Free Rad. Res. Comms., Vol. 5, No. 6, pp. 305-314
Reprints available directly from the publisher
Photocopying permitted by license only

INVITED COMMENTARY

CONCEPTS RELATED TO THE STUDY OF REACTIVE OXYGEN AND CARDIAC REPERFUSION INJURY

JAMES P. KEHRER

Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA 78712-1074

(Received June 28, 1988, Accepted August 24, 1988)

The phenomenon of reperfusion injury remains poorly defined. Questions remain about whether injury occurs in addition to that produced by hypoxia or ischemia, or whether the observed changes simply reflect the unmasking of an underlying injury. Various pathological processes which occur upon the return of oxygen to hypoxic and ischemic heart tissue have been quantitated to assess the extent of reperfusion injury, yet it is not known if they reflect identical or different processes. In addition, the mechanism(s) responsible for these diverse changes may not be the same in the various model systems used to study reperfusion injury. Although reactive oxygen species clearly are formed at reperfusion, conclusive evidence that they are producing injury, particularly during the first seconds, is not available. Several sources of these reactive oxygen species have been proposed but none have been clearly linked with injury in several species or model systems. As research in the field of reperfusion injury continues, it is imperative for scientists to clearly define the system they are using so that studies examining mechanisms of cell lysis at reperfusion are not confused with those assessing the occurrence and mechanisms of damage in addition to that produced by oxygen deprivation.

KEY WORDS: Heart, reperfusion injury, oxygen radicals, reactive oxygen species

INTRODUCTION

Despite the publication of literally hundreds of scientific papers over the past several years, the phenomenon known as reperfusion injury remains an enigma. In fact, there continues to be considerable debate about whether the changes which occur upon reperfusion of hypoxic or ischemic heart tissue reflect new injury, or whether this is simply the unmasking of damage which occurred during the period of oxygen deprivation which was not evident in quiescent cells.

The 1988 meeting of the Federation of the American Societies for Experimental Biology had reperfusion injury as one of its themes. The controversies surrounding this research field were readily evident in the various presentations. Most of the work on reperfusion injury has dealt with the heart since, with the advent of a variety of revascularization treatments, this is the area of greatest clinical interest. Nevertheless, it is necessary to remember that other organs including lung, liver, kidney, brain, intestine, stomach, pancreas and skin also undergo reperfusion injury. Hypotheses regarding the mechanism of injury in one tissue may not necessarily apply to others. Furthermore, a variety of endpoints are used to quantitate the injury and are unlikely to represent the same basic processes. The purpose of this commentary is to reflect on

some of the conflicting ideas regarding the existence and mechanisms of cardiac reperfusion injury.

Definition of Reperfusion Injury

Just what is this phenomenon which is commonly referred to as reperfusion injury? A wide variety of terms have been coined to describe the events which surround the return of oxygen into a hypoxic or ischemic organ. These include stunned myocardium, hibernating myocardium, stuttering ischemia, posthypoxic-reoxygenation injury, jeopardized myocardium, lethal ischemia, infarct size, zone at risk, and reversible and irreversible damage, among others[1]. While useful in various contexts, the proliferation of descriptive terms can lead to the assumption that the biochemical processes responsible for the different endpoints used to assess injury are related.

Before one can adequately define reperfusion injury, a definition of injury must be available. At its simplest, injury is any alteration which adversely affects normal cell function. This suggests that the existence of "injury" is directly related to the sensitivity of the index employed. In practice, a wide range of endpoints with differing sensitivities have been used to study reperfusion injury (Table 1). The original definition of reperfusion injury was the sudden release of intracellular constituents (mainly measured as creatine kinase or lactate dehydrogenase) from heart tissue upon reperfusion or reoxygenation after a prolonged period of hypoxia or ischemia. However, this effect clearly represents cell lysis and is probably unrelated to more subtle indices of injury. Thus, for reperfusion injury it becomes important to distinguish between studies which are examining: 1) viable cells killed by contracture-dependent rupture, 2) the unmasking of biochemically dead cells, 3) polymorphonuclear leukocyte (PMN)-dependent tissue damage at late time points, and 4) true reflow-induced cell death related to oxidant stress or some other mechanism.

