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Direct Measurement of Myocardial Free Radical Generation in an In Vivo Model: Effects of Postischemic Reperfusion and Treatment With Human Recombinant Superoxide Dismutase

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Objectives. The purpose of this study was to determine whether postischemic reperfusion of the heart in living rabbits induces a burst of oxygen free radical generation that can be attenuated by recombinant human superoxide dismutase administered at the moment of reflow.

Background. This phenomenon was previously demonstrated in crystalloid perfused, globally ischemic rabbit hearts.

Methods. Thirty-two open chest rabbits were assigned to one of four groups of eight animals each: Group I (control animals), no coronary artery occlusion; Group II, 30 min of circumflex marginal coronary artery occlusion without reperfusion; Group III, 30 min of coronary occlusion followed by 60 s of reperfusion, and Group IV, 30 min of coronary occlusion followed by treatment with recombinant human superoxide dismutase (a 20-mg/kg body weight bolus 90 s before reperfusion and a 0.17-mg/kg infusion during 60 s of reperfusion). Full thickness biopsy specimens taken from the ischemic region were then rapidly freeze clamped and electron paramagnetic resonance spectroscopy was performed at 77°K.

Results. Three radical signals similar to those previously identified in the isolated, crystalloid perfused rabbit heart were observed: an isotropic signal with $g = 2.004$ suggestive of a semiquinone, an anisotropic signal with $g_{\parallel} = 2.033$ and $g_{\perp} = 2.005$ suggestive of an oxygen-centered alkyl peroxy radical, and a triplet with $g = 2.000$ and $a_N = 24$ G suggestive of a nitrogen-centered radical. In addition, a fourth signal consistent with an iron-sulfur center was seen. The oxygen-centered free radical concentration during normal perfusion (Group I) was 1.8 ± 0.8 μmol compared with 4.4 ± 0.9 μmol after 30 min of regional ischemia without reperfusion (Group II) and 13.0 ± 2.5 μmol after 60 s of reperfusion (Group III) ($p < 0.05$ among all three groups). In contrast, superoxide dismutase treated-rabbits (Group IV) demonstrated a peak oxygen radical concentration of only 5.9 ± 1.2 μmol ($p < 0.05$ vs. Group III).

Conclusions. This study demonstrates that reperfusion after regional myocardial ischemia in the intact rabbit is associated with a burst of oxygen-centered free radicals. The magnitude of this burst is greater than that seen after a comparable duration of global ischemia in the isolated, buffer-perfused rabbit heart preparation and is significantly reduced by superoxide dismutase administration begun just before reflow.

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The development of thrombolytic agents has allowed early reperfusion of myocardium jeopardized by acute coronary thrombosis to become a clinical reality. Recent studies have demonstrated that such early restoration of myocardial blood flow can preserve regional cardiac function (1–3) and decrease mortality (4–8). However, despite these apparent benefits, there is evidence suggesting that postischemic reperfusion can also cause further tissue damage (9–12). Thus, the overall positive effect of reperfusion may be the difference between the benefit obtained by terminating the ischemic period and the deleterious effects of "reperfusion injury." The generation of oxygen free radicals at the time of reperfusion is one of the mechanisms that has been proposed to explain this phenomenon (13). This hypothesis is supported by animal studies that demonstrate that superoxide dismutase, an oxygen radical scavenger, administered at the moment of reperfusion can decrease myocardial infarct size (14–16) and enhance functional and metabolic recovery (17–21) after an ischemic insult. However, several studies (22–24) have failed to demonstrate such benefit from superoxide dismutase administration, indicating that the dose, blood level, timing of enzyme administration and severity of the ischemic

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insult, alone or in combination, may be critical for such benefit to be realized.

Electron paramagnetic resonance spectroscopy was recently used to demonstrate that a burst of oxygen radical generation occurs in the isolated, crystalloid perfused, globally ischemic rabbit heart during the 1st min of postischemic reperfusion (25) and that this burst can be attenuated by the administration of recombinant human superoxide dismutase (26). However, there are important differences between the isolated heart preparation and the intact animal. Crystalloid perfusate does not contain red blood cells, leukocytes or plasma components and has a high partial pressure of oxygen (Po_2). Clinical myocardial infarction also entails regional, not global, ischemia. Finally, superoxide dismutase might not be as efficacious in the intact animal as it is in the isolated heart. Therefore, the purpose of this study was 1) to develop an in vivo model of regional myocardial ischemia and reperfusion in which free radical concentrations could be measured directly, 2) to determine whether the oxygen radical burst associated with reperfusion in the isolated heart preparation also occurs in the intact animal, and 3) to determine the effectiveness of superoxide dismutase in scavenging postischemic oxygen radical generation in vivo.

