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Endothelial Superoxide Production in the Isolated Rat Heart During Early Reperfusion After Ischemia

A Histochemical Study

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This paper describes a histochemical study of superoxide generation in buffer-perfused, isolated rat hearts during the first 2 minutes of reperfusion after 60 minutes of warm ischemia. Superoxide radical production was demonstrated by a modification of Karnovsky's manganese/diaminobenzidine technique, in which superoxide oxidizes Mn^{++} to Mn^{+++} ions, which in turn oxidize diaminobenzidine to form amber, osmiophilic polymers, observable by light or electron microscopy. Isolated hearts were rendered ischemic, reperfused with oxygen equilibrated buffer containing Mn^{++} and diaminobenzidine, fixed by perfusion with Trump's solution, and processed for light and electron microscopy. The method consistently demonstrated evidence of superoxide generation near the luminal surfaces of arterial, capillary, and venular endothelial cells during the first 2 minutes of reoxygenation after ischemia. The histochemical reaction was absent or markedly reduced in non–manganese-treated or nonischemic hearts, as well as in hearts perfused with calcium-free or oxygen-free buffers. The histochemical differences were statistically significant on quantitative morphometric analysis. These results provide direct, visual evidence of the existence and endothelial localization of a burst of superoxide radicals in intact, postischemic myocardium and suggest the pathophysiologic importance of calcium-dependent endothelial cell activation in the initiation of reperfusion injury. (Am J Pathol 1991 139:1069–1080)

The clinical significance, underlying mechanisms, and indeed the very existence of reperfusion injury in cardiac tissue have been much discussed and debated[1–9] since the initial description by Hearse et al[10] in 1973 of oxygen-dependent enzyme release from previously ischemic rat hearts. The potential of the myocardium to sustain a form of oxygen-mediated injury on reperfusion, which amplifies primary hypoxic injury during ischemia, has been referred to as the "oxygen paradox"[11,12]: namely, that although the reintroduction of oxygen to ischemic tissue is obviously necessary to restore normal function, postischemic tissues may simultaneously suffer from a form of oxygen toxicity that is thought to be caused by oxygen-centered free radicals.[2,13–19] Free radicals such as $O_2^-\bullet$ and HO• are extremely reactive chemical species that contain an unpaired electron in an outer orbital, represented herein by the symbol •. The primary and most abundant free radical thought to be formed during reperfusion is superoxide ($O_2^-\bullet$), which is subsequently converted to other partially reduced, reactive oxygen species: hydrogen peroxide (H_2O_2) and the highly toxic hydroxyl radical (HO•) by way of the superoxide-driven Fenton reaction.[13,14,20] Superoxide radicals may be generated in postischemic tissues by the reduced form of nicotinamide-adenine dinucleotide phosphate oxidase in activated leukocytes,[21] by nicotinamide-adenine dinucleotide (reduced form) dehydrogenase within mitochondria,[22,23] or possibly by the action of xanthine oxidase on xanthine and hypoxanthine substrates that accumulate during ischemia.[2,24–26] The sudden rise in oxygen tension during reperfusion, perhaps especially if oxygen therapy is given, has been proposed to drive these enzymatic sources to generate a burst of $O_2^-\bullet$ radicals that exceeds the scavenging capacity of antioxidant defense systems, which may be weakened during ischemia.[27]

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Ischemia/reoxygenation injury of heart, in particular, has been rather intensively investigated during the past decade.[4,28–33] In most studies, the evidence that superoxide radicals actually occur *in vivo* is largely indirect. Treatments such as allopurinol to block superoxide production by xanthine oxidase, or superoxide dismutase to destroy superoxide ions, are given, and improved survival or function of treated animals is observed. Recently, however, electron spin resonance (ESR) techniques have been employed to provide spectroscopic evidence of a burst of free radical generation within moments of reperfusion of previously ischemic hearts.[34–37] These methods are nonetheless limited in that they cannot distinguish for certain the particular free radical species generated, and they cannot show which cell types within the heart are sources of free radicals.

Seeking a more visual and direct demonstration of the elusive oxygen radicals in tissues during the reperfusion phase, we explored a number of histochemical approaches in experimental models that would demonstrate not only the presence of oxygen radicals but also the sites of their formation. After testing a variety of previously published and novel routes to the deposition of a satisfactory histochemical reaction product in the presence of either superoxide, hydrogen peroxide, organic hydroperoxides, or the hydroxyl radical, we finally obtained excellent results with a relatively simple modification of Karnovsky's manganese/diaminobenzidine technique,[38] previously employed to demonstrate superoxide generation by leukocytes.

The principle of Karnovsky's method is as follows. Living cells that produce superoxide are exposed to a physiologic solution containing added divalent manganese and diaminobenzidine (DAB). Then

$$O_2^-\bullet + Mn^{++} + 2\,H^+ \rightarrow H_2O_2 + Mn^{+++}$$
$$Mn^{+++} + DAB \rightarrow \text{osmiophilic polymer.}$$

Nascent superoxide, which can act as either an oxidant or a reductant,[39] in the present case rapidly oxidizes divalent manganese ($k = 6 \times 10^6\ M^{-1}sec^{-1}$)[40] to the trivalent state in the presence of proton donors, with concomitant generation of hydrogen peroxide.[21] The Mn^{+++} ion, in turn, is able to oxidize diaminobenzidine to form an amber, osmiophilic polymer, widely exploited as a sensitive histochemical marker.[41,42] The accumulation of polymeric reaction product in vital tissue in the presence of Mn^{++} and DAB is a measure of the time integral of $O_2^-\bullet$ generation. This histochemical reaction for $O_2^-\bullet$ has now been shown to be inhibited by the addition of exogenous superoxide dismutase to preparations of neutrophils[38] and rat pulmonary vascular endothelium,[43] thus strongly implicating its specificity for superoxide *in vivo*. Mn^{++} is not oxidized by hydrogen peroxide, as may be readily demonstrated in simple test tube experiments.

In optimizing the original manganese/diaminobenzidine histochemical method[38] for use in myocardium, we realized that higher manganese concentrations might be required for efficient trapping of superoxide in intact tissues containing superoxide dismutase (SOD). The rationale is based on the fundamental competition between the introduced manganese and native SOD for reaction with $O_2^-\bullet$:

$O_2^-\bullet + Mn^{++} + 2\,H^+ \rightarrow H_2O_2 + Mn^{+++}$
($k_1 = 6 \times 10^6\ M^{-1}sec^{-1}$)

$O_2^-\bullet + SOD \rightarrow \frac{1}{2}H_2O_2 + \frac{1}{2}O_2 + SOD$
($k_2 = 10^9\ M^{-1}sec^{-1}$).

Because the pseudo–first-order rate constant for the latter reaction is so large, the proportion of nascent superoxide scavenged by Mn^{++}, namely

$$p = \frac{k_1[Mn^{++}][O_2^-\bullet]}{k_1[Mn^{++}][O_2^-\bullet] + k_2[SOD][O_2^-\bullet]}$$

may be substantially less than unity. Taking published values for rate constants k_1 and k_2[40,44] and estimated tissue SOD at 10^{-6} to 10^{-5} mol/l,[45] the scavenging efficiency of divalent manganese, p, would be approximately 9%, 50%, and 91%, respectively, for 1, 10, and 100 mmol/l (millimolar) Mn^{++}. Accordingly, we adopted the use of higher manganese concentrations than the 0.5 mmol/l originally described for studies of leukocytes and found the most clear and satisfactory results with 40 mmol/l manganese. We refer to this modification as the high manganese/diaminobenzidine method.

