1992

Histochemical demonstration of endothelial superoxide and hydrogen peroxide generation in ischemic and reoxygenated rat tissues

Charles F. Babbs
Purdue University, babbs@purdue.edu

Meloney D. Cregor

Stephen F. Badylak

Histochemical demonstration of endothelial superoxide and hydrogen peroxide generation in ischemic and reoxygenated rat tissues

Charles F. Babbs, Meloney D. Cregor, and Stephen F. Badylak

Biomedical Engineering Center, Purdue University, West Lafayette, Indiana, USA.

[Cardiovascular Research 1992; Vol 26: 593-602]

Abstract

Objective: The aims were to test and evaluate two novel and independent histochemical methods for detecting the initial postischemic burst of superoxide and hydrogen peroxide in buffer perfused rat tissues during reflow after 60 min warm ischemia.
Methods: The first is a high manganese/diaminobenzidine technique, in which superoxide oxidises Mn^{2+} to Mn^{3+}, which in turn oxidizes diaminobenzidine to form amber colored polymers, observable by light microscopy. The second is a high iron/diaminobenzidine technique, in which hydrogen peroxide oxidizes diethylenetriaminepenta-acetate chelated Fe^{2+} to form intermediate species, which in turn oxidize diaminobenzidine similarly to Mn^{3+}. Various isolated organs of the rat were rendered ischemic for 60 min, and reperfused with oxygen or air equilibrated buffers containing diaminobenzidine and either Mn^{2+} or Fe^{2+}. Tissues were fixed by perfusion with Trump's solution and processed for light microscopy.
Results: Both manganese and iron methods consistently showed the appearance of reaction product on the luminal surfaces of arterial, capillary, and venular endothelial cells in lung, heart, and intestine of the rat during the first 2 to 3 min of reoxygenation after ischemia. The histochemical reactions were nearly absent in non-manganese treated and non-iron-treated controls. Superoxide dismutase strongly inhibited Mn^{2+}/diaminobenzidine reaction product formation and catalase strongly inhibited Fe^{2+}/diaminobenzidine reaction product formation, when tested in specially perfused lung preparations in which these specific antioxidant enzymes were concentrated.
Conclusions: These histochemical techniques provide direct, visual evidence that a burst of reactive oxygen species is generated in postischemic rat tissues. The Mn^{2+}/diaminobenzidine and Fe^{2+}/diaminobenzidine techniques permit investigation of the endothelium derived reactive oxygen by simple laboratory procedures available to almost any investigator at low marginal cost. The endothelial oxidants so revealed may be of pathophysiological significance in a variety of cardiovascular disorders.

Key words: endothelium; histochemistry; hydrogen peroxide; iron; ischemia; LDL; manganese; oxidants; reperfusion injury; superoxide.

Supported by grant HL-42015 from the National Heart. Lung. and Blood Institute and by a grant from Johnson and Johnson.

Introduction

The intriguing concept of oxygen dependent reperfusion injury has been much discussed and debated [1-6] since the initial description by Hearse et a1. in 1973 of oxygen dependent enzyme release from previously ischemic rat hearts [7]. The potential of the myocardium and other tissues to sustain a form of oxygen mediated injury upon reperfusion, which amplifies primary hypoxic injury during ischemia, has been referred to as the "oxygen paradox" [8]: 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 initiated by partially reduced, reactive oxygen species, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO) [10-14].

The deleterious chemical reactions proposed to occur during reperfusion after ischemia begin with the enzymatic production of superoxide radicals, which either spontaneously or in the presence of superoxide dismutase (SOD) combine to form hydrogen peroxide and dioxygen. Superoxide radicals may be generated in postischemic tissues by NADH dehydrogenase within mitochondria [15, 16], by NADPH oxidase in activated leukocytes [17], or possibly[2, 18, 19], but not necessarily [20], by the action of xanthine oxidase upon xanthine and hypoxanthine substrates that accumulate during ischemia. According to the oxygen radical hypothesis of reperfusion injury, the reintroduction of oxygen during reflow drives these enzymatic reactions to generate a burst of O_2^- radicals and H_2O_2 that may initiate a variety of pathological processes, including lipid peroxidation [14, 21], alterations in capillary permeability [22, 23], chemotaxis of leukocytes [24, 25], and inactivation of mitochondria1 enzymes [26].

Such undesirable effects are especially likely when the increased rate of oxidant formation exceeds the scavenging capacity of physiological defense systems, such as superoxide dismutase and catalase, which may be partially degraded by the ischemic event [27]. Sstudies of isolated perfused hearts using electron spin resonance techniques have provided evidence that increased numbers of oxygen centered free radicals are indeed generated during the first two to three minutes of reoxygenation after ischemia [28-31]. To complement these electron spin resonance studies, we explored a number of histochemical approaches to the detection of reactive oxygen species in perfused organs that would potentially reveal not only the presence of specific oxidants, but also the sites of their formation.

Highly reactive oxygen metabolites are intrinsically difficult to detect, owing to their exceedingly short life times, ranging from roughly a nanosecond for HO, to a millisecond for O_2^- to a few seconds for H_2O_2 [32-33]. Accordingly we concluded that conventional histochemical techniques, in which special stains are applied after the tissue is fixed and sectioned, would be unlikely to demonstrate the sources of reactive oxygen. Instead, we adopted supravital or intravital techniques using perfused organs. The organs were perfused with histochemical reagents which, in the presence of reactive oxygen species, react to form insoluble precipitates that can be observed by light microscopy.

After testing a variety of other routes to the formation of such reaction products we found two independent methods that have given remarkably consistent and mutually confirmatory results. The first is a modification of Karnovsky's manganese/diaminobenzidine technique [34],

previously reported to show superoxide generation by leukocytes. The second is a modification of the high iron/ diamine technique for peroxides [35], suitable for studies of living tissue at physiological pH. In this paper we describe application of these modified histochemical methods to characterize the burst of oxidant production by endothelial cells during reoxygenation of a variety of previously ischemic tissues. The results of our initial survey, obtained using simple light microscopy, have provided internally consistent evidence of endothelial superoxide and hydrogen peroxide generation in postischemic lung, heart, and intestine of the rat.

Methods

Approach to O_2^- histochemistry

In principle, Karnovsky's method [34] for superoxide works as follows. Cells stimulated to produce superoxide are exposed to a physiological solution containing added divalent manganese and diaminobenzidine (DAB). Then,

$$O_2^-\bullet + Mn^{2+} + 2H^+ \rightarrow H_2O_2 + Mn^{3+} \qquad (1)$$

$$Mn^{3+} + nDAB \rightarrow \text{osmiophilic polymer.} \qquad (2)$$

Superoxide, which can act as either an oxidant or a reductant [36], in the present case oxidizes divalent manganese quite rapidly ($k = 6 \times 10^6\ M^{-1}sec^{-1}$) [37] to the trivalent state, with concomitant generation of hydrogen peroxide. This reaction is readily demonstrated in simple spot tests by the rapid color change visible upon addition of granular potassium superoxide to solutions of manganous chloride. Similarly, the ability of manganic ions to oxidize diaminobenzidine may be readily observed by the addition of manganic acetate (in ethanol) to test solutions of diaminobenzidine, reaction (2) probably proceeding by a radical chain mechanism [38, 39]. In optimizing the original manganese/diamine histochemical method for use in perfused organ preparations, we realized that higher manganese concentrations might be required for efficient trapping of superoxide in intact tissues containing superoxide dismutase. The rationale is based upon the fundamental competition between the introduced manganese and native superoxide dismutase for reaction with O_2^-:

$$O_2^-\bullet + Mn^{2+} + 2H^+ \xrightarrow{k_1} H_2O_2 + Mn^{3+}$$

$$(k_1 = 6 \times 10^6\ mol^{-1} \cdot litre \cdot s^{-1}) \qquad (1)$$

$$O_2^-\bullet + SOD \xrightarrow{k_2} 1/2H_2O_2 + 1/2O_2 + SOD$$

$$(k_2 = 1 \times 10^9\ mol^{-1} \cdot litre \cdot s^{-1}). \qquad (3)$$

Because the pseudo-first order rate constant for the latter reaction (3) is three orders of magnitude greater than that for reaction of O_2^- with Mn^{2+} (1), the proportion of nascent superoxide scavenged by Mn^{2+}, namely

$$p = \frac{k_1[Mn^{2+}][O_2^-\bullet]}{k_1[Mn^{2+}][O_2^-\bullet] + k_2[SOD][O_2^-\bullet]} \qquad (4)$$

may be substantially less than unity if the manganese concentration is small. Taking published values for rate constants k_1 and k_2 [37, 40] and estimated tissue superoxide dismutase at 10^{-6} to 10^{-5} M [41, 42], the scavenging efficiency of divalent manganese, p, would be approximately 9%, 50%, and 91% respectively for 1 , 10, and 100 mM Mn^{2+}. Accordingly, we adopted the use of higher manganese concentrations than the 0.5 mM originally described for studies of leukocytes and found the most clear and satisfactory results with 40 mM manganese. We refer to this modification as the high manganese/diamine method.