It is interesting that despite a decade of work on the phenomenon of reperfusion injury, there is still disagreement among researchers whether the changes seen at reoxygenation represent damage in addition to that attributable to the preceding hypoxia or ischemia. When subjected to oxygen deprivation, all cells will minimize their metabolic demands. Ultimately this results in an essentially quiescent cell which, while perhaps severely damaged, does not exhibit many of the classic signs of injury. Upon the introduction of oxygen the cell again becomes metabolically active and at that point the injury to various cellular components becomes evident. An analogous

TABLE I
Endpoints Used to Assess Cardiac Reperfusion Injury

- release of intracellular enzymes
- arrhythmias
- contractility and other mechanical parameters
- infarct size (at various times after reperfusion)
- myocardial edema; capillary permeability
- ultrastructural and histochemical changes
- peak and resting tension
- mitochondrial function and oxygen utilization
- energy metabolism (ATP/creatine phosphate content & synthesis)
- calcium transport and content
- coronary flow
- cardiac output; systemic blood pressure

situation may be the cytotoxic effects of cancer chemotherapeutic agents which are much more evident in rapidly metabolizing cells.

Poole-Wilson[1] has recently written an excellent review summarizing many of the pitfalls associated with interpreting the voluminous research published in this field. Proof that reperfusion injury exists has relied on demonstrating that the phenomenon can be prevented through pharmacologic or other types of interventions at the moment of reperfusion or reoxygenation. Unfortunately, conflicting results have been reported for virtually every intervention. The most convincing evidence of additional injury at reperfusion has been suggested to come from experiments which altered the calcium concentration at the time of reperfusion[1]. However, the protective effect of decreasing calcium, which is most beneficial when performed during the ischemic period,[2] may be explained by increases in cellular levels of high energy phosphates which this manipulation causes.[3]

Schaper and Schaper[4] have suggested that reperfusion is beneficial for reversibly injured tissue but accelerates the necrosis of irreversibly injured tissue, and recently have presented electron microscopic data indicating that, at the subcellular level, reperfusion does not damage the myocardium in addition to the effects of ischemia.[5] However, the concept of reversible and irreversible injury is murky at best. There is as yet no way to tell whether irreversible injury is a consequence of the ischemia or the result of additional damage at reperfusion since tissue which is not reperfused must eventually die. Thus, the conclusion that the reperfusion process is itself injurious remains suspect. Nevertheless, when limiting the discussion to selected indices of injury there is an abundance of evidence that the introduction of oxygen to an ischemic or hypoxic tissue induces damage beyond that caused by the absence of respiration.

Mechanisms of reperfusion injury

Despite questions about whether additional injury occurs at reperfusion, there are undoubtedly dramatic changes associated with this event. These include myocardial cell swelling and contracture, the accumulation of cytosolic and mitochondrial calcium, and the massive loss of intracellular constituents. Numerous mechanisms to explain these effects have been proposed (Table 2) and research continues to determine the extent to which the individual mechanisms contribute to the result.

The loss of energy seems to be a critical factor in determining whether myocardial cells are damaged during hypoxia or ischemia.[6] Irreversible injury has not been reported when the myocardial ATP content is at least partially preserved and treatments, such as hypothermia,[7] which delay the loss of ATP provide protection to heart tissue from both ischemic and reperfusion injury. It is interesting, therefore, that the only treatment at the time of reoxygenation which will completely prevent the release of intracellular constituents from hypoxic heart tissue is with agents which inhibit mitochondrial ATP formation and the subsequent contracture-induced rupture of myocytes made fragile by anoxic injury.[3,8,9] Such treatments obviously do not protect heart tissue, but do demonstrate that the mere introduction of oxygen is not sufficient to initiate cell lysis.

The energy-dependency of myocardial cell lysis has been extensively studied, and seems to be a unifying factor between several models of cardiac damage including the oxygen and calcium paradoxes and the perfusion of hypoxic heart tissue with calcium-free medium.[3,8,10,11] A hypothesis outlining the events which may contribute to the cell

TABLE II
Potential Explanations For Myocardial Cell Changes Upon Reperfusion

Effect	Mechanisms	Result
1. Loss of energy	Decreased ATP production	●Loss of ability to maintain ion gradients
	Loss of adenine nucleotides	
	Decreased NAD	●Injury to critical cell components
	Acidosis	
2. Contracture	Mechanical (energy-dependent)	●Cell rupture
Osmotic swelling	Increased membrane fragility	
3. Membrane damage	Oxygen radicals (leukocytes, mitochondria, xanthine oxidase, other?)	●Alterations in membrane permeability to ions
	Phospholipases	●Blebs
	Prostaglandins	●Altered ion channels
	Accumulation of toxic metabolites	●Arrhythmias
	Proteases	
	Increased intracellular calcium	
	Electrical instability	
4. No-reflow	Neutrophil; platelets	●Plugging
	Oxygen radicals	●Vessel constriction

lysis upon reoxygenation of isolated-perfused heart tissue is shown in Figure 1. Although these events have not been fully explored and may only apply to the lytic event seen following prolonged periods of hypoxia or ischemia, they provide an intriguing basis for additional research in this field. In particular, the ability of mitochondria to resume respiration even after prolonged periods of hypoxia suggests that if contracture-mediate rupture were prevented it may be possible to salvage portions of the myocardium.