Methods

In vivo model of regional myocardial ischemia. New Zealand White male or female rabbits, 4 to 5 kg, were anesthetized with intravenous pentobarbital, 50 to 80 mg/kg body weight, and intraperitoneal phenobarbital, 100 mg/kg. The animals then underwent tracheostomy and mechanical ventilation supplemented with a mixture of 95% oxygen and 5% carbon dioxide. Respiratory settings and gas mixtures were adjusted to maintain an arterial Po_2 of 90 to 150 mm Hg and an arterial pH of 7.30 to 7.45. A carotid artery was cannulated with a fluid filled catheter for arterial pressure and blood gas monitoring. Rabbits that were to receive superoxide dismutase also had a jugular vein cannulated with a fluid-filled catheter. A left lateral thoracotomy was performed and the heart suspended in a pericardial cradle. Regional myocardial ischemia was induced by ligating the proximal portion of the left circumflex marginal artery (the first large epicardial branch of the left coronary artery in the rabbit) over a small piece of PE-250 polyethylene tubing. Reperfusion could then be easily accomplished by cutting the ligature. Control animals underwent a similar thoracotomy but did not undergo any manipulation of their coronary arteries. The procedures employed in this study conformed to the guiding principles of the American Physiological Society regarding animal experimentation.

Experimental protocol. Pilot experiments were initially carried out to determine the effect of duration of regional ischemia on oxygen free radical generation after reperfusion in the intact rabbit. Previous studies (27) from this laboratory had demonstrated in the globally ischemic, crystalloid perfused rabbit heart model that maximal oxygen free radical generation occurred when reperfusion followed 30 min of ischemia. Other laboratories have also demonstrated that infarction in the in vivo rabbit remains subendocardial after 30 min of regional ischemia but is fully transmural after 60 min (28). For these reasons and because a pilot study demonstrated that 75 and 90 min of regional myocardial ischemia resulted in even less oxygen radical generation, 30- and 50-min periods of ischemia were chosen for the present study. In another series of pilot experiments, the peak oxygen radical concentration occurred after 60 s of reperfusion in rabbit hearts subjected to either 30 or 50 min of regional ischemia.

Thirty-two rabbits were then assigned to one of four experimental groups of eight rabbits each:

Group I. Control animals that underwent thoracotomy but no coronary artery manipulation (n = 8).

Group II. Animals subjected to 30 min of regional myocardial ischemia without reperfusion (n = 8).

Group III. Animals subjected to 30 min of regional myocardial ischemia followed by 60 s of reperfusion (n = 8).

Group IV. Animals subjected to 30 min of regional myocardial ischemia followed by 60 s of reperfusion with superoxide dismutase treatment. Ninety seconds before reperfusion, 20 mg/kg body weight of recombinant human superoxide dismutase dissolved in normal saline solution was administered as a bolus through the jugular vein catheter. During the 60 s of reperfusion preceding myocardial biopsy, the animal received a 0.17-mg/kg infusion of recombinant human superoxide dismutase.

Recombinant human superoxide dismutase was expressed in *Escherichia coli* and supplied courtesy of Bio-Technology General Corporation (BTG). The enzyme's *N*-terminal amino acid is not acetylated, but it is otherwise structurally identical with the natural human enzyme.

To document the effect of a longer duration of ischemia on the magnitude of radical generation, an additional 10 rabbits were subjected to 50 min of regional ischemia with (n = 5) and without (n = 5) 60 s of reperfusion.