In the present report, we describe application of the high manganese/diaminobenzidine method to explore superoxide radical production during reoxygenation of previously ischemic hearts at sites that can be visualized by both light and electron microscopy. The results show, with remarkable sharpness and clarity, the appearance of superoxide radicals on the luminal face of vascular endothelium in postischemic arterioles, capillaries, and venules of the isolated rat heart.

Materials and Methods

Chemicals

Reagent grade calcium chloride, manganese chloride, potassium chloride, sodium azide, sodium chloride, sodium phosphate, and 37% formaldehyde stock solution were obtained from standard sources. Glutaraldehyde, and Tris (hydroxymethylaminomethane) were obtained from Sigma Chemical Company (St. Louis, MO) and 3-3′

diaminobenzidine tetrahydrochloride dihydrate (DAB) and acetylsalicylic acid (ASA) from Aldrich Chemical Company (Milwaukee, WI).

Solutions

Buffers containing 40 mmol/l divalent manganese, in combination with either 40 mmol/l citrate or 80 mmol/l acetylsalicylate chelator anions, were prepared as indicated in Table 1. Manganous salts were tested in the presence of either acetylsalicylate or citrate in preliminary studies. The greatest amount of reaction product was formed with citrate, which was used in the majority of experiments. The acetylsalicylate, which can form binary chelates with manganous ions, was tested to provide a manganous complex with greater lipid solubility and hence greater membrane penetration. Nonmanganese containing control buffer contained physiologic concentrations of sodium, potassium, calcium, chloride, and 4 mmol/l Tris-HCl (Table 1). Sodium azide (1 mmol/l) was added, as specified by Briggs et al,[38] to minimize spurious formation of reaction product by peroxidase,[46] catalase,[47] and mitochondrial cytochrome oxidase.[48] This concentration of azide does not, however, substantially inhibit copper-zinc or manganese superoxide dismutases.[49] The pH was adjusted to 7.4 with dropwise addition of 0.5 N NaOH, with rapid stirring, and the solutions filtered. Buffer solutions were sparged either with 100% argon gas, to initiate the ischemic/anoxic phase, or with 100% oxygen gas, to accomplish reoxygenation. To avoid microbubble emboli, flasks of perfusate were bubbled with gas through a $\frac{1}{4}$-inch diameter tube for 30 minutes before the experiment, stoppered, and brought to 37°C in a temperature-controlled water bath.

Diaminobenzidine stock solution was made by dissolving 10 mg DAB-tetrahydrochloride per milliliter distilled water and slowly adjusting the pH to 5 with dropwise addition of 0.5 N NaOH (about 2 ml/100 ml) and vigorous stirring to prevent precipitation of DAB by clouds of concentrated base. If precipitate did form, it was redissolved by addition of HCl. An isotonic working solution of Mn^{++}/DAB buffer, containing 2.5 mmol/l DAB (1 mg/ml) then was prepared by mixing the 10 mg/ml DAB stock and manganese-containing buffer in a ratio of 1:9. After perfusion of hearts with DAB-containing solutions, the collected effluent was detoxified before disposal by the addition of saturated potassium dichromate solution, as described by Morrell et al.[50]

Isolated Heart Preparation

Healthy male Wistar rats weighing approximately 450 g were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally). The chest was opened with heavy scissors, and the sternum and ventral portions of the ribs were removed to expose the heart and lungs. A #2 silk suture was placed loosely around the aortic root, and the aorta was incised and cannulated with a blunted 17-gauge needle, excluding air bubbles, such that the tip of the cannula rested 1 to 2 mm above the aortic valve. The heart was quickly removed and placed in a dish of ice cold 0.9% saline solution. Myocardial contractions ceased within 5 seconds. The cannula was secured with the silk suture and mounted on a stopcock. Then the heart was flushed with 30 ml warm, oxygen-equilibrated TRIS-buffered Ringer's solution (without Mn^{++} or DAB, Table 1) to clear coronary circulation of blood.

Ischemia/Reoxygenation Protocol

To initiate the ischemic anoxia, the heart was manually perfused with 15 ml warm (37°C) argon-equilibrated, Tris-buffered Ringer's solution that did not contain manganese or DAB. The measured partial pressure of oxygen (PO_2) of argon-equilibrated perfusate solutions, determined with the aid of a Corning blood gas analyzer, was less than 3 mmHg. The hearts with attached cannulas and stopcocks then were placed in capped 4-oz. specimen jars containing 100 ml warmed, argon-sparged Ringer's solution under argon gas. The jars then

Table 1. *Solutions*

Solution	Salt concentration (mM)*			
	Manganese chloride	Sodium chloride	Sodium citrate	Sodium acetylsalicylate (ASA)
Tris buffered Ringer's	0	140	0	0
Manganese citrate	40	0	40	0
Sodium citrate	0	60	40	0
Manganese ASA	40	0	0	80
Sodium ASA	0	60	0	80

* All buffers contained $CaCl_2$ 2 mM, KCl 4 mM, Tris 4 mM, NaN_3 1 mM, pH 7.4 adjusted with NaOH.

were sealed and transferred to a 37°C nonshaking water bath for 60 minutes to provide a period of ischemic anoxia.

After the ischemic period, the hearts were removed from the capped, oxygen-depleted jars, and reoxygenation was performed by coronary perfusion through the aortic root with warm, 37°C to 39°C, oxygen-saturated buffer solutions containing histochemical reagents (Table 1) at a flow rate of 30 ml/2 min, using a hand-held syringe. This reperfusion interval was selected in view of earlier studies with electron spin resonance spectroscopy,[34–37] indicating that the maximal formation of an SOD-inhibitable free radical signal occurred during the first 2 minutes of reperfusion. By limiting histochemical perfusion to this interval, we hoped to maximize the "signal-to-noise ratio" of the method and to minimize any toxic effects of the manganese on the isolated heart preparation. In the present series of hearts, the manganese and DAB probes were introduced only at the onset of reoxygenation and not during the prior ischemic interval. In initial experiments, perfusion pressure was monitored with a Harvard Apparatus pressure monitor, equipped with a calibrated meter readout and the flow rate adjusted to maintain 100 mm Hg aortic pressure at the onset of perfusion. This value proved to be 15 ml/minute, which was then standardized. Staining reactions were stopped and the tissues fixed by subsequent perfusion, first with 10 ml cold, 0.9% saline, followed by 10 ml cold Trump's fixative (4% formaldehyde, 1% glutaraldehyde, pH 7.4), each perfusion lasting approximately 45 seconds. Tissues were stored in vials of chilled Trump's fixative overnight and prepared for light or electron microscopic examination.

Controls

Control experiments were performed to probe the veracity of the assumed mechanism of reaction product formation, namely

$$\text{substrates} + O_2 \xrightarrow[\text{DAB}]{\text{enzymes}} O_2^-\bullet \xrightarrow{Mn^{++}} Mn^{+++} \rightarrow \text{histochemical marker},$$

by interrupting the causal chain of reaction product formation at various steps. These controls included hearts reoxygenated with plain, non-Mn^{++}/non-DAB buffer solution, non-Mn^{++} containing DAB solution, non–DAB-containing Mn^{++} solution, and non–oxygen-containing (argon-sparged) Mn^{++}/DAB solution. Isotonic sodium chloride replaced manganese chloride in nonmanganese control buffers (Table 1). Because of previous reports suggesting the calcium dependence of $O_2^-\bullet$ generation by neutrophils,[51] and endothelial cells,[52] control hearts perfused with calcium free buffer (both before ischemia and during reoxygenation) also were studied. The nonischemic control hearts were prepared in cold saline and then immediately perfused with 30 ml warm, oxygen-equilibrated Mn^{++}/DAB buffer.

