
<tr>
<td>Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL.</td>
<td>Ischemic preconditioning decreases apoptosis in rat hearts in vivo.</td>
</tr>
<tr>
<td>Qin Y, Tang XL, Park SW, Sun JZ, Kalya A, Bolli R.</td>
<td>The early and late phases of ischemic preconditioning: a comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40-minute coronary occlusion.</td>
</tr>
<tr>
<td>Ranjadayalan K, Umachandran V, Davies SW, Syndercombe-Court D, Gutteridge CN, Timmis AD.</td>
<td>Thrombolytic treatment in acute myocardial infarction: neutrophil activation, peripheral leucocyte responses, and myocardial injury.</td>
</tr>
<tr>
<td>Raschke P, Becker BF, Leipert B, Schwartz LM, Zahler S, Gerlach E.</td>
<td>Posts ischemic dysfunction of the heart induced by small numbers of neutrophils via formation of hypochlorous acid.</td>
</tr>
<tr>
<td>Reimer KA, Murry CE, Richard VJ.</td>
<td>The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and controversy?</td>
</tr>
<tr>
<td>Repine JE.</td>
<td>Oxidant-antioxidant balance: some observations from studies of ischemia-reperfusion in isolated perfused rat hearts.</td>
</tr>
</tbody>
</table>
Bibliography of One Hundred Key Papers

Schaiff WT, Eisenberg PR. Direct induction of complement activation by pharmacologic activation of plasminogen. 
*Cor Art Dis.* 1997;8:9-18.

Schultz JE, Rose E, Yao Z, Gross GI. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. 

Schultz JJ, Hsu AK, Gross GI. Ischemic preconditioning is mediated by a peripheral opioid receptor mechanism in the intact rat heart. 

*J Mol Cell Cardiol.* 1993;25:927-938.

Semb AG, Vaage J, Sorlie D, Lie M, Mjos OD. Coronary trapping of a complement activation product (C3a des-Arg) during myocardial reperfusion in open-heart surgery. 

*Circulation.* 1993;87:536-546.

Simpson PJ, Lucchesi BR. Free radicals and myocardial ischemia and reperfusion injury. 


Smith EF 3d, Griswold DE, Hillegass LM, Silvjak MJ, Davis PA, DiMartino MJ. Cardioprotective effects of the vasodilator/β-adrenoceptor blocker, carvedilol, in two models of myocardial infarction in the rat. 

*Eur Heart J.* 1993;14(suppl I):82-86.


*Science.* 1990;249:146-151.


Weyrich AS, Buerke M, Albertine KH, Lefer AM. Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium. 
**Williams FM.**

*Neutrophils and myocardial reperfusion injury.*


---

**Yao Z, Gross GJ.**

*Activation of ATP-sensitive potassium channels lowers threshold for ischemic preconditioning in dogs.*


---

**Yao Z, Mizumura T, Mei DA, Gross GJ.**

*K<sub>ATP</sub> channels and memory of ischemic preconditioning in dogs: synergism between adenosine and K<sub>ATP</sub> channels.*


---

**Youker K, Smith CW, Anderson DC, et al.**

*Neutrophil adherence to isolated adult cardiac myocytes. Induction by cardiac lymph collected during ischemia and reperfusion.*


---

**Youker KA, Hawkins HK, Kukiela GL, et al.**

*Molecular evidence for a border zone vulnerable to inflammatory reperfusion injury.*


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**Zweier JL.**

*Measurement of superoxide-derived free radicals in the reperfused heart: evidence for a free radical mechanism of reperfusion injury.*