Approach to H_2O_2 histochemistry

The principle of the iron/diamine technique is that hydrogen peroxide rapidly oxidizes aqueous diethylenetriamine- N,N,N,N",N"-penta-acetate (DTPA) chelated ferrous iron ($k = 1.4 \times 10^3\ M^{-1}sec^{-1}$) [43], which is soluble and relatively stable at physiological pH. Unlike ethylenediaminetetraacetate (EDTA)-Fe^{2+}, nitrilotriacetate- Fe^{2+}, and citrate- Fe^{2+}, the DTPA-ferrous complex does not auto-oxidize rapidly in the presence of molecular oxygen [44]. Thus the ferrous chelate of DTPA can be made fresh prior to an experiment and will remain in the ferrous oxidation state until it is oxidized by H_2O_2 to the ferric state. Ferric iron, in turn, is then able to oxidize diaminobenzidine to form a satisfactory histochemical marker [.44, 45]. The melanin-like diaminobenzidine polymers, created by both the Mn^{2+}/DAB and Fe^{2+}/DAB methods, are widely exploited as sensitive histochemical markers [39, 46]. They are insoluble in aqueous or organic solvents and do not migrate from their original sites of deposition.

In the initial studies reported here, various organs of the rat, including lungs, heart, kidneys, pancreas, and intestine, were perfused with blood free buffer solutions and subjected to a cycle of ischemia and reperfusion with either oxygen or air equilibrated buffers containing Mn^{2+} salts or Fe^{2+}-DTPA and 2.5 mM diaminobenzidine (1 mg/ml). Staining reactions were stopped and the

tissues fixed by perfusion with cold 0.9% saline, followed by cold Trump's fixative, and the tissues were subsequently processed for routine light microscopic examination.

Materials

All inorganic salts were of reagent grade or better. Catalase, DTPA, glutaraldehyde, HEPES (N[2-hydroxyethyl]piperazine- N'-[2-ethanesulfonic acid]), superoxide dismutase (3000 U/mg), and Tris(hydroxymethylaminomethane), were obtained from Sigma Chemical Company (St Louis, MO, USA). 3-3'Diaminobenzidine tetrahydrochloride dihydrate was obtained from Aldrich Chemical Company (Milwaukee, WI, USA).

Solutions

Buffers containing 40 mM divalent manganese in combination with either 80 mM acetylsalicylate or 40 mM trisodium citrate, and buffers containing 10 mM $FeSO_4$ in combination with 10 mM DTPA, were prepared as indicated in Table 1. In the manganese/diaminobenzidine experiments sodium azide (1 mM) was added, as specified by Karnovsky [34] to minimize the spurious formation of diaminobenzidine reaction product by peroxidase [47, 48] and mitochondria1 cytochrome oxidase [39]. In the Fe^{2+}/DTPA experiments 10 mM sodium azide was added to inhibit endogenous catalase, which appeared otherwise to compete strongly with Fe^{2+} for reaction with H_2O_2. The pH was adjusted to 7.4 with drop by drop addition of 0.5 N NaOH with stirring, and the solutions were filtered just before use.

Diaminobenzidine stock solution was made by dissolving 10 mg/L in distilled water and slowly adjusting pH to neutrality with drop by drop addition of 0.5 N NaOH (about 3 ml/100 m1) with vigorous stirring to prevent precipitation of diaminobenzidine by drops of concentrated base. The molarity of the stock solution was 25 mM, and its osmolarity, when titrated to near neutral pH, was approximately 225 mOsmol/L. A working solution containing 2.5 mM diaminobenzidine (1 mg/ml) and either manganous or ferrous salts was prepared by mixing the 10 mg/ml diaminobenzidine stock solution and buffer in a ratio of 1:9. The calculated osmolarity of the working solutions was in the physiological range of 280 to 300 mOsmol/L. After organ perfusion, the collected effluent was detoxified before disposal by the addition of saturated potassium dichromate solution to oxidize residual diaminobenzidine [49].

Transendothelially perfused lungs

According to a protocol approved the Purdue animal care and use committee, male Wistar derived rats, weighing 350 to 400 g, were anaesthetized with intraperitoneal pentobarbitol sodium (60 mg/kg). The trachea of each rat was intubated with a Teflon cannula. The chest was opened with heavy scissors and the sternum and ventral portions of the ribs removed to expose the heart and lungs. The pericardium was incised and the pulmonary artery cannulated with a blunted 16 g needle via a puncture wound in the right ventricle. A solution of 0.9% NaCl at 37 ^{o}C, previously bubbled and equilibrated with 100% oxygen, was perfused through the pulmonary artery cannula, at which time the right atrial appendage was excised to promote systemic venous drainage. Perfusion continued for a total volume of 100 ml, the lungs at this point being completely white and free of blood.

The aortic root was then ligated to oppose left heart outflow. Pulmonary artery perfusion was then continued at a rate of *5* ml/min with 30 ml of warm, oxygen saturated buffer until a steady, clear flow of effluent from the tracheal cannula was observed, during which time both lungs became opalescent and completely fluid filled. At this point "transendothelial perfusion" of the lung was established, during which gas in the alveoli and airways was displaced with protein free perfusate that drained through the tracheobronchial tree. To minimize the volume and cost of perfusate in the experiments using superoxide dismutase, the right pulmonary hilus was ligated, and only the left lung was subsequently perfused.

Table 1 Compositions and osmolarities of blood free buffer solutions for histochemical studies of superoxide and hydrogen peroxide generation in perfused organs of the rat. Values in mmol/L, mOsm/L.#

Salt	*Fe-DTPA*	*High Mn ASA*	*No Fe DTPA*	*No Mn ASA*
NaCl	80(160)	0(0)	90(180)	60(120)
$MnCl_2$	0(0)	40(120)	0(0)	0(0)
$FeSO_4$ and/or DTPA	10(80)	0(0)	10(80)	0(0)
ASA sodium salt	0(0)	80(160)	0(0)	80(160)
$CaCl_2$	2(6)	2(6)	2(6)	2(6)
KCl	4(8)	4(8)	4(8)	4(8)
Tris	0(0)	4(7)	0(0)	4(7)
HEPES	4(6)	0(0)	4(6)	0(0)
NaN_3	10(20)	1(2)	10(20)	1(2)
Calculated osmolarity	(290)	(303)	(290)	(303)

Values in parentheses are osmolarities at pH 7.4. Abbreviations: ASA = acetylsalicylic acid; DTPA = diethylenetriamine-N,N,N',N",N"-pentaacetate; HEPES= N[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]; Tris = trishydroxymethylaminomethane.Tris and HEPES buffers were used in lieu of phosphate buffer to avoid precipitation of Mn^{2+} and Fe^{2+} ions by phosphate. HEPES was used in the ferrous DTPA experiments to avoid precipitate formation that occurred in preliminary experiments with Tris. In a minority of experiments iso-osmotic trisodium citrate was substituted for ASA sodium salt, without influence on the results.

We employed this "transendothelial" perfusion scheme because it offers two specific advantages in the histochemical study of pulmonary ischemia and reoxygenation. First, because air is excluded from the alveoli, it is possible to create well defined states of profound hypoxia and reoxygenation by manipulation of the oxygen tension in the perfusate. Second, because the transendothelial perfusion scheme guarantees contact of histochemical reagents with both endothelial and epithelial cell types, the model is particularly well suited to identifying the various lung cell types that form superoxide. In a classical perfused and ventilated lung model, the epithelial cells might not have been sufficiently exposed to the histochemical reagents.

Buffer perfused hearts

Rats were anaesthetized as in perfused lung preparations and the hearts excised as follows. A number 2 silk suture was placed loosely around the aortic root, and the aorta 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% NaCl solution. Myocardial contractions ceased within 5 sec. The cannula was secured with the silk suture and mounted on a stopcock. Then the heart was flushed with 30 ml of warm oxygenated Tris buffer solution (without Mn^{2+}, Fe^{2+}, or diaminobenzidine) to clear coronary circulation of blood.

Buffer perfused abdominal organs

The chest and abdomen of each anaesthetized rat were opened in the midline with heavy scissors. The descending thoracic aorta was cannulated with a blunted 16-guage needle, such that the catheter tip was at the level of the diaphragm. The abdominal circulation was perfused with 100 ml of Tris buffer solution at 37 ^{o}C, the right atrial appendage having been excised to promote drainage of venous effluent, which was removed by suction.