Microscopy

Sections of all hearts were processed for light microscopy by dehydration in graded alcohol solutions, infiltration with xylene, embedding in paraffin wax, sectioning, dewaxing, and mounting in the usual way. Six-micron-thick sections were stained routinely with either hematoxylin and eosin (H&E) or methyl green.

Specimens for transmission electron microscopy (TEM) were postfixed in osmium, dehydrated in graded ethanol, infiltrated with propylene oxide, and embedded in Poly/Bed 812 epoxy resin. One-micron-thick sections were cut and stained with methylene blue and Azure II. Areas for thin sectioning were identified by light microscopic examination, and thin sections (50–80 nm) were cut with a Reichert Ultracut E ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-100CX transmission electron microscope. Many earlier experiments had convinced us that the oxidized DAB polymer is not altered by fixation or subsequent tissue processing for either light or electron microscopy, making for routine and economical morphologic examination.

Quantitative Morphometry

Vascular profiles of H&E-counterstained slides were examined by light microscopy at 100× magnification by an observer who was unaware of the treatment or control group designation of the specimen. Diaminobenzidine reaction product appeared as amorphous, amber-brown deposits within blood vessels, which were scored as positive if reaction product was clearly visible and in contact with the vessel wall. Counts of profiles of positively stained arteries, capillaries, and veins were made within 800 × 800 μ^2 test areas, delimited by a micrometer eyepiece on the light microscope. Profiles intersected by the left and upper margins of the counting frame were included and those intersected by the right and lower margins were excluded to avoid systematic bias, as previously described.[53,54] The tissue on each 1 in. × 3 in. glass slide was scanned in vertical strips at successive 2.5-mm horizontal increments from left to right. Such sys-

tematic sampling with a random start is superior to purely random sampling, because it yields smaller errors.[53]

The mean numerical densities of positively stained arteries, capillaries, and veins per square centimeter then were tabulated for hearts undergoing the various experimental and control treatments and compared by a one-way analysis of variance. If a Bartlett's chi-square test for homogeneity of variance failed, a suitable transformation was found, as described by Anderson,[55] and the analysis of variance was repeated on the transformed data to test the null hypothesis that the density of stained vessels is the same in hearts from the various experimental and control groups. Specific comparisons of the effects of experimental versus control treatments were made using a Scheffe multiple comparison test, which allows comparison of multiple lumped control groups with one or more experimental groups. The number of observations in each group need not be the same. (In the case of only two groups, the Scheffe test is mathematically equivalent to the *t*-test). A *P*-value of 0.05 or less was considered significant.

Results

Histologic sections of perfused, isolated hearts that were studied by light microscopy after ischemia and reoxygenation with plain buffered Ringer's solution (without manganese or DAB), fixation in Trump's solution, routine tissue processing, and staining showed completely normal architecture, except for the absence of formed blood elements. All sections showed good fixation and the characteristic tinctorial appearance of the counterstain employed.

Light Microscopic Histochemistry

During the first 2 minutes of reperfusion with oxygen-equilibrated Mn^{++}/DAB containing Ringer's solution, the larger coronary arteries of the isolated hearts became noticeably brownish black in color to the naked eye, indicating reaction product formation that was subsequently confirmed microscopically. No grossly visible darkening of non–manganese-treated control hearts was observed. By light microscopy, the histochemical marker for superoxide production was also readily visible after routine tissue processing and counterstaining with H&E. The reaction product appeared in amorphous, amber deposits against the pink background of blood vessel walls. Counterstaining with methyl green intensified the contrast of the reaction product, rendering it brown against a green background. The methyl–green-stained sections were most suitable for black-and-white photomicroscopy (Fig. 1).

The photomicrographs in Figure 1 show selective staining of the luminal surfaces of vascular endothelium in coronary arterioles and myocardial capillaries and venules in the presence of manganese and DAB. In many instances, the accumulation of amorphous reaction product was sufficient to completely (A, bottom) or partially (B, bottom) fill the lumens of smaller capillaries.

The use of citrate as a manganese chelator produced more voluminous amounts of reaction product, whereas acetylsalicylate chelator appeared to produce somewhat sharper localization to vascular endothelium at the light microscopic level. The numerical densities of positively stained vessels per square centimeter obtained with manganese ASA buffer (n = 3), however, were not significantly different from those obtained with manganese citrate buffer (n = 5). Accordingly morphometric results from both buffers were pooled for later analysis. Generally many more positively stained arteries, veins, and capillaries were formed in the subepicardium than in the subendocardium. This finding correlated with diminished subendocardial perfusion after 60 minutes' ischemia in similar postischemic rat hearts, which was readily demonstrated by adding India ink to the perfusate.

Controls

The isotonic substitution of sodium for manganese in DAB containing Ringer's solution almost completely abolished reaction product formation (Figure 2A). There was virtually no staining of vascular structures when all aspects of the ischemia/reoxygenation protocol were followed except for the omission of manganese from the perfusate. This control experiment excludes simple polymerization of DAB by tissue peroxidases or mitochondrial cytochrome oxidase[48] as a major mechanism of endothelial reaction product formation. The non–manganese-treated control also rules out the plausible conclusion that ischemic capillaries take up diaminobenzidine preferentially simply by virtue of having been ischemic.

The possibility that Mn^{++} might participate in a Fenton-like reaction with endogenous H_2O_2 was considered and excluded, because molar concentrations of manganous ions do not react readily with hydrogen peroxide *in vitro* and because addition of 3% hydrogen peroxide to the Mn^{++}/DAB solution used in the experiments did not produce a visible reaction product. In contrast, positive control spot tests performed with either ferrous sulfate and hydrogen peroxide solutions or with granular potassium superoxide and working Mn^{++}/DAB solutions produced immediate, dark color reactions.