Many researchers have assumed that a hypothesis developed to explain a phenomenon observed in one tissue or species may be readily applied to others. This is clearly illustrated in the field of reperfusion injury with the enzyme xanthine oxidase. Granger *et al.*[12] first reported that xanthine oxidase was apparently an important factor in mediating increases in capillary permeability upon reperfusion of ischemic feline intestine. These data formed the basis of the well-known hypothesis involving the D to O conversion of xanthine oxidase in ischemic tissue, and the subsequent formation of oxygen radicals during reperfusion. However, while data both for and against this hypothesis in various tissues have appeared in the literature,[13-20] investigators have lost sight of the original basis of this theory, and have all too often cited it as an established fact rather than the hypothesis that it remains. In reality, it is clear that, while xanthine oxidase may play a role in reperfusion injury in certain tissues and species such as cat intestine, it is certainly not necessary for this phenomenon in heart since species which lack this enzyme in cardiac tissue, such as rabbit, pig and human,[21-23] still develop reperfusion injury. Furthermore, the infusion of adenine nucleotides into isolated rat[24] and dog[25] hearts (which contain large amounts of xanthine oxidase), and rabbit hearts[26] (which lack this enzyme), enhanced recovery following a period of ischemia, the opposite of what one would expect if xanthine oxidase were an important damaging mechanism.

Models of Reperfusion Injury

Disparities among the wide range of models used to study reperfusion injury could account for some of the conflicting data regarding the existence, cause and mechan-