Ischemia-reoxygenation protocol

In all three organ preparations ischemic anoxia was initiated by first perfusing with warm, 37 ^{o}C, argon equilibrated, 4 mM Tris or HEPES buffer solution without manganese or iron (Table 1). Perfusion was then stopped for 60 min, during which time the tissue was surrounded by warm argon equilibrated buffer solution, which either was irrigated through the thoracic or abdominal cavity for lung and abdominal organ preparations, or surrounded the heart in a sealed specimen jar at 37 ℃ for isolated heart preparations.

After 60 min ischemia, histochemical staining was performed in situ by vascular perfusion with either Mn^{2+}/ DAB buffer, or Fe^{2+}/DAB buffer (Table 1), previously bubbled vigorously for *5* min with 100% oxygen gas in the Mn^{2+}/DAB experiments (to give an oxygen tension of 93 kPa) [50] or shaken with room air in the Fe^{2+}/DAB experiments (to give an oxygen tension of 20 kPa). Room air was used in the Fe^{2+}/DAB experiments to minimize auto-oxidation of ferrous iron. Experimental and theoretical studies in a related model of ischemia and reperfusion showed little difference in oxygen mediated injury during reoxygenation at 20 vs. 93 kPa [50]. Organs were perfused with a hand held syringe at rates sufficient to give normal arterial pressures in

preliminary experiments in which pressure was measured using a solid state pressure transducer. Reperfusion with histochemical reagents was continued for 3 min in lungs and for 2 min in hearts. This 2 to 3 min time window represents the earliest phase of reoxygenation, during which others have found evidence of free radical generation by electron spin resonance spectroscopy [28-31].

After in situ histochemical staining, the perfused vascular beds were flushed with chilled (6 °C) 0.9% NaCl to remove excess diaminobenzidine, and the tissues then fixed by perfusion with chilled Trump's solution (1 % glutaraldehyde, 4% formaldehyde, 90 mM phosphate, pH 7.4). Tissues were stored in vials of fixative overnight and processed for light microscopy.

Microscopy

All tissues were dehydrated in graded alcohol solutions, embedded in paraffin wax, sectioned, dewaxed, and mounted on glass slides. Sections were stained routinely with hematoxylin and eosin and in some experiments with methyl green. Black and white photomicrographs were taken using 35 mm Kodak T-max black and white print film. Prints were made on Ilford multigrade paper.

Controls

In the present initial studies of various tissues of the rat, not all controls were done for each tissue type. Instead, tissues that were technically best suited to a particular type of control study were identified. These included (1) nonischemic hearts, in which histochemical perfusion was performed immediately after cannulation of the aortic root; (2) non-reoxygenated hearts, in which reperfusion after ischemia was done with argon saturated rather than oxygen saturated Mn^{2+}/DAB or Fe^{2+}/DAB buffers; (3) non-manganese-treated and non-iron-treated hearts or lungs, in which there was isotonic substitution of sodium for either manganese or iron in the postischemic perfusate; and (4) superoxide dismutase and catalase treated lungs, in which specific enzymatic scavengers of either O_2^- or H_2O_2 were added to the reoxygenation perfusate. The isolated heart was selected for non-ischemic controls, because this tissue can be rapidly harvested with minimal ischemia time and minimal physical disturbance of vascular endothelial cells that might cause their activation. The transendothelially perfused lung was selected for studies of superoxide dismutase and catalase, because these high molecular weight enzymes tend to become concentrated in the pulmonary vasculature of this preparation. (Note: Oxygen or argon equilibration of buffers must be done by bubbling the gas in solutions using large diameter tubing, which makes large diameter bubbles. Small bubbles tend to persist and become trapped in blood vessels, blocking continued perfusion.)

Morphometry

Vascular profiles of hematoxylin-eosin counterstained slides were examined by light microscopy at 100 X 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 (Mn^{2+}/DAB) or gray-black (Fe^{2+}/DAB) 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 a 800 x 800 μ test area, 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 [51, 52]. The tissue on a 1 v 3 inch glass slide was scanned in vertical strips at successive 2.5 mm horizontal increments from left to right. Such systematic sampling with a random start is superior to purely random sampling, because it yields smaller errors [51].

To account for possible differing degrees of lung inflation in the analysis of lung specimens, counts of vascular profiles were divided by the measured fraction of lung parenchyma (typically about 0.5) and expressed as the number of positive vessels per square centimeter of parenchymal tissue. The areal fraction of pulmonary parenchyma, defined here as all lung tissue excluding airways, alveoli, and intravascular spaces, was measured as the fraction of approximately 400 test points in the counting frame overlying parenchymal structures at systematically spaced horizontal and vertical levels of the specimen.

The mean numerical densities of positively stained arteries, capillaries, and veins per cm^2 were then tabulated for the various tissues undergoing 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 [53], and the analysis of variance repeated on the transformed data to test the null hypothesis that the observed densities of stained vessels were not different among tissues in the various experimental and control groups. Since density of positively stained capillaries produced by manganese ASA buffer was not significantly different from that of manganese citrate buffer, results from both buffers were pooled for later analysis. 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 0.01 or less was considered significant.

Results

Spot tests of the methods

Buffer solutions containing 40 mM Mn^{2+}/2.5 mM diaminobenzidine readily produced voluminous brown-black precipitates after addition of authentic granular potassium superoxide. Similarly, buffer solutions containing 10 mM Fe^{2+}-DTPA/2.5 mM diaminobenzidine readily produced voluminous brown precipitates after drop by drop addition of 3% hydrogen peroxide solution. Both of these spot test reactions caused the water clear Mn^{2+} or Fe^{2+}/DAB solutions to become completely opaque within seconds. Superoxide dismutase (1 mg/ml) inhibited formation of the Mn^{2+}/DAB reaction product in vitro. Catalase (1.2 mg/ml) greatly reduced formation of the Fe^{2+}/DAB reaction product in vitro. Reaction product did not form upon addition of hydrogen peroxide to Mn^{2+}/DAB solution. As expected on the basis of spontaneous dismutation,

however, reaction product did form upon addition of potassium superoxide to Fe-DTPA/DAB solution, but when catalase (1.2 mg/ml) was added to Fe-DTPA/DAB solution (without azide) prior to potassium superoxide, the amount of reaction product formed was markedly less. These simple tests of prepared buffer solutions confirmed the specificity of the two techniques for O_2^- and H_2O_2 respectively.

Results in lung -- production of the reaction products in situ

Figures 1 and *2* illustrate the fundamental phenomena of reaction product formation in pulmonary arteries, capillaries, and veins of the rat in sections of rat lungs rendered ischemic for one hour and reperfused, as just described, with either Mn^{2+}/DAB containing buffer (fig 1) or Fe^{2+}/DAB containing buffer (fig 2). In fig 1(A) a larger pulmonary artery is shown adjacent to epithelial lined bronchus at low magnification. A ring of dark reaction product is closely associated with the pulmonary vascular endothelium. In fig 1(B) pulmonary capillaries are shown at higher magnification. Dark granular reaction product is adherent to the walls of the capillary lumens. In fig l(C) the luminal faces of larger pulmonary veins are similarly coated with reaction product. Comparison of figs 1 and *2* reveals that remarkably similar staining patterns are created by the companion histochemical techniques for O_2^- and H_2O_2 in pulmonary arteries, capillaries, and veins.