The elimination of 2 mmol/l Ca^{++} from the perfusates

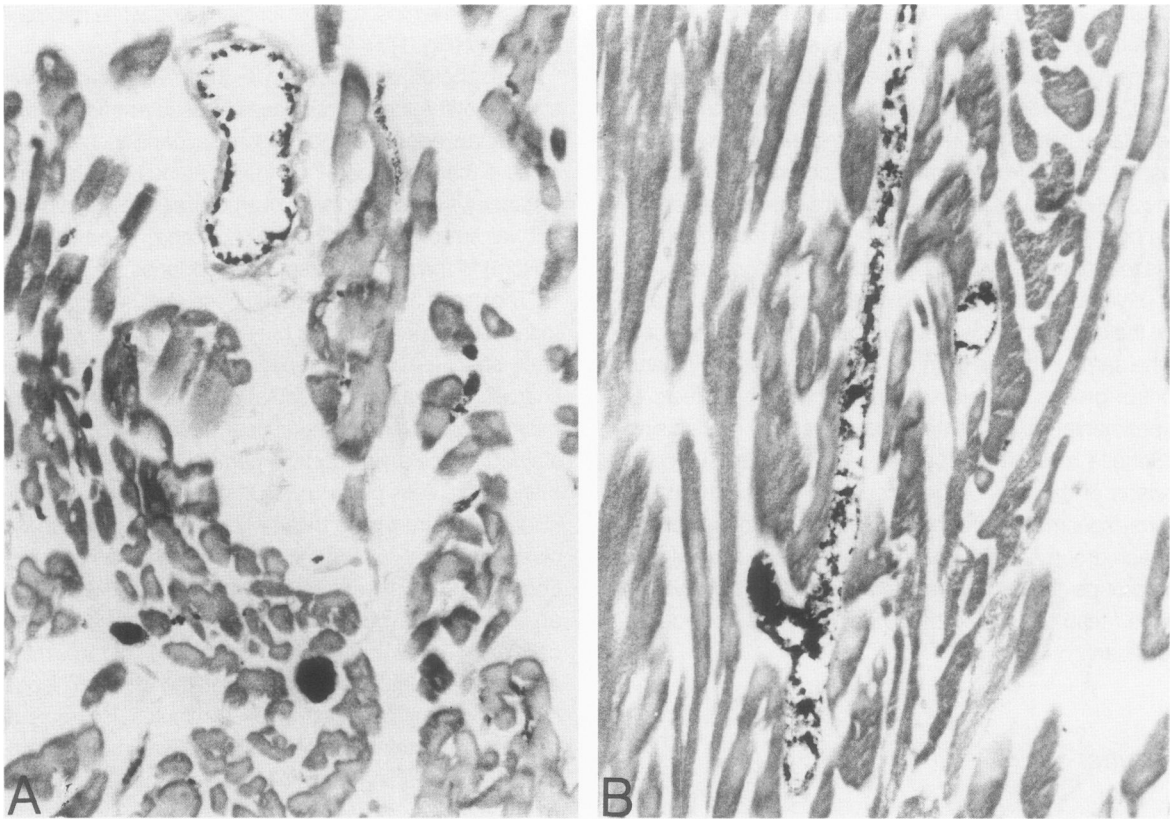

Figure 1. *Postischemic coronary arterioles and capillaries in an isolated rat heart subjected to a cycle of 60 min ischemic anoxia and 2 min reperfusion with oxygen sparged, DAB containing Mn^{++}/ASA buffer. Methyl green counterstain. Vessels are shown in transverse section* **(A)** *longitudinal section* **(B)**. *Original magnification 100×. Upon light microscopic examination, both arteries and capillaries show deposition of reaction product on the luminal surfaces of the endothelial carpet. Completely or partially filled capillaries are visible in the bottom portions of micrographs* **(A)** *and* **(B)**. *Black and white photomicrographs were taken using 35 mm Kodak T-max black and white print film, 1/8 sec exposure. Prints were made on Ilford multigrade paper (Ilford Photo Corp., Paramus, NJ).*

(both during anoxia and during reperfusion) sharply reduced reaction product formation (Figure 2B) with an effectiveness very nearly equal to the omission of manganese. This finding both suggests specific biochemical mechanisms and excludes many conceivable artifacts that would not be calcium dependent.

Similarly there was greatly reduced endothelial staining of nonischemic control tissues perfused for the same 2-minute period with Mn^{++}/DAB solution, compared with previously ischemic tissues. This control rules out nonspecific staining simply by virtue of the proximity of the endothelial cells to high concentrations of perfused staining solutions. Control hearts reperfused with anoxic, argon-saturated manganese citrate/DAB solution, followed by saline and Trump's solution, also showed negligible vascular reaction product formation, confirming the strict oxygen dependence of the phenomenon. Finally when DAB was omitted from the perfusate, no reaction product could be detected after ischemia and reperfusion.

Results of light microscopic morphometric analysis of positively stained vascular profiles (Figure 3) showed that the mean densities of stained vessels in each of the various control groups were significantly different from those observed in the ischemic and reoxygenated experimental group. An intense positive reaction using the high manganese/diaminobenzidine method required the combined presence of divalent manganese, calcium, prior ischemia, and molecular oxygen.

Electron Microscopy

The flocculent reaction product associated with the luminal surfaces of endothelial cells was readily observable in transmission electron micrographs. Figure 4 shows the localization of the Mn^{++}/DAB reaction product by transmission electron microscopy, after osmium tetroxide postfixation. The electron-dense reaction product in the vessel lumen corresponds to the endothelial associated, amber reaction product observed by light microscopy. Reaction product was always localized to the luminal rather than the anti-luminal surfaces of the endothelial