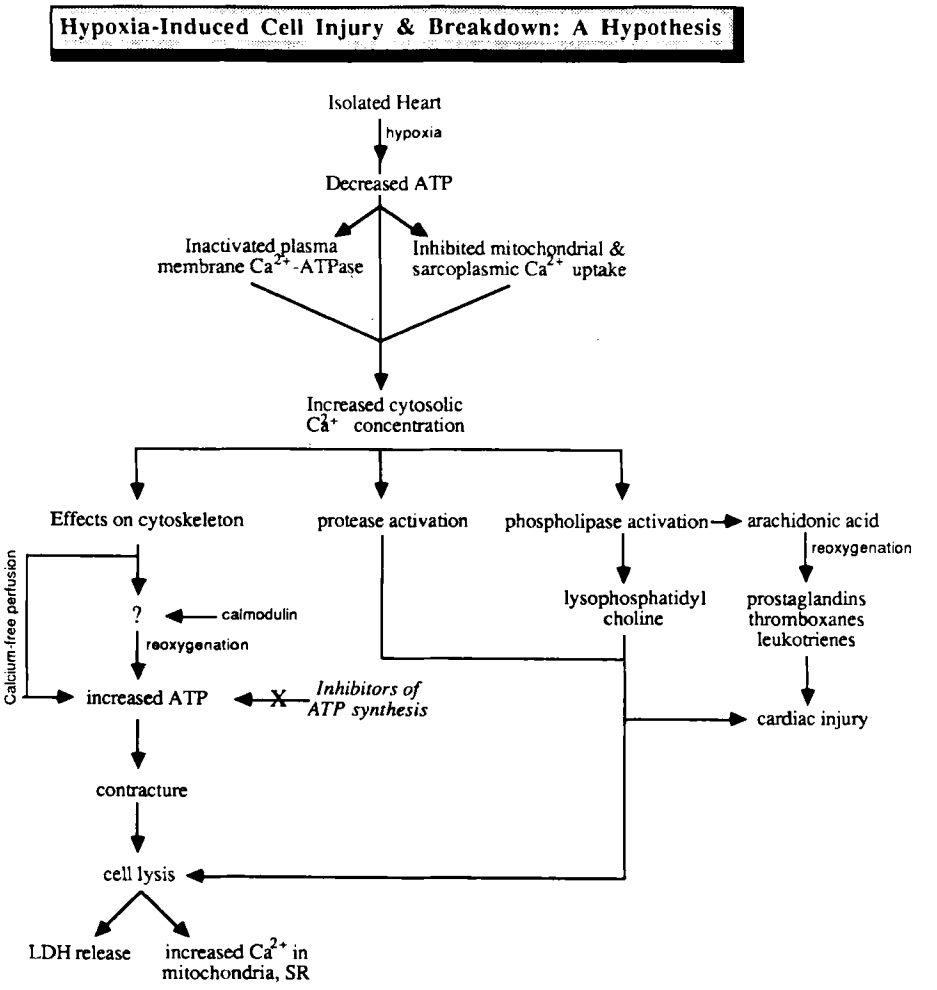

FIGURE 1 This figure illustrates the pathways which have been hypothesized to be involved in the cell injury and lysis which occurs upon reoxygenation after 30–60 minutes of hypoxia. During the hypoxic period there is a loss of ATP which either directly, or via changes in intracellular free calcium, results in "damage" to the cell. The change in free calcium may affect the cytoskeleton through an as yet undefined mechanism or it may activate some enzyme systems. Increasing cytosolic ATP after the hypoxia-induced "damage" has occurred results in rapid cell lysis. An independent pathway to cell lysis may involve effects on proteolytic enzymes or lipids subsequent to increases in intracellular free calcium levels. This latter pathway may explain the extensive cell lysis eventually seen in heart tissue perfused with hypoxic medium for several hours.

ism(s) of this phenomenon. Contracture-induced cell lysis appears to be unique to the heart which might explain some of the differences noted between various tissues. Additional model-related discrepancies are dramatically illustrated by comparing results obtained following 60 minutes of hypoxia in an isolated-perfused rat heart with those utilizing 10 minutes of ischemia in a dog heart *in vivo*. Although both models will reveal changes associated with reperfusion or reoxygenation, the underlying mechanisms may be dramatically different. For example, the hypoxic rat heart described above has been subjected to a severe insult which precludes the resumption

of contractile activity while the dog heart, which was deprived of oxygen for a much shorter period of time, may resume virtually normal function. Smaller differences in the period of oxygen deprivation may also affect the results as evidenced by the protective effects achieved with superoxide dismutase after 90 minutes[27] but not 3 hours[28] of ischemia. Alternatively, closed versus open chest models may play a role in the contrasting effects of this antioxidant enzyme.[28]

The endpoint chosen to assess damage may influence one's conclusion regarding the involvement of reactive oxygen species in reperfusion injury. Several investigators have shown that the cell lysis observed at reoxygenation after prolonged hypoxia or ischemia in dog or rat heart is unrelated to reactive oxygen species.[13,29,30] In contrast, a recent review of the literature discussed a large body of evidence suggesting that oxygen radicals, particularly those derived from xanthine oxidase, appear to be involved in the fibrillation which occurs upon reperfusion after short periods of ischemia in the rat.[31] However, multiple triggers seem to exist for reperfusion-induced arrhythmias in this species[32] and data are available in dog[33] and pig[34] suggesting oxygen radicals are not a factor in the reperfusion-induced arrhythmias occurring in these species.

Another factor to consider when interpreting results relating to reperfusion injury are the differences between ischemia and hypoxia. Both events deprive a tissue of oxygen but, unless specific changes are made in the perfusate, only ischemic tissue accumulates various metabolites and becomes acidotic. The importance of these differences is illustrated by a recent report of a study in liver which suggested that the cell injury elicited by hypoxia and ischemia may have significantly different origins.[35] Thus, comparisons between reperfusion and reoxygenation injury must be done cautiously. One final factor to consider is the length of reperfusion *in vivo*. Model systems which employ hemoperfusion and longer periods of time will begin to attract leukocytes. A large body of evidence has suggested that activated neutrophils themselves, or products they release such as peroxidized lipids or fragments, oxygen radicals, thromboxanes and leukotrienes may play a role in the pathophysiology of reperfusion injury.[36,37] More prolonged reperfusion may, therefore, provide contrasting results as has been seen with the diminished infarct size reported after 4 hours, but not 4 days, of reperfusion in dogs treated with allopurinol.[18,38]

Active Species of Oxygen

In recent years the oxygen radical hypothesis of cardiac reperfusion injury has attracted a great number of adherents. Several studies using spin traps and electron spin resonance spectroscopy have provided direct evidence supporting the formation of radicals (in particular carbon centered, superoxide and hydroxyl radicals) upon reperfusion of ischemic heart tissue of various species.[39-43] Although some of the observed ESR signals have recently been suggested to arise artifactually,[44] it seems clear that reactive oxygen species are produced in reperfused heart tissue. This is perhaps not surprising considering the highly reduced state of an oxygen deprived tissue and it has been suggested that the site of radical production is the mitochondrial respiratory chain.[44] Respiring mitochondria are known to release a variety of reactive oxygen species. In addition, it has been recently shown that submitochondrial particles isolated from ischemic heart tissue produce increased amounts of superoxide[45] and that cardiac mitochondria have a highly active superoxide generator which is

unrelated to energy-linked respiration.[46] The role, if any, of this superoxide generator in cardiac reperfusion injury remains a topic for further study.