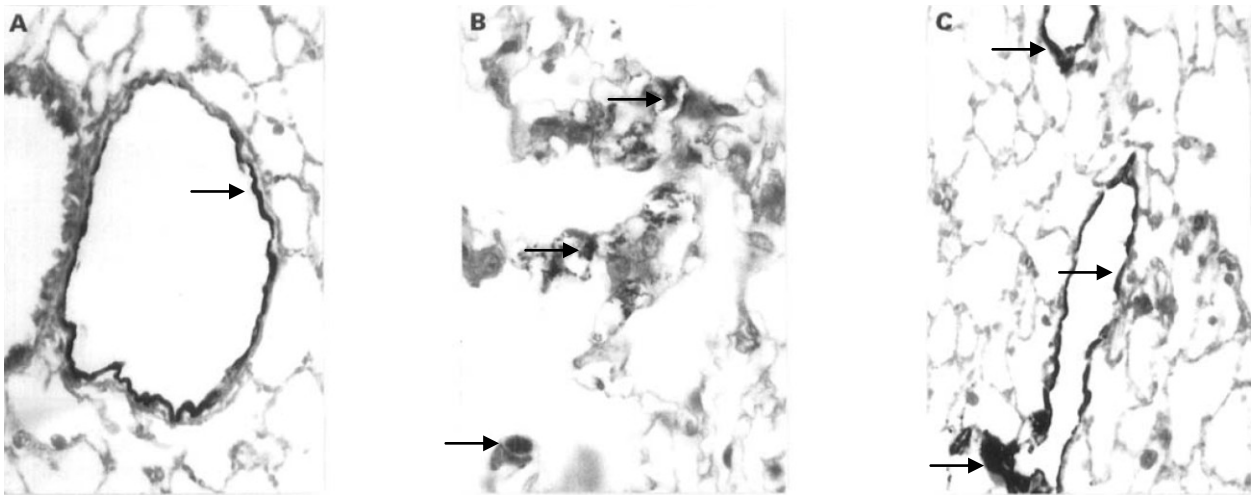

Figure 1 Photomicrographs showing results of the high manganese/diamine histochemical procedure for superoxide formation in rat lung during 3 min of reoxygenation after 60 min ischemia. Rut lungs were rendered ischemic for one hour and then reperfused with Mn and diaminobenzidine (DAB) containing buffer: (A) Pulmonary artery is shown adjacent to a bronchus. A ring of dark reaction product is closely associated with the pulmonary artery endothelium (arrow). 100X . (B) Dark granular reaction product coats walls of alveolar capillaries. 400X. (C) Dark reaction product coats the luminul surfaces of large pulmonary veins seen in longitudinal and cross sections. 100X. At the microscope the Mn/DAB reaction product was amber brown against the background of the methyl green counterstain.

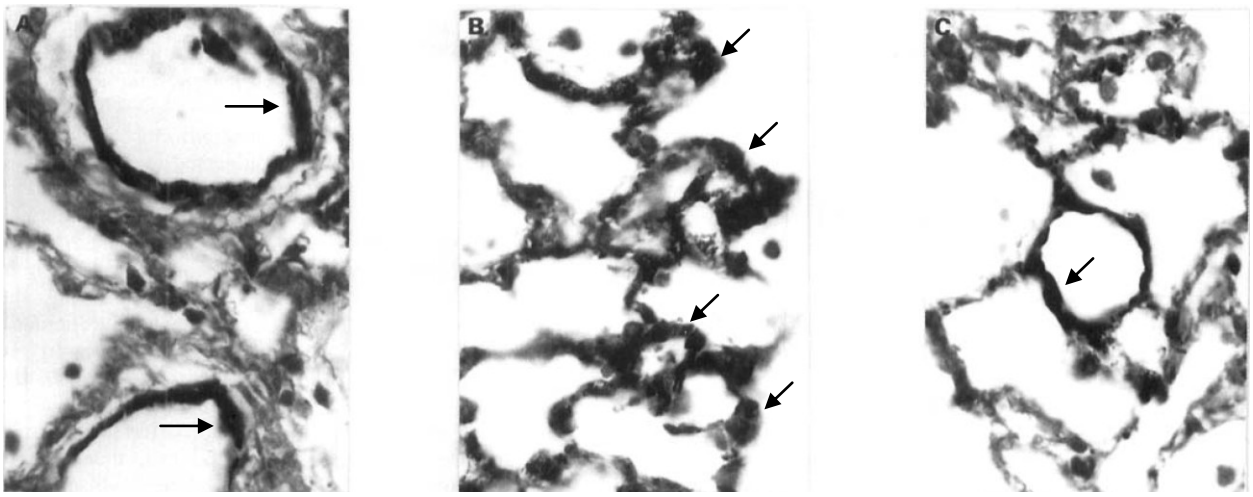

Figure 2 Photomicrographs illustrating results of the ferrous DTPA/DAB procedure for hydrogen peroxide formation in transendotheially perfused rat lungs during reoxygenation after 60 min ischemia. Dark reaction product in a branched pulmonary artery (A), pulmonary capillaries (B), and pulmonary venule and nearby capillaries (C) is similarly distributed to the Mn/DAB reaction product in fig 1. At the microscope the Fe/DAB reaction product was brownish black. Methyl green counterstain. 400X.

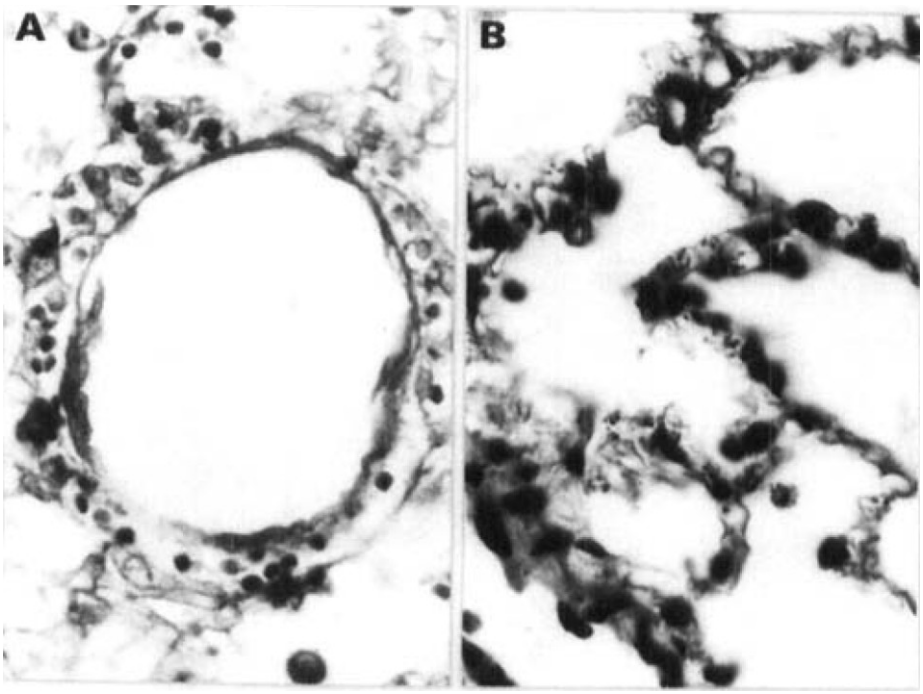

Figure 3 Superoxide dismutase (SOD) and catalase treated control lungs. (A) Pulmonary venule and nearby capillaries from a SOD treated rat lung (3000 U=l mg/ml SOD in perfusates during both ischemia and reflow) subjected to an otherwise identical ischemia-reoxygenation protocol to the animal in fig 1 using the Mn/DAB technique for superoxide. No reaction product is seen in the SOD treated control vessels, a finding characteristic of all sections of lung from this animal. Methyl green counterstain. 400X. (B) Lung from a catalase treated rat (13,000 U=1.2 mg/ml catalase in perfusates) subjected to an otherwise identical ischemia-reoxygenation protocol to the animal in fig 2 using the ferrous DTPA/DAB technique for hydrogen peroxide. Scattered punctate deposits of reaction product are seen in the catalase treated control capillaries (centre); however the density of reaction product is in capillaries markedly less than in non-catalase treated experimental lungs (fig. 2(B)). Haematoxylin-eosin counterstain. 400X.

Figure 3 shows pulmonary vessels from rats treated separately with buffer containing either superoxide dismutase (3000 Units/ml), during both ischemia and reperfusion with Mn^{2+}/DAB (A), or catalase (13,000 Units/ml and 0 mM azide), during both ischemia and reperfusion with Fe^{2+}/DAB (B). The two specific antioxidant enzymes greatly attenuated reaction product formation, providing strong evidence for the specificity of the histochemical reactions for superoxide and hydrogen peroxide, respectively.

Results of morphometric analysis of ischemic and reoxygenated rat lungs in the absence or presence of superoxide dismutase, catalase, Mn^{2+}, or Fe^{2+} are presented in fig 4*. The numbers of ischemic and reoxygenated vessels that were stained positively with the companion techniques for O_2^-. and H_2O_2 were quite similar. The addition of highly selective enzymatic scavengers of either O_2^- or H_2O_2 (superoxide dismutase and catalase) sharply attenuated reaction product formation, measured as the observed density of positively stained vessels.

There was also minimal reaction product in pulmonary parenchyma rendered ischemic for 60 min and reperfused with diaminobenzidine containing buffer, in which sodium was isotonically substituted for either Mn^{2+} (minus Mn control) or Fe^{2+} (minus Fe control). Thus when either specific probe, Mn^{2+} or Fe^{2+}, was omitted from the perfusate, reaction product formation was nearly eliminated. Similar results were obtained when Zn^{2+} was substituted for Fe^{2+} in the Fe^{2+}/DAB procedure. These control experiments exclude simple polymerization of diaminobenzidine by tissue peroxidases or mitochondrial cytochrome oxidase as a major mechanism of endothelial reaction product formation. These controls also show that ischemic endothelial cells do not take up diaminobenzidine simply by virtue of having been ischemic.