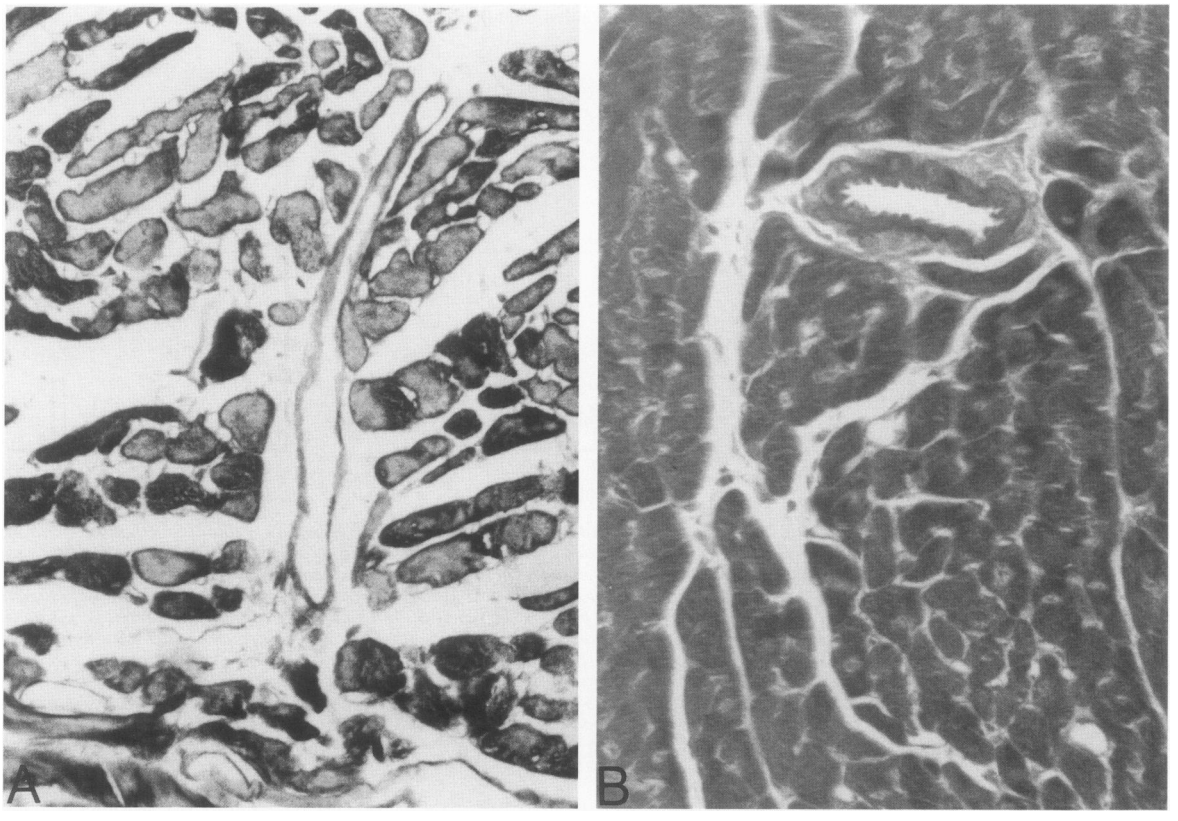

Figure 2. *No-manganese and no-calcium controls. Sections of a similarly ischemic rat heart as in Figure 1, reoxygenated with nonmanganese* (A) *or noncalcium* (B) *containing DAB buffer. Methyl green counterstain. Details of photomicroscopy similar to Figure 1. Elimination of manganese from the reperfusion medium* (A) *abolishes reaction product formation. This control excludes simple polymerization of DAB by tissue peroxidases or mitochondrial cytochrome oxidase as a major mechanism of reaction product formation in these experiments. In particular, if DAB and endogenous hydrogen peroxide were simultaneously present then peroxidase could have induced DAB polymerization, as in an ordinary peroxidase stain. The peroxidase reaction, however, is not manganese dependent. The darkly stained regions of myocytes in* (A) *are not reaction product but rather inhomogeneities in the methyl green counterstain. Elimination of calcium from the perfusate* (B) *similarly abolishes reaction product formation in the presence of DAB and manganese. This control excludes nonbiological mechanisms of reaction product formation that would not be dependent upon calcium ions. Only methyl green counterstaining was observed in this field.*

cells, in many cases appearing in pockets or caveolae on the cell surface (Figure 4B).

Ultrastructural evidence of reaction product formation was not observed in non–manganese-treated control hearts (Figure 5). Interestingly the mitochondrial swelling of the non–manganese-treated heart is more pronounced than in the manganese-treated heart—a phenomenon that perhaps could be related either to the superoxide dismutase mimetic effect of manganese[40,56] or perhaps to its calcium entry blocking effect.

Discussion

The rapid growth of investigative activity in the field of free radicals in biology and medicine has raised the intriguing possibility that oxygen radicals may constitute a major new class of pathogens that can be placed in the sequence—bacteria, viruses, auto-antibodies, free radicals—in order of both decreasing physical size and increasing difficulty of detection. Historically the understanding of the behavior of newly discovered pathogens has been advanced by microscopic techniques able to demonstrate their presence in tissue: light microscopy for bacteria, electron microscopy for viruses, and immunofluorescence for auto-antibodies. This paper demonstrates the application of a simple microscopical technique to study the generation of oxygen radicals during early reperfusion of the ischemic isolated rat heart, which we selected as a model suitable for initial investigation for a number of reasons. The isolated heart is the classical model of reperfusion injury[10] that is reversible by antioxidant drugs, given either before[1,12,57] or just after[31,32] the ischemic period; it is a technically straightforward tissue preparation; and previous studies using ESR spectroscopy have shown signals indicative of free radical generation in the isolated rat heart during the first few minutes of reperfusion.[34,37,58] Thus we were fairly certain that if free radicals could be demonstrated histochemically by the high manganese/DAB method, they would be found in the isolated rat heart.

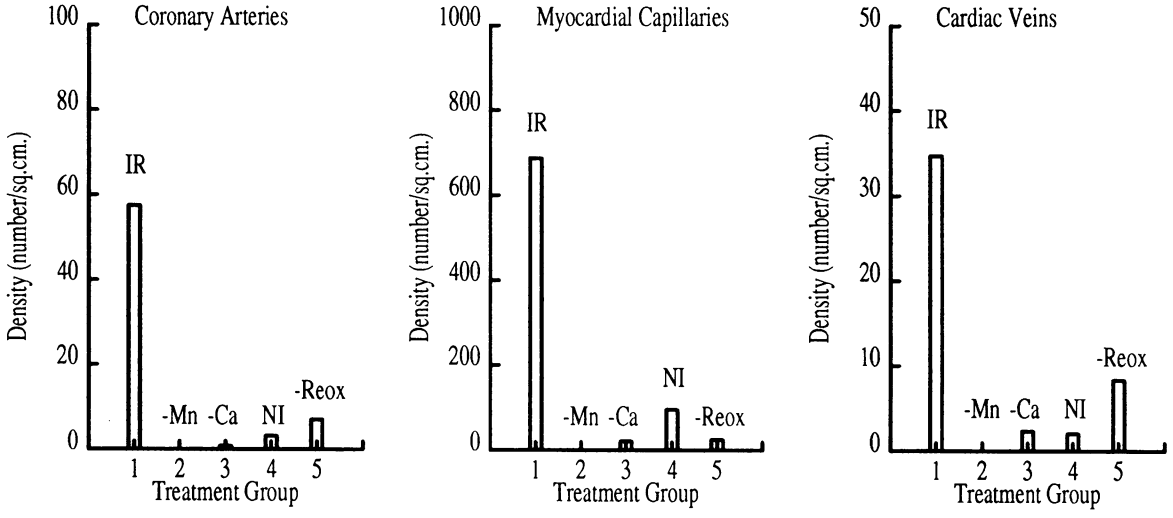

Figure 3. *Results of quantitative morphometric analysis of ischemic rat hearts reperfused with high manganese/diaminobenzidine solutions for 2 min. Vascular profiles of hematoxylin-eosin counterstained slides were examined by light microscopy at 100× magnification and counted in non-overlapping fields. Means values are plotted. IR = ischemia followed by reoxygenation with 100% oxygen equilibrated buffer (n = 6). −Mn = IR controls minus manganese (replaced with equimolar sodium chloride, n = 6). −Ca = IR minus calcium in perfusate (n = 4). NI = non-ischemic perfusion (n = 4). −Reox = ischemia followed by reperfusion with anoxic, argon equilibrated perfusate (n = 2). The total number of positively stained vessels (arteries + capillaries + veins) in the IR group is significantly greater than that in any other group (P < 0.01 vs. −Mn, −Ca, −Reox; P < 0.05 vs. NI) and significantly greater than that in the pooled control groups 2 through 5 (P < 0.01). Note: steriological theory predicts that the measured intersection densities, plotted here, are exactly one half the length densities (total length per unit volume) of stained vessels, assuming that they are randomly oriented in space and that they assume all orientations with equal likelihood. These assumptions would appear approximately true for all but the largest coronary vessels, which have a trivial influence on the final results. Both quantities have dimension cm^{-2}.*

The results of the high manganese/DAB technique provide clear visual evidence that a burst of superoxide radicals actually happens during the first 2 minutes of reoxygenation in postischemic rat hearts, in contrast to hearts that have not been subject to ischemic anoxia, or those that have been rendered ischemic, but not reoxygenated. As suggested by previous studies of pulmonary artery endothelial cells studied both in tissue culture[52,59] and *in situ*,[43] apparent superoxide production in the present experiments proved to be calcium dependent. The calcium dependence of endothelial $O_2^-\bullet$ production *in situ*, which we observed, is consistent with an extracellular to intracellular calcium flux and activation of protein kinase C[60,61] in a general scheme similar to excitation–contraction and stimulus–secretion coupling in other types of cells. The involvement of cyclooxygenase seems unlikely, however, because the activation response in our studies is preserved in the presence of ASA, a well-known cyclooxygenase inhibitor.[62]

The specific localization of initial postischemic free radical production to endothelial cells helps to suggest hypotheses about the pathophysiology of oxygen–radical-mediated processes that would be difficult to formulate from data obtained strictly from bulk myocardium. Early endothelial superoxide generation may be of special pathophysiologic importance, because a variety of secondary mechanisms and biochemical cascades—including edema, microthrombosis, the "no-reflow phenomenon" of Ames et al,[63,64] and leukocyte infiltration[65–69]—could be readily initiated by endothelial activation. Thus endothelial production of oxygen radicals may be a key triggering mechanism for the initiation of reperfusion injury in the heart. This conclusion is consistent with published studies of endothelial cells grown in culture,[36,70,71] suggesting that endothelial cells, although not "professional phagocytes," can nonetheless become activated to produce bursts of oxygen radicals.