Although the magnitude of oxygen radical production appears to be quite low in reperfused heart tissue, it can continue above basal levels for up to three hours.[39] The infusion of superoxide dismutase can decrease the tissue levels of these radicals and improve myocardial contractility in an isolated-perfused preparation.[43] It remains unclear, however, whether these reactive oxygen species are producing significant amounts of tissue injury *in vivo*, particularly as it relates to the indices of cell death which are employed. A number of studies have reported the formation of products of lipid peroxidation in reperfused heart tissue.[47-49] However, conflicting data have also been reported[50] and since lipid peroxidation may be a result as well as cause of tissue injury, the significance of these findings is unclear. Cells and tissues have a normal oxidant tone, and the idea that all oxidation is bad is perhaps a bit shortsighted. Rat liver has a tremendous capacity to withstand an oxidative stress.[51] Thus, in addition to increases in reactive oxygen above background, one must consider both species and tissue variability when determining whether the increase is sufficient to overwhelm tissue defense mechanisms.

The protective effects observed with various antioxidants have been used as the primary evidence supporting the involvement of reactive oxygen species in cardiac reperfusion injury. However, conflicting data are available for most of these findings. In particular, Jennings and coworkers failed to find protection in dogs treated with superoxide dismutase.[52] These workers also found that multiple short episodes of ischemia protected heart tissue against subsequent prolonged ischemia and reperfusion, and attributed this to either the preservation of ATP levels or the reduction of catabolite levels.[53] Findings by other groups have also supported the absence of protective effects with superoxide dismutase and catalase.[28] Studies in reperfused rat liver (which admittedly may not apply to the heart) strongly suggest that hypoxic injury is a prerequisite to reactive oxygen formation during reoxygenation[54] and only minor amounts of reactive oxygen species are formed.[55] However, data implicating oxygen radicals in hepatic reperfusion injury are also available[56] and the large number of studies showing some protection of heart tissue following treatment with antioxidants, including spin traps,[39] cannot be dismissed. It is possible reactive oxygen species play a role in selected tissue changes in certain model systems. Alternatively, antioxidant compounds may assist recovery through direct tissue interactions rather than via their ability to scavenge reactive oxygen species.

SUMMARY

It was not possible in this brief commentary to cover all of the research which has addressed the issue of the role of oxygen radicals in cardiac reperfusion injury. Although the cited studies were selected to make specific points, it is of interest that after the initial flurry of supporting evidence, more recent data tended to minimize the injury directly attributable to the reactive oxygen species formed at reperfusion. There is no doubt that exogenously generated reactive oxygen species can damage heart tissue,[57,58] but evidence that the levels produced endogenously are producing significant injury is not available.

The total injury measured in reperfused heart tissue will be the sum of that

produced by the ischemia and that produced by the reperfusion. It has been suggested that the relative contribution of ischemia to the total injury increases with time, while that of reperfusion, and in particular oxygen radicals, decreases.[59] Thus, the time when interventions with oxygen radical scavengers have an effect will be limited. In some tissues, such as kidney or brain, this "window" of therapeutic opportunity appears to be quite long, while in the heart it is disappointingly short.

In general, it seems to be established that, even if reactive oxygen species are involved in cardiac reperfusion injury, xanthine oxidase is a significant source in only selected species.[14] The generation of various oxidation products by leukocytes at late times in heart tissue reperfused with blood appears to be a likely contributor to the final damage. However, evidence that oxygen radicals produced during the first seconds of reperfusion are a significant cause of injury is minimal, and it seems likely that the extent of cardiac damage caused by oxygen deprivation is vastly in excess of that caused by oxygen radicals.

As research into the mechanisms of the various changes observed upon reperfusion continues, it is imperative for scientists in this field to clearly define the system they are using. Studies examining mechanisms of cell lysis must not be confused with those assessing the mechanism of hypoxic or ischemic injury, or the occurrence and mechanism of further damage upon reperfusion. Such efforts will be rewarded by a better understanding of the seemingly contradictory literature accumulating on this topic.