---

* Note: stereological theory predicts that the measured intersection densities in figures 4 and 7 are exactly one half the length densities (total length per unit volume) of stained vessels, assuming that the various vessels are randomly orientated in space and that they assume all orientations with equal likelihood. These assumptions would appear approximately true for all but the largest vessels, which have a trivial influence on the final results.

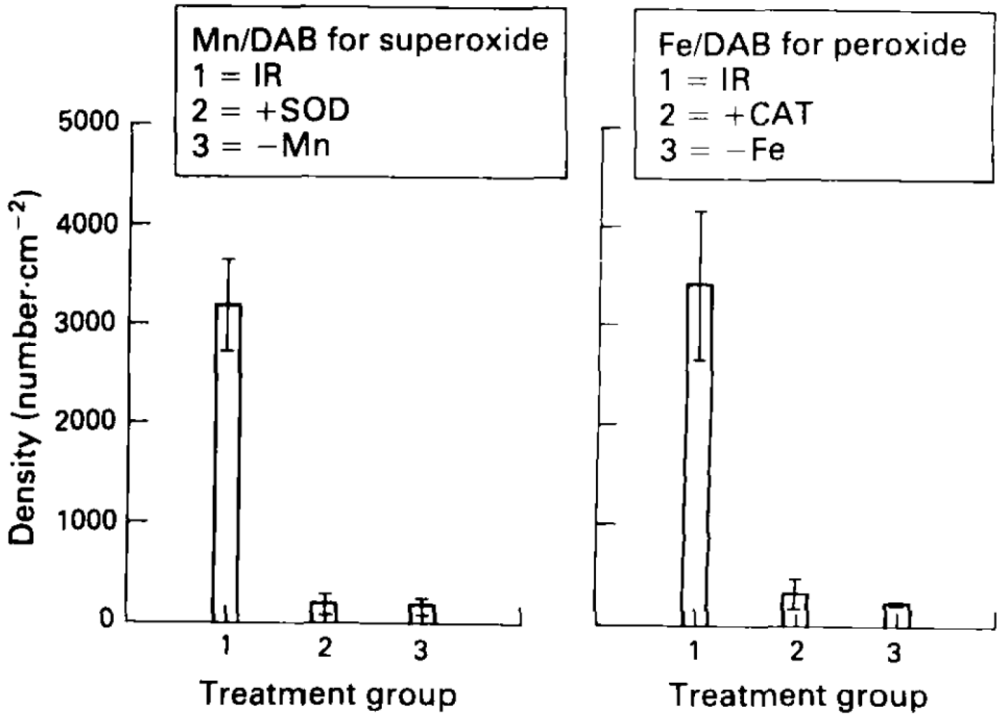

Figure 4 Quantitative morphometric results for transendotheially perfused rat lungs subjected to a cycle of ischemic anoxia and reperfusion with histochemical reagents to detect the production of superoxide or hydrogen peroxide. Treatment groups include ischemia and reperfusion (IR). IR with superoxide dismutase (+SOD), IR with catalase (+CAT), IR minus manganese (-Mn), and IR minus ferrous iron (-Fe). Vascular profiles of hematoxylin and eosin counterstained sections were examined by light microscopy at l00X magnification. Blood vessels containing histochemical reaction product were counted in non-overlapping fields. With the Mn^{2+}/DAB technique for superoxide 9% of all positive vessels were pulmonary arteries, 67% were capillaries, and 24% were venules or veins. With the Fe^{2+}/DAB technique for hydrogen peroxide 2% of all positive vessels were pulmonary arteries, 92% were capillaries and 6% were veins. Columns are mean values for n > 3 rats in each group, bars =standard errors. The total numbers of positively stained vessels (arteries + capillaries +veins) in the ischemia and reoxygenation groups are significantly greater than those in the corresponding antioxidant enzyme treated groups and significantly greater than those in the groups treated without manganese or ferrous probes ($p < 0.01$ for Mn^{2+}/DAB, and $p < 0.01$ for Fe^{2+}/DAB; square root transformed data).

Results in heart - effects of prior ischemia and reoxygenation

Figure *5* (A and B) shows postischemic myocardial capillaries in isolated rat hearts subjected to a cycle of 60 min ischemic anoxia and 2 min reperfusion with either oxygen equilibrated Mn^{2+}/DAB buffer or air equilibrated Fe^{2+}/DAB buffer, respectively. Upon light microscopic examination coronary vessels show similar deposition of endothelium associated reaction product overlying the luminal surfaces of the endothelium with both techniques.

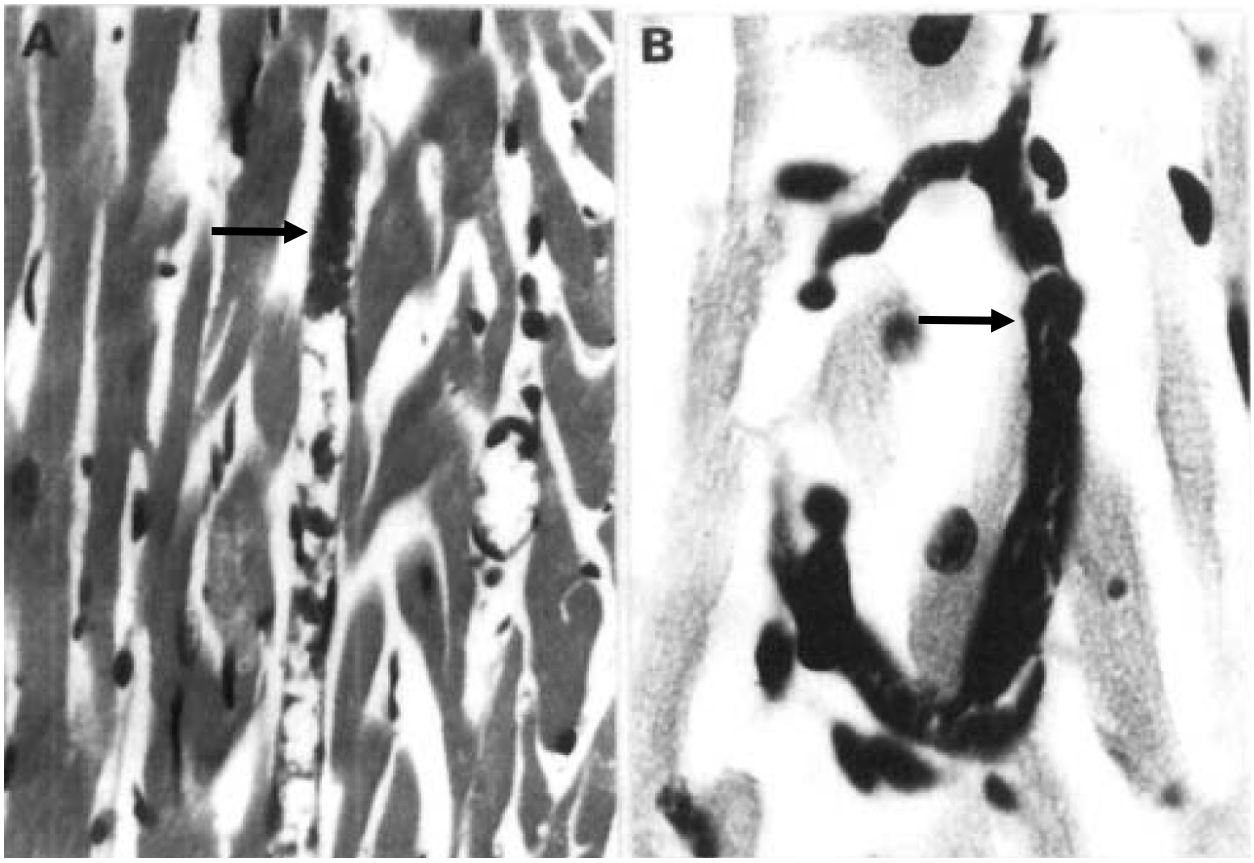

Figure 5 Postischemic myocardium in an isolated rat heart subjected to a cycle of 60 min ischemic anoxia and 2 min reperfusion with oxygen equilibrated, DAB containing buffer, to which was added the molecular probe Mn^{2+} f or superoxide (A) r Fe^{2+}-DTPA for hydrogen peroxide (B). Capillaries containing granular reaction product are shown in longitudinal section. Similar results are obtained with the two histochemical probes. Upon light microscopic examination, coronary vessels show deposition of reaction product on the luminal surfaces of the endothelium. In the examples shown reaction product completely or partially filled the capillary lumens. Haematoxylin-eosin counterstain. Original magnification 400X . Specimen (B) was illuminated with red light for photomicroscopy.