The ultrastructural pattern of diaminobenzidine reaction product along the blood-facing sides of endothelial cells is particularly interesting and worthy of comment. It is possible that superoxide is only visible in the vascular lumens because superoxide dismutase destroys nascent superoxide within cells, or because there was limited penetration of manganese and DAB into cells, which was insufficient to demonstrate superoxide at intracellular sites—especially given the brief, 2-minute period of reperfusion. This caveat is especially pertinent to the lack of reaction product observed in cardiomyocytes in the present study. Strictly speaking, if one sees reaction product with the high manganese/DAB technique, it suggests that superoxide is being made, but if one does not see reaction product, it does not mean that superoxide is not being made.

Nevertheless we suspect that the limitation of superoxide production specifically to the luminal surface of the endothelium may prove to be quite correct in view of the agreement of our present results with those of Shlafer and co-workers,[72] using a completely independent technique

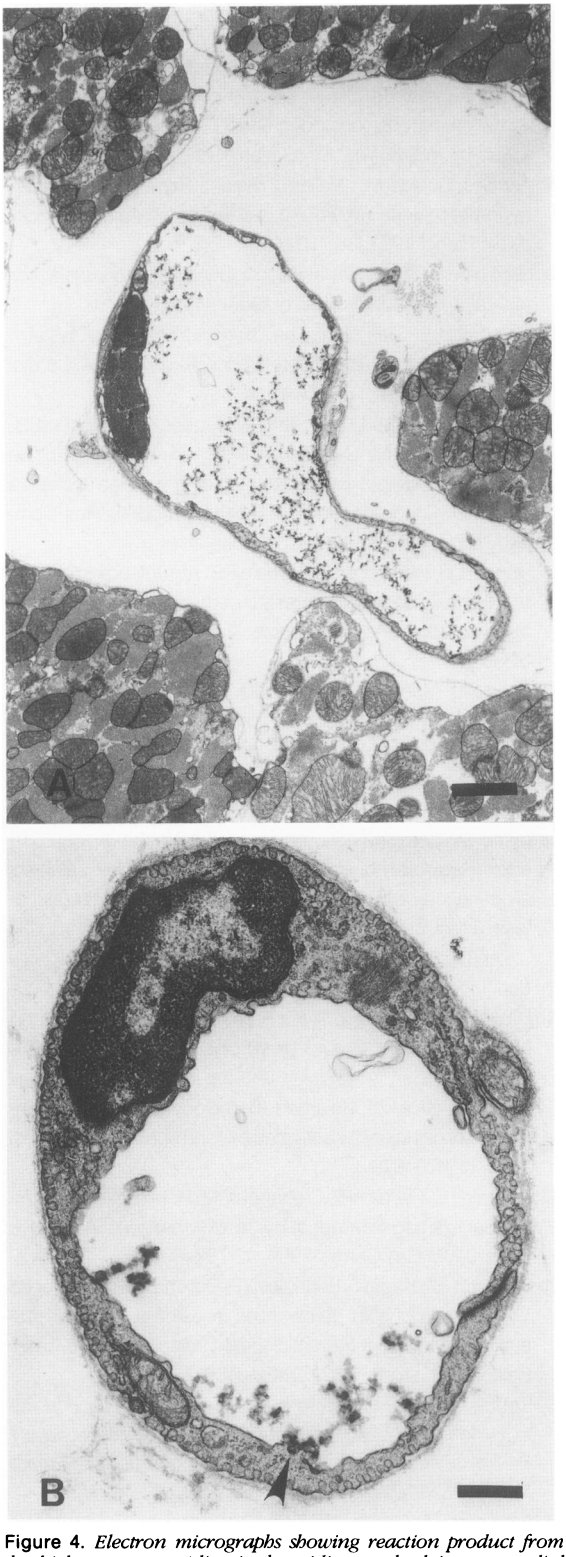

Figure 4. *Electron micrographs showing reaction product from the high manganese/diaminobenzidine method in myocardial capillaries.* **A**: *Reaction product appears as a flocculent precipitate within the capillary lumen. Mitochondrial swelling in this manganese treated heart is less pronounced than in the non-manganese treated control heart (Figure 5); magnification* × *4,400. Bar = 2 μm.* **B**: *Reaction product is associated with the luminal surface only and often appears in small invaginations (arrow); magnification,* × *7,500. Bar = 0.5 μm.*

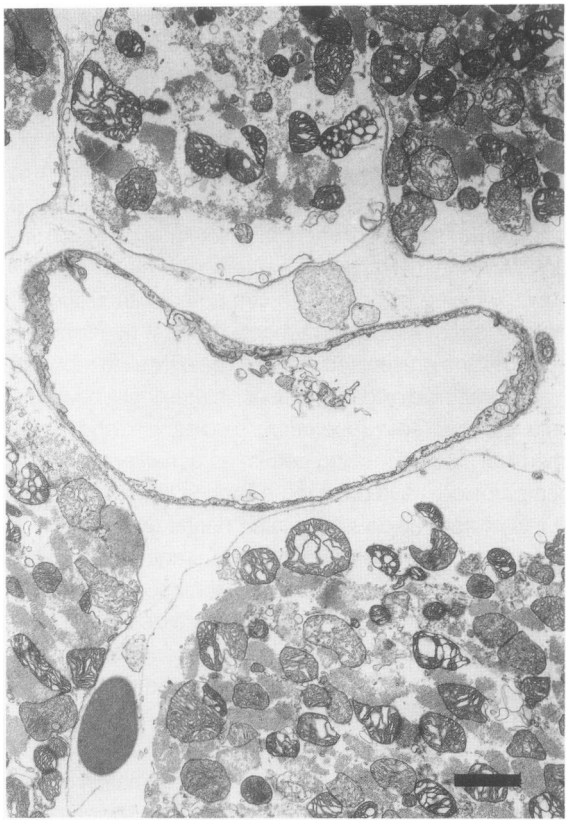

Figure 5. *Electron micrographs showing absence of reaction product in a non-manganese treated control heart. There is no reaction product in the capillary lumen, which was typical, and mitochondrial swelling in the adjacent myocytes is more pronounced than in Figure 4; magnification,* × *4,400. Bar = 2 μm.*

for histochemical localization of hydrogen peroxide in ischemic and reperfused rabbit hearts. These investigators found a distribution of cerium reaction product, indicating the presence of H_2O_2, on the luminal surfaces—but not the abluminal surfaces—of vascular endothelial cells in a pattern that was virtually identical to the distribution of Mn^{++}/DAB reaction product in the present study. Formation of the cerium reaction product in Shlafer's study was profoundly inhibited by catalase. Because either spontaneous or SOD-catalyzed dismutation of $O_2^-\bullet$ to H_2O_2 (namely, $2O_2^-\bullet + 2H^+ \rightarrow H_2O_2 + O_2$) is certain to occur, the congruent results provided by the cerium technique and the high manganese/DAB technique suggest that the same fundamental process is at work.