Acknowledgments

JPK is the Gustavus Pfeiffer Centennial Fellow of Pharmacology and is supported by Research Career Development Award HL01435 from the National Institutes of Health. Thanks go to Dr. Charles V. Smith for his helpful comments regarding this manuscript.

References

1. Poole-Wilson, P.A. Reperfusion damage in heart muscle: Still unexplained but with new clinical relevance. *Clin. Physiol.* **7:** 439–453 (1987).
2. Tosaki, A. and Hearse, D.J. Protective effect of transient calcium reduction against reperfusion induced arrhythmias in rat hearts. *Am. J. Physiol.* **253:** H225-H233 (1987).
3. Kehrer, J.P. Park, Y. and Sies, H. Energy-dependence of enzyme release from hypoxic isolated perfused rat heart tissue. *J. Appl. Physiol.* **65:** 000–000 (1988).
4. Schaper, J. and Schaper, W. Reperfusion of ischemic myocardium: Ultrastructural and histochemical aspects. *J. Am. Coll. Cardiol.* **1:** 1037-1046 (1983).
5. Schaper, J. Heinrichs, C.M. and Schaper, W. The effects of reperfusion on ischemic myocardium. *FASEB J* **2:** A701 (1988).
6. Hearse, D.J. and Chain, E.B. The role of glucose in the survival and recovery of the anoxic isolated perfused rat heart. *Biochem. J.* **128:** 1125–1133 (1972).
7. Hearse, D.J. Humphrey, S.M. and Bullock, G.R. The oxygen paradox and the calcium paradox: Two facets of the same problem? *J. Mol. Cell. Cardiol.* **10:** 641–668 (1978).
8. Ganote, C.E. Worstell, J. and Kaltenbach, J.P. Oxygen-induced enzyme release after irreversible myocardial injury. Effects of cyanide in perfused rat hearts. *Am. J. Pathol.***84:**327–350 (1976).
9. Vander Heide, R.S. and Ganote, C.E. Increased myocyte fragility following anoxic injury. *J. Mol. Cell. Cardiol.***19:**1085–1103 (1987).
10. Ganote, C.E. Liu, S.Y. Safavi, S. and Kaltenbach, J.P. Anoxia, calcium and contracture as mediators of myocardial enzyme release. *J. Mol. Cell. Cardiol.***13:**93–106 (1981).
11. Ruigrok, T.J.C. Boink, A.B.T.J. Spies, F. Blok, F.J. Maas, A.H.J. and Zimmerman, A.N.E. Energy dependence of the calcium paradox. *J. Mol. Cell. Cardiol.***10:** 991–1002 (1978).
12. Granger, D.N. Rutili, G. and McCord, J.M. Superoxide radicals in feline intestinal ischemia. *Gastroenterology***81:** 22–29 (1981).