Concretions of reaction product often appeared so voluminous as to nearly fill the lumens of many smaller vessels. With hematoxylin and eosin counterstain Mn^{2+}/ DAB reaction product, indicative of superoxide formation, is amber, and the Fe^{2+}/DAB reaction product, indicative of hydrogen peroxide formation, is charcoal gray to black. Both reaction products are loosely, rather than tightly, associated with the vascular wall and appear to behave physically like a semi-rigid coat of paint on the endothelial surface. Although some functional differences in pulmonary versus systemic vascular endothelial cells are known, for example in angiotensin converting enzyme activity, oxidant formation by rat coronary vessels appears to be similar that of rat pulmonary vessels.

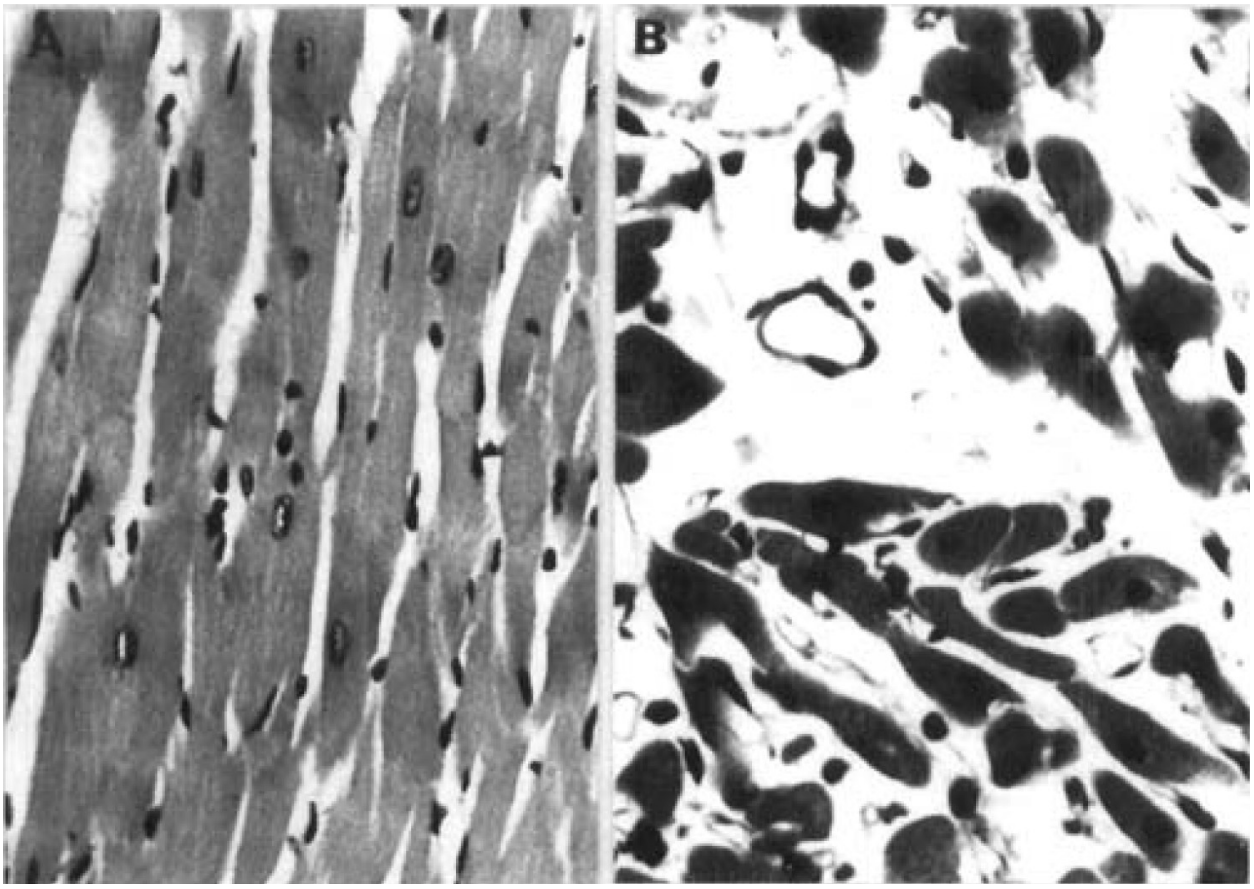

Figure 6 Non-ischemic myocardium. Sections of non-ischemic rat hearts otherwise treated similarly to hearts shown in fig 5 with (A) Mn^{2+}/DAB or (B) Fe^{2+}/DAB histochemical probes. Open capillaries without reaction product are shown in longitudinal section (A) and in transverse section (B). Hematoxylin-eosin counterstain. Absence of prior ischemia sharply reduces endothelial reaction product formation in comparison to that seen in postischemic tissues.

As controls, sections of non-ischemic rat hearts, perfused with the histochemical probes immediately after harvesting, are illustrated in fig 6. Similarly, ischemic hearts reperfused with oxygen depleted (argon bubbled and equilibrated) buffers, containing either Mn^{2+}/DAB or Fe^{2+}/DAB, showed markedly reduced accumulation of reaction product, indicating the oxygen dependence of reaction product formation. In hearts, as in lungs, the quantitative pattern of vascular staining in experimental versus control tissues was similar for the companion histochemical methods (fig 7).

Results in abdominal organs

In similar experiments involving 60 min ischemia and reperfusion of abdominal organs we found abundant postischemic reaction product formation in the microvascular channels of rat small intestine. Postischemic renal golmerular capillaries also appeared to be sources of O_2^- and H_2O_2, but to a lesser extent than pulmonary, coronary, and intestinal vessels. Capillaries within the islets of Langerhans in the pancreas showed moderate postischemic reaction product formation. Thus our initial surveys of various tissues in the rat suggest that endothelial production of superoxide and hydrogen peroxide is a widespread phenomenon, occurring in arterial, venous, and capillary endothelium of pulmonary vessels and in systemic vessels as well.