A biologic rationale for endothelial generation of $O_2^-\bullet$ also seems apparent. If such a free radical burst were to function either as a chemoattractant or as a defense against intravascular invaders, it would be adaptive for radical production to occur only on the blood-facing side of the cell. Otherwise two undesirable results could occur: damage to the underlying somatic cells, causing a self-inflicted cardiomyopathy, or damage to the underly-

ing basement membrane, causing a vascular permeability defect. Accordingly the polarity of free radical production that we observed may well be indicative of the normal functional role of the activated endothelial cell, either in microbial killing or in cell signaling.

Xanthine oxidase, an enzyme that may be present in capillary endothelium,[73] has been widely discussed as a potential source of oxygen radicals in reperfusion injury.[14,25,28,30,74] Rat tissues express especially high levels of xanthine oxidase,[74–76] compared with tissues of other species, including humans,[77] and it might be argued that the present results could be difficult to replicate in a low xanthine oxidase species. Interestingly, however, both electron spin resonance studies[35,36] and histochemical studies,[72] using cerium as a marker for hydrogen peroxide, have provided evidence for postischemic bursts of free radicals in isolated hearts of the rabbit, a species with very little myocardial xanthine oxidase. Thus, although superoxide generation and xanthine oxidase activity have been separately localized to capillary endothelium, the precise biochemical sources of superoxide and the species dependence of the present findings remain to be determined.

The high manganese/DAB technique works exceedingly well to disclose sites of superoxide formation during early reperfusion of the previously ischemic rat heart. The technique allows one to see the footprints of heretofore putative superoxide radicals, using otherwise routine light and electron microscopy, to provide direct visual evidence that oxygen radicals are indeed generated in the initial moments of reperfusion in the perfused rat heart model. The present study was limited to very early times after the onset of reoxygenation, when electron spin resonance experiments had shown free radical production to be maximal. These results by no means exclude $O_2^-\bullet$ generation by activated leukocytes in subsequent stages of reperfusion injury. The present study, however, clearly demonstrates the potential role of endothelial cells as discrete free radical sources *in situ,* as well as the calcium dependence of endothelial cell activation.

References

1. Gauduel Y, Duvelleroy MA: Role of oxygen radicals in cardiac injury due to reoxygenation. J Mol Cell Cardiol 1984, 16:459–470
2. McCord JM: Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985, 312:159–163
3. Burney RE, Walsh D, Kaplan LR, Fraser S, Tung B, Overmyer J: Reperfusion arrhythmia: Myth or reality? Ann Emerg Med 1989, 18:240–243
4. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR: Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 1984, 54:277–285
5. Gallagher KP, Buda AJ, Pace D, Gerren RA, 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 1986, 73:1065–1076
6. Aznar J, Santos MT, Valles J, Sala J: Serum malondialdehyde-like material (MDA-LM) in acute myocardial infarction. J Clin Pathol 1983, 36:712–715
7. Nayler WG, Elz JS: Reperfusion injury: Laboratory artifact or clinical dilemma? Circulation 1986, 74:215–221
8. Hammond B, Hess ML: The oxygen free radical system: Potential mediator of myocardial injury. J Am Coll Cardiol 1985, 6:215–220
9. Lenz ML, Michael LH, Smith CV, Hughes H, Shappell SB, Taylor AA, Entman ML, Mitchell JR: Glutathione disulfide formation and lipid peroxidation during cardiac ischemia and reflow in the dog in vivo. Biochem Biophys Res Commun 1989, 164:722–727
10. 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
11. Hess ML, Manson NH: Molecular oxygen: Friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox, and ischemia/reperfusion injury. J Mol Cell Cardiol 1984, 16:969–985
12. Myers CL, Weiss SJ, Kirsh MM, Shlafer M: Involvement of hydrogen peroxide and hydroxyl radical in the oxygen paradox: Reduction of creatinine kinase release by catalase, allopurinol, or deferoxamine, but not by superoxide dismutase. J Mol Cell Cardiol 1985, 17:675–684
13. Slater TF: Free Radical Mechanisms in Tissue Injury. London, Pion Limited, 1972
14. Halliwell B, Gutteridge JMC: Free Radicals in Biology and Medicine. Oxford, Oxford University Press, 1987, pp 206–234
15. Farber JL, Kyle ME, Coleman JB: Biology of disease: Mechanisms of cell injury by activated oxygen species. Lab Invest 1990, 62:670–679
16. Guarnieri C, Flamigni F, Caldarera CM. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J Mol Cell Cardiol 1980, 12:797–808
17. Meerson FZ, Kagan VE, Kozlov YP, Belkina LM, Arkhipenko YV: The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. Basic Res Cardiol 1982, 77:465–485
18. Babbs CF: Role of iron ions in the genesis of reperfusion injury following successful cardiopulmonary resuscitation: Preliminary data and a biochemical hypothesis. Ann Emerg Med 1985, 14:777–783
19. Freeman BA, Crapo JD: Biology of disease—Free radicals and tissue injury. Lab Invest 1982, 47:412–426
20. Michelson AM: Toxic effects of active oxygen, Biochemical and Medical Aspects of Active Oxygen. Edited by O Hayaishi, K Asada. Baltimore, University Park Press, 1977, pp 155–170
21. Curnutte JT, Karnovsky ML, Babior BM: Manganese-

dependent NADPH oxidation by granulocyte particle: The role of superoxide and the nonphysiological nature of the manganese requirement. J Clin Invest 1976, 57:1059–1067