13. Kehrer, J.P. Piper, H.M. and Sies, H. Xanthine oxidase is not responsible for reoxygenation injury in isolated-perfused rat heart. *Free Rad. Res. Commun.***3:**69–78 (1987).
14. Downey, J.M. Hearse, D.J. and Yellon, D.M. The role of xanthine oxidase during myocardial ischemia in several species including man. *J. Mol. Cell. Cardiol.***20:**(suppl. II) 55–63 (1988).
15. Bindoli, A. Cavallini, L. Rigobello, M.P. Coassin, M. and Di Lisa, F. Modification of the xanthine-converting enzyme of perfused rat heart during ischemia and oxidative stress. *Free Rad. Biol. Med.***4:**163–167 (1988).
16. Metzger, J. Dore, S.P. and Lauterburg, B.H. Oxidant stress during reperfusion of ischemic liver: No evidence for a role of xanthine oxidase. *Hepatology***8:** 580–584 (1988).
17. Marotto, M.E. Thurman, R.G. and Lemasters, J.J. Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: Protection by allopurinol. *Hepatology***8:**585–590 (1988).
18. Reimer, K.A. and Jennings, R.B. Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. *Circulation***71:**1069–1075 (1985).
19. Richard, V.J. Murry, C.E. Jennings, R.B. and Reimer, K.A. The xanthine oxidase inhibitor oxypurinol does not limit myocardial infarct size after 90 minutes of ischemia followed by 4 days of reperfusion in dogs. *FASEB J.***2:**A488 (1988).
20. Puett, D.W. Forman, M.B. Cates, C.U. Wilson, B.H. Hande, K.R. Friesinger, G.C. and Virmani, R. Oxypurinol limits myocardial stunning but does not reduce infarct size after reperfusion. *Circulation***76:** 678–686 (1987).
21. Downey, J.M. Miura, T. Eddy, L.J. Chambers, D.E. Mellert, T. Hearse, D.J. and Yellon, D.M. Xanthine oxidase is not a source of free radicals in the ischemic rabbit heart. *J. Mol. Cell. Cardiol.***19:**1053–1060 (1987).
22. Eddy, L.J. Stewart, J.R. Jones, H.P. Engerson, T.D. McCord, J.M. and Downey, J.M. Free radical-producing enzyme, xanthine oxidase, is undetectable in human hearts, *Am. J. Physiol.***253:**H709–H711 (1987).
23. Muxfeldt, M. and Schaper, W. The activity of xanthine oxidase in heart of pigs, guinea pigs, rabbits, rats, and humans. *Basic Res. Cardiol.***82:** 486–492 (1987).
24. Lasley, R.D. Ely, S.W. Berne, R.M. and Mentzer, R.M. Allopurinol enhanced adenine nucleotide repletion after myocardial ischemia in the isolated rat heart. *J. Clin. Invest.***81:**16–20 (1988).
25. Devous, M.D. and Jones, C.E. Effect of inosine on ventricular regional perfusion and infarct size after coronary occlusion. *Cardiology***64:** 659–667 (1971).
26. Takeo, S. Tanonaka, K. Miyake, K. and Imago, M. Adenine nucleotide metabolites are beneficial for recovery of cardiac contractile force after hypoxia. *J. Mol. Cell. Cardiol.* **20:** 187–199 (1988).
27. Jolly, S.R. Kane, W.J. Bailie, M.B. Abrams, G.D. and Lucchesi, B.R. Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. *Circ. Res.* **54:** 277–285 (1984).
28. Gallagher, K.P. Buda, A.J. Pace, D. Gerran, R.A. and Shlafer, M. Failure of superoxide dismutase and catalase to alter size of infarction in conscious dogs after 3 hours of occlusion followed by reperfusion. *Circulation* **73:** 1065–1076 (1986).
29. Vander Heide, R.S. Sobotka, P.A. and Ganote, C.E. Effects of the free radical scavenger DMTU and mannitol on the oxygen paradox in perfused rat hearts. *J. Mol. Cell. Cardiol.* **19:** 615–625 (1987).
30. Lenz, M. Hughes, H. Smith, C. Michael, L. Entman, M. and Mitchell, J. No evidence for reactive oxygen damage in ischemia-reflow injury in canine heart. *The Pharmacologist* **29:** 194 (1987).
31. Manning, A.S. Reperfusion-induced arrhythmias: do free radicals play a critical role? *Free Rad. Biol. Med.* **4:** 305–316 (1988).
32. Hearse, D.J. and Tosaki, A. Free radicals and calcium: simultaneous interacting triggers as determinants of vulnerability to reperfusion-induced arrhythmias in the rat heart. *J. Mol. Cell. Cardiol.* **20:** 213–223 (1988).
33. Parratt, J.R. and Wainwright, C.L. Failure of allopurinol and a spin trapping agent N-t-butyl-α-phenyl nitrone to modify significantly ischaemia and reperfusion-induced arrhythmias. *Brit. J. Pharmacol.***91:** 49–59 (1987).
34. Podzuweit, T. Braun, W. Müller, A. and Schaper, W. Arrhythmias and infarction in the ischemic pig heart are not mediated by xanthine oxidase-derived free oxygen radicals. *Basic Res. Cardiol.* **82:** 493–505 (1987).
35. Jaeschke, H. Smith, C.V. and Mitchell, J.R. Different initiating events in hypoxia and ischemia/reflow damage in rat liver. *FASEB J.* **2:** A1157 (1988).
36. Werns, S.W. and Lucchesi, B.R. Leukocytes, oxygen radicals, and myocardial injury due to ischemia and reperfusion. *Free Rad. Biol. Med.* **4:** 31–37 (1988).
37. Mehta, J.L. Nichols, W.W. and Mehta, P. Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion. *J. Am. Coll. Cardiol.* **11:** 1309–1316 (1988).