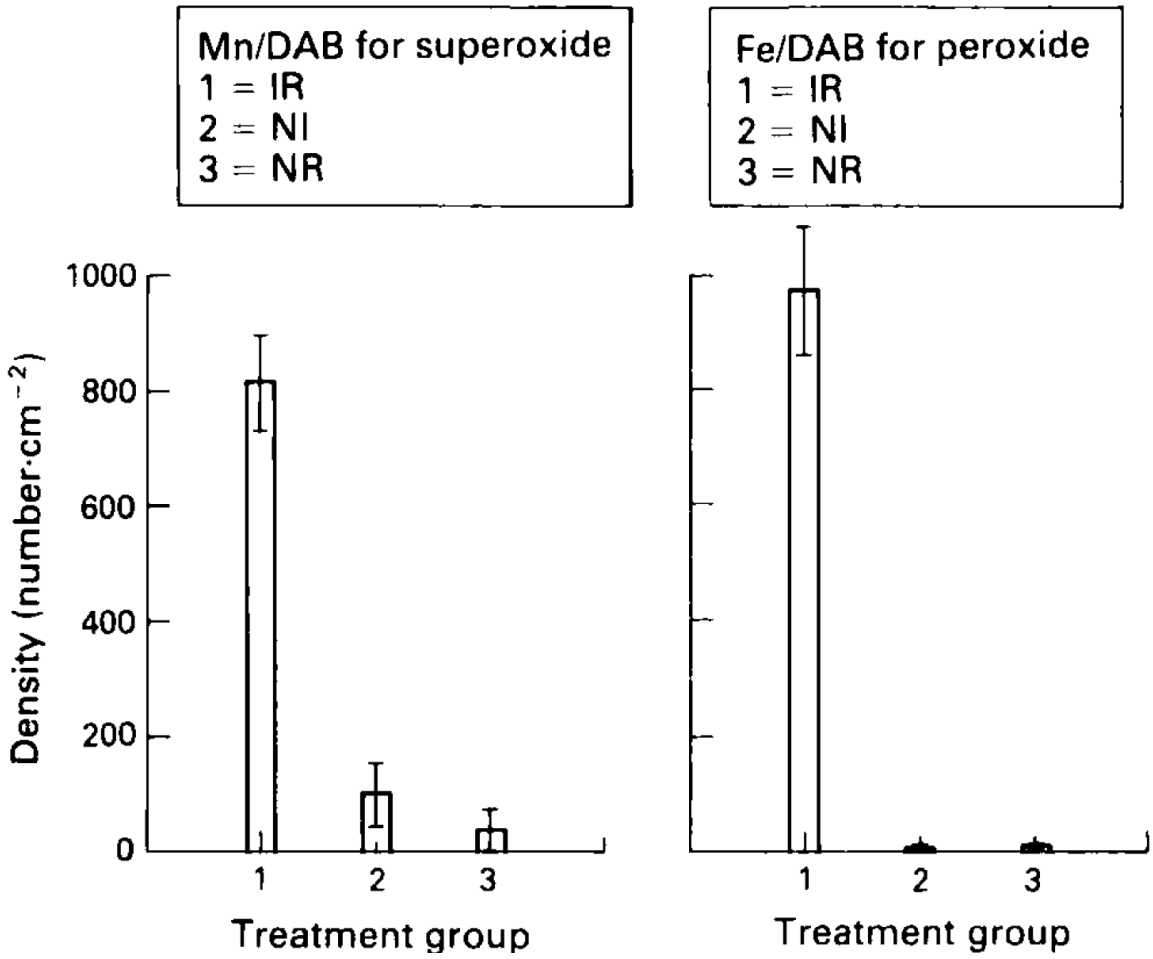

Figure 7 Quantitative morphometric results for isolated perfused rat hearts subjected to a cycle of ischemic anoxia and reperfusion with histochemical reagents to detect the production of superoxide or hydrogen peroxide. Treatment groups include hearts subjected to ischemia and reperfusion (IR), non-ischemic hearts (NI), and non-reoxygenated hearts that were reperfused with argon equilibrated buffer (NR). With the Mn/DAB technique for superoxide, 10% of all positive vessels in IR hearts were coronary, arteries. 85% were capillaries, and 5% were veins. With the Fe/DAB technique for hydrogen peroxide, 4% of all positive vessels were coronary arteries, 91 % were capillaries and 5% were veins. The total numbers of positively stained vessels (arteries + capillaries + veins) in the ischemia and reoxygenation groups are significantly greater than those in the corresponding non-ischemic and non-reoxygenated groups ($p<0.01$ for Mn/DAB, and $p < 0.01$ for Fe/DAB; square root transformed data). Other details similar to fig 4.

Discussion

A major scientific hurdle to the study of free radical mechanisms in the genesis of reperfusion injury has been the lack of a simple, direct demonstration of the existence of reactive oxygen species in affected tissues. As estimated by recently developed computer models of ischemic and reoxygenated tissues [55], the instantaneous, volume averaged concentrations of superoxide and hydrogen peroxide are likely to be extremely low (in the range of a few micromolar) and the half lives of these species are likely to be extremely short (a few seconds at most). To detect these short lived oxidants in tissue we used the preplaced molecular probes, Mn^{2+} and Fe^{2+}, which, when oxidized, could initiate polymerization of diaminobenzidine to form a nearly ideal histochemical marker. This strategy has revealed endothelial sources of potentially toxic oxidants at the light microscopic level with clarity and specificity previously unachieved.

The agreement between the two techniques for O_2^- and H_2O_2 both qualitatively and quantitatively is noteworthy. Since spontaneous or superoxide dismutase catalyzed dismutation of O_2^- to H_2O_2 is certain to occur, the correlation of the results of the manganese/diamine technique with those of the iron/diamine technique strongly suggests that the same biological process is being detected and tends to dispel doubts about possible non-specific interferences that might have created false positive results with either technique alone.

Technically, the high manganese/diamine and high iron/diamine techniques have several desirable features, compared to other potential approaches for studying oxygen radicals in biology. High cost and physically complex equipment are not required. The necessary apparatus is readily available to almost any scientist. The methods are easy to perform, and experiments can be done in a few hours. Tissue processing by routine methods can be done at minimal expense. The tissue markers for superoxide and hydrogen peroxide formation are clearly distinguishable from counterstains and remain stable after mounting, providing permanent records for study. The use of histochemical end points allows the investigator to see and understand discrete cellular sources of reactive oxygen species in structural context. Moreover, although the present studies used light microscopy, the polymeric diaminobenzidine reaction product is osmiophilic and readily observable by electron microscopy as well [34, 47].

The histochemical findings reported here for intact tissues support the previous work of Ryan, Ratych, Zweier, and their respective coworkers in cell cultures [30, 56, 57], suggesting that endothelial cells, although not “professional phagocytes”, can nonetheless both produce and be damaged by bursts of toxic oxidants and can function effectively as phagocytic cells [58]. This specific localization of initial postischemic free radical production to endothelial cells that we observed in situ may be of special pathophysiological importance, because a variety of secondary mechanisms and biochemical cascades -- including edema, microthrombosis, the no reflow phenomenon of Ames [59], and leukocyte infiltration -- could be initiated by such endothelial activation. The importance of leukocyte infiltration, perhaps triggered by endothelial oxidants, has been suggested by recent work of Granger [60, 61] showing reduced reperfusion injury of intestine in animals either treated with anti-leukocyte antibodies or rendered leukopenic. Although in the present study we used a bloodless perfusate, free of leukocytes, platelets, clotting factors, and endogenous inflammatory mediators, we imagine that amplification loops,

mediated by leukocytes and other components of whole blood, could greatly multiply tissue effects of endothelial superoxide and hydrogen peroxide generation.

The localization of superoxide and hydrogen peroxide generation to the outer luminal surfaces of endothelial cells is in agreement with the recent studies of Shlafer and coworkers [62], who used a completely independent technique for histochemical localization of hydrogen peroxide in ischemic and reperfused isolated rabbit hearts. They found a confluent extracellular distribution of cerium reaction product, indicating the presence of H_2O_2, on the luminal surfaces but not on the abluminal surfaces of vascular endothelial cells in a pattern that was virtually identical to the distribution of Mn/DAB and Fe/DAB reaction products in our studies. Formation of the cerium reaction product was profoundly inhibited by added catalase. One technical advantage of the high iron/diamine technique for hydrogen peroxide compared to the cerium technique, however, is that the reaction product is readily visible with the light microscope at minimal expense, whereas the cerium reaction product is readily detected only by electron microscopy.

The most straightforward explanation of these findings is that superoxide is made by a directional cell surface enzyme, and essentially secreted into the extracellular space. Of course, it is possible that superoxide and hydrogen peroxide were detected only in the vascular lumens either because superoxide dismutase and peroxidases (in peroxisomes) destroy these toxic oxidants within cells, or because there was limited penetration of the manganese and ferrous ion probes into cells. Logically, however, if one sees reaction product with the high manganese/diamine or ferrous DTPA/diamine techniques, it suggests that oxidants are being made, but if one does not see reaction product, it does not mean that oxidants are not being made.

Nevertheless, the concept of extracellular superoxide production does help to resolve a paradox in the literature: namely that the high molecular weight proteins, superoxide dismutase and catalase, given intravenously, have been effective in many experiments in protecting against reperfusion injury [63-67], even though it is unlikely that these enzymes efficiently cross the cell membranes to enter intracellular sites of action. If indeed a major fraction of oxygen radical mediated injury is initiated by an intravascular cloud of reactive oxygen species, then intravenous high molecular weight scavengers would constitute rational antiradical therapy.

Conclusion

Superoxide and hydrogen peroxide generation by vascular endothelial cells in the lungs, heart, kidney, intestine, and pancreatic islets of the rat can be demonstrated in situ by two independent histochemical methods during the first two to three minutes of reoxygenation following 60 minutes of ischemia. The results of the present histochemical studies provide direct visual evidence of a burst of reactive oxygen species in a number of postischemic tissues of the rat.

Although the response to endothelial oxidant production in a given in vivo setting will depend upon a balance of many factors present in plasma and in formed blood elements, the ability to visualize the sites of formation of oxygen radicals in simple experimental models by straightforward light microscopic techniques provides a route to the exploration of a variety of

interesting hypotheses about endothelial cell function in health and disease. Quite possibly, a variety of important pathophysiological processes, including microvascular thrombosis, leukocyte infiltration, and low density lipoprotein oxidation may be triggered by endothelium derived reactive oxygen species.

References

1 Gauduel Y, Duvelleroy MA. Role of oxygen radicals in cardiac injury due to reoxygenation. J Mol Cell Cardiol 1984;16:459-70.

2 McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Eng J Med 1985;312:159-63.

3 Burney RE, Walsh D. Kaplan LR, Fraser S, Tung B, Overmyer J . Reperfusion arrhythmia: myth or reality'? Ann Emerg Med 1989:18:240-3.

4 Nayler WG, Elz JS. Reperfusion injury: laboratory artifact or clinical dilemma'? Circulation l986;74:2 15-2 1.

5 Hammond B, Hess ML. The oxygen free radical system: potential mediator of myocardial injury. J Am Coll Cardiol 1985;6: 215-20.

6 Lenz ML, Michael LH, Smith CV. et al. Glutathione disulfide formation and lipid peroxidation during cardiac ischemia and reflow in the dog in vivo. Biocheni Biophys Res Commun 1989;164:722-7.

7 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.

8 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;16:969-85.

9 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;17:675-84.