Left ventricular biopsy specimens. After the assigned period of either ischemia or postischemic reperfusion, full thickness myocardial biopsy specimens were obtained by inserting a scalpel into the center of that portion of the left ventricle that had become cyanotic and hypocontractile during coronary ligation and then hyperemic in those hearts undergoing reperfusion. The myocardium adjacent to the site of scalpel entry was then grasped with forceps, and the scalpel was used to rapidly excise a full thickness biopsy specimen. Myocardial blood flow measurements, as described later, were used to confirm ischemia, reperfusion and proper biopsy tissue selection. In rabbits not undergoing coronary occlusion, a comparable region of the left ventricle was removed at biopsy. All biopsy specimens were quickly freeze clamped at 77°K with Wollenberger tongs that had been precooled in liquid nitrogen. With this technique, the time interval from the onset of the biopsy procedure to freeze clamping the tissue was consistently 4 to 5 s. Biopsy

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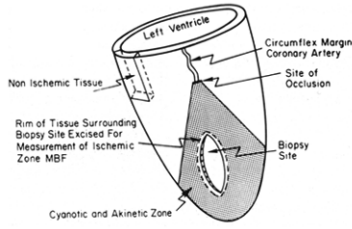


Figure 1. Schematic diagram of left ventricular biopsy method. Both the biopsy site and the surrounding rim of tissue used for measurement of ischemic zone myocardial blood flow (MBF) are located within the region that became cyanotic and akinetic (**shaded area**) during coronary artery ligation. Tissue distant from the ischemic zone (**dashed area at upper left**) was used to measure nonischemic myocardial blood flow.

specimens were stored in liquid nitrogen until spectra were recorded.

Regional myocardial blood flow. The radioactive microsphere technique was used to determine regional myocardial blood flow. Radiolabeled microspheres, diameter 13 μm, suspended in 10% dextran were agitated vigorously for 2 min before injection. Approximately 250,000 microspheres suspended in 0.5 ml of normal saline solution were injected into the left atrium during the control period in Group I rabbits and 15 min after coronary ligation in rabbits in Groups II, III and IV. To confirm reperfusion, rabbits in Groups III and IV received a second injection of microspheres with a different radiolabel, 15 to 20 s after release of the coronary artery ligature. Similarly, in all rabbits subjected to 50 min of ischemia, radioactive microspheres were injected into the left atrium midway through the ischemic period and, in the group undergoing reperfusion, 15 to 20 s after release of the coronary artery ligature. Beginning 10 s before each injection and continuing for 90 s afterward, a reference blood sample was withdrawn from the cannulated carotid artery at a constant rate of 1.4 ml/min with use of a Harvard infusion/withdrawal pump.

After myocardial biopsy, the heart was removed and a 1 to 2-mm rim of tissue was cut adjacent to and surrounding the biopsy site (Fig. 1). Because this tissue was well within the ischemic region (shaded area), its measured blood flow was assumed to be representative of the biopsy specimen. In rabbits undergoing coronary ligation, a tissue sample was also obtained from a nonischemic region distant to the biopsy site. The tissue was weighed and, together with the reference blood samples, counted for radioactivity at the appropriate energy windows in a Packard 5986 scintillation counter. Myocardial blood flow was expressed as ml/min per 100 g of left ventricle.

Electron paramagnetic resonance spectroscopic methods. Biopsy specimens were kept under liquid nitrogen while they were ground into coarse (1.5- to 2.5-mm) particles and loaded into precision 3-mm internal diameter quartz electron paramagnetic resonance tubes. Electron paramagnetic resonance spectra were then recorded by an investigator unaware of sample identity using a Varian model E-9 spectrometer, with care taken to use nonsaturating microwave power.

Previous studies (25) used temperature annealing to isolate the individual spectral components by their temperature stability (Fig. 2). After obtaining an initial spectrum at 77°K, samples were warmed to −80°C for 1 min, recooled to 77°K, and a second spectrum was then recorded. This process removed the nitrogen-centered free radical signal from the spectrum, which could be recovered by computer subtraction of the spectrum obtained after temperature annealing from the spectrum measured before temperature annealing. Similarly, warming the specimen to −80°C for an additional 60 min followed by subtraction of the resultant spectrum from the previous one allowed isolation of the oxygen-centered free radical component of the spectrum, leaving a carbon-centered, semiquinonelike free radical signal behind. The individual nitrogen-, oxygen- and carbon-centered free radical signals could then be quantified by comparing their double integral with that of a potassium peroxylamine free radical standard of known concentration.