38. Chambers, D.E. Parks, D.A. Patterson, G. Roy, R. McCord, J.M. Yoshida, S. Parmley, L.F. and Downey, J.M. Xanthine oxidase as a source of free radical damage in myocardial ischemia. *J. Mol. Cell. Cardiol.* **17:** 145–152 (1985).
39. Bolli, R. Patel, B.S. Jeroudi, M.O. Lai, E.K. and McCay, P.B. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl-N-tert-butylnitrone. *J. Clin. Invest.* **82:** 476–485 (1988).
40. Arroyo, C.M. Kramer, J.H. Dickens, B.F. and Weglicki, W.B. Identificaiton of free radical sin myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.* **221:** 101–104 (1987).
41. Zweier, J.L. Flaherty, J.T. and Weisfeldt, M.L. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci. USA* **84:** 1404–1407 (1987).
42. Garlick, P.B. Davies, M.J., Hearse, D.J. and Slater, T.F. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ. Res.* **61:** 757–760 (1987).
43. Zweier, J.L. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J. Biol. Chem.* **263:** 1353–1357 (1988).
44. Baker, J.E. Felix, C.C. Olinger, G.N. and Kalyanaraman, B. Myocardial ischemia and reperfusion: Direct evidence for free radical generation by electron spin resonance spectroscopy. *Proc. Natl. Acad. Sci. USA* **85:** 2786–2789 (1988).
45. Guarnieri, C. Muscari, C. Ventura, C. and Mavelli, I. Effect of ischemia on heart submitochondrial superoxide production. *Free Rad. Res. Commun.* **1:** 123–128 (1985).
46. Nohl, H. A novel superoxide radical generator in heart mitochondria. *FEBS Lett.* **214:** 269–273 (1987).
47. Meerson, F.Z. Kagan, V.E. Kozlov, Y.P. Belkina, L.M. and Arkhipenko, Y.V. The role of lipid peroxidation in the pathogenesis of ischemic damage and the antioxidant protection of the heart. *Basic Res. Cardiol.* **77:** 465–485 (1982).
48. Guarnieri, C. Flamigni, F. and Caldarera, C.M. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. *J. Mol. Cell. Cardiol.* **12:** 797–808 (1980).
49. Romaschin, A.D. Rebeyka, I. Wilson, G.J. and Mickle, D.A.G. Conjugated dienes in ischemic and reperfused myocardium: an *in vivo* chemical signature of oxygen free radical mediated injury. *J. Mol. Cell. Cardiol.* **19:** 289–302 (1987).
50. Julicher, R.H.M. Tijbury, L.B.M. Sterrenberg, L. Bast, A. Koomen, J.M. and Noordoek, J. Decreased defense against free radicals in rat heart during normal reperfusion after hypoxic, ischemic and calcium-free perfusion. *Life Sci.* **35:** 1281–1288 (1984).
51. Smith, C.V. Hughes, H. Lauterburg, B.H. and Mitchell, J.R. Oxidant stress and hepatic necrosis in rats treated with diquat. *J. Pharmacol. Exptl. Therap.* **235:** 172–177 (1985).
52. Uraizee, A. Reimer, K.A. Murry, C.E. and Jennings, R.B. Failure of superoxide dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs. *Circulation* **75:** 1237–1248 (1987).
53. Murry, C.E. Jennings, R.B. Richard, V.J. and Reimer, K.A. Ischemic preconditioning protects myocardium from subsequent ischemic injury. *FASEB J.* **2:** A1478 (1988).
54. Jaeschke, H. Smith, C.V. and Mitchell, J.R. Hypoxic damage generates reactive oxygen species in isolated perfused rat liver. *Biochem. Biophys. Res. Commun.* **150:** 568–574 (1988).
55. Jaeschke, H. Smith, C.V. and Mitchell, J.R. Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. *J. Clin. Invest.* **81:** 1240–1246 (1988).
56. Younes, M. and Strubelt, O. The involvement of reactive oxygen species hypoxic injury to rat liver. *Res. Commun. Chem. Pathol. Pharmacol.* **59:** 369–381 (1988).
57. Burton, K.P. Evidence of direct toxic effects of free radicals on the myocardium. *Free Rad. Biol. Med.* **4:** 15–24 (1988).
58. Ytrehus, K. Muyklebust, R. Olsen, R. and Mjøs O.D. Ultrastructural changes induced in the isolated rat heart by enzymatically generated oxygen radicals *J. Mol. Cell. Cardiol.* **19:** 379–389 (1987).
59. Hoshino, T. Maley, W.R. Bulkley, G.B. and Williams, G.M. Ablation of free radical-mediated reperfusion injury for the salvage of kidneys taken from non-heartbeating donors. *Transplantation* **45:** 284–289 (1988).

Accepted by Prof. H. Sies