10 Slater TF. Free radical mechanisms in tissue injury. London: Pion, 1972.

11 Halliwell B. Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press, 1987:206-34.

12 Farber JL, Kyle ME, Colenian JB. Biology of disease: mechanisms of cell injury by activated oxygen species. Lib Invest 1990;62:670-9.

13 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;77:465-85.

14 Freeman BA, Crapo JD. Biology of disease -- free radicals and tissue injury. Lab Invest 1982;47:412-26.

15 Boveris A, Turrens JF. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 1980:191:421-7.

16 Nohl H. A novel NADH-linked superoxide radical generator in heart mitochondria. Fed Eur Biochem Soc 1987:214:269-73.

17 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;57:1059-67.

18 Bucher JR, Tien M, Morehouse LA. Aust SD. Three mechanisms for the formation of an initiator of lipid peroxidation by xanthine oxidase. In: Cohen G. Greenwald RA, eds. Free radicals and their scavenger systems. vol 1. New York: Elsevier Biomedical. 1983:296-9.

19 Chambers DE, Parks DA. Patterson G. et a/. Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol 1985;17: 145-52.

20 Babbs CF, Salaris SC, Turek JJ. Cytochemical studies of hydrogen peroxide generation in postischemic hepatocytes. Am J Physiol 1991;260 (Heart Circ Physiol 29):H123-9.

21 Aust SD, Svingen BA. The role of iron in enzymatic lipid peroxidation. In: Free radicals in biology, vol V. New York: Academic Press, 1982:1-28.

22 Grisham MB, Gaginella TS. VonRitter C, Tamai H, Be RM, Granger DN. Effects of neutrophil-derived oxidants on intestinal permeability, electrolyte transport, and epithelial cell viability. Inflammation 1990;14:531-42.

23 Korthuis RJ, Granger DN, Townsley ML. Taylor AE. The role of oxygen-derived free radicals in ischemia-induced increases in canine skeletal muscle vascular permeability. Circ Res 1985;57:599-609.

24 Curzio M, Esterbauer H, DiMauro C, Cecchini G. Dianzani MU. Chemotactic activity of the lipid peroxidation product 4-hydroxynonenal and homologous hydroxyalkenals. Biol Chem Hoppe Seyler 1986;367:321-9.

25 McCord JM. Oxygen-derived radicals: a link between reperfusion injury and inflammation. Fed P roc 1987;46:2402-6.

26 Southard JH, Senzig KA, Hoffmann RM, Belzer FO. Toxicity of oxygen to mitochondria1 respiratory activity in hypothermically perfused canine kidneys. Transplantation 1980:29:459-61.

27 Ferrari R, Ceconi C, Curello S, et al. Oxygen-mediated myocardial damage during ischemia and reperfusion: role of the cellular defenses against oxygen toxicity. J Mol Cell Cardiol 459-70.

28 Garlick PB. Davies MI. Hearse DJ. Slater TF. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ Res 1987;61:757-60.

29 Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium Proc Natl Acrid Sci USA I987;84:1404-7.

30 Zweier JL. Kuppusamy P. Lutty GA. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tisisue. Proc Natl Acad Sci USA 1988;85:4046-50.

31 Baker JE, Felix CC. Olinger GN. Kalyanaranian B. Myocardial ischemia and reperfusion: direct evidence for free radical generation by electron spin resonance spectroscopy. Proc Natl Acad Sci USA 1988:85:2786-9.

32 Dorfman LM. Adams GE. Reactivity of the hydroxvl radical in aqueous solutions. National Standard Reference Data Series 46 (NSRDS-NBS46). Washington DC: US Government Printing Office, 1973. US National Bureau of Standards.

33 Halliwell B, Grootveld M. Gutteridge JMC. Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. Metthod Biochem Anal 1987;33:59-90.

34 Briggs RT, Robinson JM. Karnovsky ML. Karnovsky MJ. Superoxide production by polymorphonuclear leukocytes. Hisrochernistry 1986;84:37 1-8.

35 Pearse AGE. Histochemistry theoretical and applied, 3rd ed. London: Churchill Livingstone, 1972.

36 Aust SD. Morehouse LA, Thomas CE. Hypothesis paper -- role of metals in oxygen radical reactions. J Free Rad Biol Med 1985;1:3-25.

37 Kono Y, Takahashi M. Asada K. Oxidation of manganous pyrophosphate by superoxide radicals and illuminated spinach chloroplasts. Arch Biochem Biophys 1976:174:454-62.

38 Walling C. Free radicals in solution. New York: John Wiley and Sons, 1957.

39 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.

40 Steinman HM. Superoxide dismutases: protein chemistry and structure-function relationships. In: Oberley LW, ed. Superoxide dismutase, vol 1. Boca Raton: CRC Press, 1982:11-68.

41 Fridovich 1. Superoxide dismutases. Annu Rev Biochem 1975:44:147-59.

42 Marklund SL. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. Biochem J 1984:222:649-55.

43 Rahhal S, Richter HW. Reduction of hydrogen peroxide by the ferrous iron chelate of diethylenetriamine-N,N,N',N",N"- pentaacetate. J Am Chern Soc 1988;110:3 126-33.

44 Cohen G. Lewis D. Oxygen consumption during the Fenton-type reaction between hydrogen peroxide and ferrous-chelate (Fe^{2+}- DTPA). J Inorg Biochem 1981:15:143-51.

45 Cohen G. The Fenton reaction. In: Greenwald RA, ed. Handbook of methods for oxygen radical research. Boca Raton: CRC Press. 1985:55-64.

46 Burstone MS. New histochemical techniques for the demonstration of tissue oxidase (cytochrome oxidase). J Histochem Cytochem 1959;7:112-22.

47 Graham 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 Histoehem Cytochem 1966;14:291-301.

48 Fahimi HD. Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). J Histochem Cytochem 1968;16:547-50.

49 Morrell JI. Greenberger LM. Pfaff DW. Comparison of horseradish peroxidase visualization methods. J Histoehem Cytochem 1981:29:903-16.

50 Salaris SC, Babbs CF. The effect of oxygen concentration on the formation of malondialdehyde-like material in a model of tissue ischemia and reoxygenation. Free Radical Biol Med 1989:7: 603-9.

51 Weibel ER. Stereological methods 1. London: Academic Press, 1979.

52 Elias H, Hennig A, Schwartz DE. Stereology: applications to biomedical research. Physiol Rev 1971;51:158-200.

53 Anderson VL, McLean RA. Transformations of Y. In: Design of experiments: a realistic approach. Marcel Dekker. New York: Marcel Dekker. 1974:23.

54 Smirnov VN. Vadim S, Tkachuk VA, Chazov El. Vascular endothelium and atherosclerosis: a multidisciplinary approach. In: Ryan US, ed. Endothelial Cells vol III. Boca Raton: CRC Press, 1988:139-216.

55 Babbs CF, Steiner MG. Simulation of free radical reactions in biology and medicine: a new two-compartment kinetic model of intracellular lipid peroxidation. Free Radical Biol Med 1990;8:471-85.

56 Ryan US, Vann JM. Endothelial cells: a source and target of oxidant damage. In: Simic MG, ed. Oxygen radicals in biology and medicine. New York: Plenum Press, 1988:963-74.

57 Ratych RE, Chuknyiska RS, Bulkley GB. The primary localization of free radical generation after anoxia-reoxygenation in isolated endothelial cells. Surgery 1987;102:122-31.

58 Ryan US. Endothelial cell activation responses. In: Pulmonary endothelium in health and disease. Basel: Marcel Dekker, 1987:3-33.

59 Ames A, Wright L, Kowada M, Thurston JM, Majno G. Cerebral ischemia II: The no-reflow phenomenon. Am J Pathol 1968;52:437-8.

60 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:H699-703.

61 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;67:1016-23.

62 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.

63 Parks DA, Bulkley GB, Granger DN. Role of oxygen free radicals in shock, ischemia, and organ preservation. Surgery 1983;94:428-32.

64 Parks DA, Bulkley GB, Granger DN. Hamilton SR, McCord JM. Ischemic injury in the cat small intestine, role of superoxide radicals. Gastroenterology 1982;82:9-15.

65 Dalsing MC, Grossfeld JL, Shiffler MA, et al. Superoxide dismutase: a cellular protective enzyme in bowel ischemia. J Surg Res 1983;34:589-96.

66 Fincke U, Schneider J, Friderichs E, Giertz H, Flohe L. Recombinant human superoxide dismutase (R-HSOD) enhances the myocardial salvage after fibrinolytic recanalization in canine coronary artery thrombosis (abstract). 4th International Congress on Oxygen Radicals.

University of California at Sun Diego La Jolla, June 27-July 3, contributed abstracts, 1987: 142-3.

67 Jolly SR, Kane WJ, Baillie MB, Abrams GD, Lucchesi BR. Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 1984;54:277-85.