22. Boveris A, Turrens JF: Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 1980, 191:421–427
23. Nohl H: A novel NADH-linked superoxide radical generator in heart mitochondria. Federation of European Biochemical Societies 1987, 214:269–273
24. Bucher JR, Tien M, Morehouse LA, Aust SD: Three mechanisms for the formation of an initiator of lipid peroxidation by xanthine oxidase, Oxy Radicals and Their Scavenger Systems. Vol I. Edited by G Cohen, RA Greenwald. New York, Elsevier Biomedical, 1983, pp 296–299
25. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM: Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol 1985, 17:145–152
26. Babbs CF, Salaris SC, Turek JJ: Cytochemical studies of hydrogen peroxide generation in postischemic hepatocytes. Am J Physiol 1991, 260 (Heart Circ Physiol 29):H123–H129
27. Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O: Oxygen-mediated myocardial damage during ischemia and reperfusion: Role of the cellular defenses against oxygen toxicity. J Moll Cell Cardiol 1985, 17:937–945
28. Akizuki S, Yoshida S, Chambers DE, Eddy LJ, Parmley LF, Yellon DM, Downey JM: Infarct size limitation by the xanthine oxidase inhibitor, allopurinol, in closed-chest dogs with small infarcts. Cardiovasc Res 1985, 19:686–692
29. Przyklenk K, Kloner RA: Superoxide dimustate plus catalase improve contractile function in the canine model of the "stunned myocardium." Circ Res 1986, 58:148–156
30. Werns SW, Shea MJ, Mitsos SE, Dysko RC, Fantone JC, Schork MA, Abrams GD, Pitt B, Lucchesi BR: Reduction of the size of infarction by allopurinol in the ischemic-reperfused canine heart. Circulation 1986, 73:518–524
31. Badylak SF, Simmons A, Turek J, Babbs CF: Protection from reperfusion injury in the isolated rat heart by postischemic deferoxamine and oxypurinol administration. Cardiovasc Res 1987, 21:500–506
32. Ambrosio G, Zweier JL, Jacobus WE, Weisfeldt ML, Flaherty JT: Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: The role of iron in the pathogenesis of reperfusion injury. Circulation 1987, 75:906–915
33. Babbs CF: Reperfusion injury of postischemic tissues. Ann Emerg Med 1988, 17:1148–1157
34. Garlick PB, Davies MJ, Hearse DJ, Slater TF: Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ Res 1987, 61:757–760
35. Zweier JL, Flaherty JT, Weisfeldt ML: Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proc Natl Acad Sci USA 1987, 84:1404–1407
36. Zweier JL, Kuppusamy P, Lutty GA: Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissue. Proc Natl Acad Sci USA 1988, 85:4046–4050
37. Baker JE, Felix CC, Olinger GN, Kalyanaraman B: Myocardial ischemia and reperfusion: Direct evidence for free radical generation by electron spin resonance spectroscopy. Proc Natl Acad Sci USA 1988, 85:2786–2789
38. Briggs RT, Robinson JM, Karnovsky ML, Karnovsky MJ: Superoxide production by polymorphonuclear leukocytes. Histochemistry 1986, 84:371–378
39. Aust SD, Morehouse LA, Thomas CE: Hypothesis paper—Role of metals in oxygen radical reactions. Journal of Free Radicals in Biology & Medicine 1985, 1:3–25
40. Kono Y, Takahashi M, Asada K: Oxidation of manganous pyrophosphate by superoxide radicals and illuminated spinich chloroplasts. Arch Biochem Biophys 1976, 174:454–462
41. Karnovsky MJ, Robinson JM, Briggs RT, Karnovsky ML: Oxidative cytochemistry in phagocytosis: The interface between structure and function. Histochemical J 1981, 13:1–22
42. Litwin JA: Transition metal-catalyzed oxidation of 3,3′-diaminobenzidine [DAB] in a model system. Acta Histochem 1982, 111–117
43. Babbs CF, Cregor MD, Turek JJ, Badylak SF: Endothelial superoxide production in buffer perfused rat lungs, demonstrated by a new histochemical technique. Laboratory Investigation 1991 (In press)
44. Steinman HM: Superoxide dismutases: protein chemistry and structure-function relationships. Superoxide Dismutase. Vol 1. Edited by LW Oberley. Boca Raton, FL, CRC Press, 1982, pp 11–68
45. Fridovich I: Superoxide dismutases. Ann Rev Biochem 1975, 44:147–159
46. Graham-Jr RC, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966, 14:291–301
47. Fahimi HD: Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). J Histochem Cytochem 1968, 16:547–550
48. Seligman AM, Karnovsky MJ, Wasserkrug HL, Hanker JS: Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, Diaminobenzidine (DAB). J Biol Chem 1968, 38:1–14
49. Misra HP, Fridovich I: Inhibition of superoxide dismutases by azide. Arch Biochem Biophys 1978, 189:317–322
50. Morrell JI, Greenberger LM, Pfaff DW: Comparison of horseradish peroxidase visualization methods. J Histochem Cytochem 1981, 29:903–916
51. Robinson JM, Badwey JA, Karnovsky ML, Karnovsky MJ: Superoxide release by neutrophils: synergistic effects of a phorbol ester and a calcium ionophore. Biochem Biophys Res Commun 1984, 122:734–739
52. Matsubara T, Ziff M: Superoxide anion release by human endothelial cells: Synergism between a phorbol ester and a calcium ionophore. J Cell Physiol 1986, 127:207–210
53. Weibel ER: Stereological Methods 1. London, Academic Press, 1979

54. Elias H, Hennig A, Schwartz DE: Stereology: Applications to biomedical research. Physiol Rev 1971, 51:158–200
55. Anderson VL, McLean RA: Transformation of Y, Design of Experiments: A Realistic Approach. New York, Marcel Dekker, 1974, p 23
56. Archibald FS, Fridovich I: The scavenging of superoxide radical by manganous complexes in vitro. Arch Biochem Biophys 1982, 214:452–463
57. Shlafer M, Kane PF, Kirsh MM. Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischemic, reperfused heart. J Thorac Cardiovasc Surg 1982, 83:830–839
58. Arroyo CM, Kramer JH, Dickens BF, Weglicki WB: Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. FEBS Lett 1987, 221:101–104
59. Johns A, Lategan TW, Lodge NJ, Ryan US, vanBreemen C, Adams DJ: Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. Tissue Cell 1987, 19:1–13
60. Ward PA, Johnson KJ, Till GO: Animal models of oxidant lung injury. Respiration 1986, 50:5–12
61. Ryan US: The macrophage-like properties of endothelial cells. News Physiol Sci 1988, 3:93–96
62. Campbell WB: Lipid-derived autacoids: Eicosanoids and platelet-activating factor. Chapter 24, Goodman and Gilman's The Pharmacological Basis of Therapeutics. 8th edition. Edited by AG Gilman, TW Rall, AS Nies, P Taylor. New York, Permagon, 1990, 600–617
63. Ames A, Wright L, Kowada M, Thurston JM, Majno G: Cerebral ischemia: II. The no-reflow phenomenon. Am J Pathol 1968, 52:437–438
64. Johnston WH, Latta H: Glomerular mesangial and endothelial cell swelling following temporary renal ischemia and its role in the no-reflow phenomenon. Am J Pathol 1977, 89:153–166
65. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork A, Lucchesi BR: Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 1983, 67:1016–1023
66. McCord JM: Oxygen-derived radicals: A link between reperfusion injury and inflammation. Fed Proc 1987, 46:2402–2406
67. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN: Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am J Physiol 1987, 253:H699–H703
68. Engler R: Granulocytes and oxidative injury in myocardial ischemia and reperfusion. Fed Proc 1987, 46:2395–2396
69. Rinaldo JE, Basford RE: Neutrophil-endothelial interactions: Modulation of neutrophil activation responses by endothelial cells. Tissue Cell 1987, 19:599–606
70. Rosen GM, Freeman BA: Detection of superoxide generated by endothelial cells. Proc Natl Acad Sci USA 1984, 81:7269–7273
71. Ryan US: Phagocytic properties of endothelial cells, Endothelial Cells. Vol III. Edited by US Ryan. Boca Raton, FL, CRC Press, 1988, pp 33–49
72. Shlafer M, Brosamer K, Forder JR, Simon RH, Ward PA, Grum CM: Cerium chloride as a histochemical marker of hydrogen peroxide in preperfused ischemic hearts. J Mol Cell Cardiol 1990, 22:83–97
73. Jarasch ED, Grund C, Bruder G, Heid HW, Keenan TW, Franke WW: Localization of xanthine oxidase in mammary-gland epithelium and capillary endothelium. Cell 1981, 25:67–82
74. Parks DA, Granger DN: Xanthine oxidase: Biochemistry, distribution, and physiology. Acta Physiol Scand Suppl 1986, 548:87–99
75. Battelli MG, Della-Corte E, Stirpe F: Xanthine oxidase type D (dehydrogenase) in the intestine and other organs of the rat. Biochem J 1974, 126:747–749
76. Muxfeldt M, Schaper W: The activity of xanthine oxidase in heart and liver of rats, guinea pigs, pigs, rabbits, and human beings. Circulation 76 (Supp IV) 1987, 198
77. Eddy LJ, Stewart JR, Jones HP, Engerson TD, McCord JM, Downey JM: Free radical-producing enzyme, xanthine oxidase, is undetectable in human hearts. Am J Physiol 1987, 253:H709–H711