Temperature annealing of several of the biopsy specimens demonstrated the presence of the same three electron paramagnetic resonance signals in this in vivo model. An additional signal consistent with an iron-sulfur center was also identified. This signal, like the carbon-centered radical signal, remained essentially unchanged after 60 min of warming to −80°C. Because the absorption peaks of the iron-sulfur center and carbon radical signals do not overlap, they could be easily separated (Fig. 2).

A computer simulation method (29) was then used to allow component signal quantification by producing best fit simulations of the observed spectrum with linear combinations of the component spectra. The computer-generated component spectra were then quantified by comparing their double integral with that of the potassium peroxylamine standard. The results obtained with this method were comparable (±10%) to those obtained by temperature annealing. In the present study all reported free radical concentrations were obtained by using the computer simulation method. Because the source of the iron-sulfur signal is not a free radical, its absolute concentration cannot be determined by comparison with the free radical standard. However, relative changes in concentration are still reflected by alterations in the signal's intensity and are arbitrarily expressed as the intensity, which would correspond to a given concentration of the free radical standard.

Statistical analysis. Data are expressed as the mean value ± 1 SEM. Statistical analysis was performed using an analysis of variance (ANOVA). When the overall ANOVA

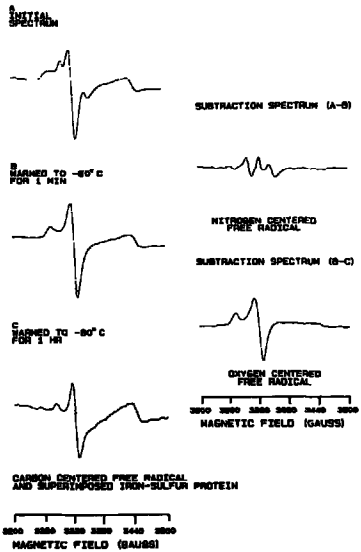


Figure 2. The temperature annealing method used to isolated free radical component spectra is illustrated. Spectrum A is the initial spectrum obtained at 77°K. Warming the tissue to −80°C for 1 min yields spectrum B. Computer subtraction of the two spectra (A minus B) allows recovery of a triplet signal most consistent with a nitrogen-centered radical. Additional warming to −80°C for 1 h yields spectrum C. Subtraction of spectrum C from B allows recovery of a signal demonstrating axial symmetry consistent with an oxygen-centered radical. Spectrum C is itself composed of two signals, an isotropic signal consistent with a carbon-centered radical and a signal with a $g_\perp$ of 1.94, which is most consistent with an iron-sulfur center. Spectra were recorded at a microwave frequency of 9.278 GHz, microwave power of 1.0 mW and modulation amplitude of 2.5 G.

revealed statistical significance of $p < 0.05$, differences among the groups were tested by unpaired *t* tests.

Results

Hemodynamic monitoring. Arterial pressure, O_2 and pH of arterial blood both before and after thoracotomy, as well as the arterial pressure midway through the ischemic period, were not different among the experimental groups.

Regional myocardial blood flow. Measurements of myocardial blood flow from distant, nonischemic myocardium and from ischemic myocardium surrounding the biopsy site are presented in Table 1. In Groups II, III and IV, the mean transmural blood flow in the ischemic region during coronary artery ligation was ≤16% of the value in the control group.

The myocardial blood flow measurements made after reperfusion must be considered semiquantitative because they were made under the rapidly changing, nonsteady state conditions of early reperfusion and because the reference blood sample could only be collected during the 1st 60 s of reperfusion (that is, until the time of left ventricular biopsy). Nonetheless, these measurements allowed us to document that reperfusion of the distal myocardial bed occurred in all Group III and Group IV rabbits, as well as in those rabbits that underwent reperfusion after 50 min of ischemia.

Identification of free radical signals with electron paramagnetic resonance spectroscopy. In this regionally ischemic in vivo preparation, the same three distinct free radical signals previously reported (25) in globally ischemic, buffer-perfused rabbit hearts were identified. In addition, a prominent iron-sulfur signal was seen (27).

The first free radical signal is isotropic with a g value of 2.004, identical with the carbon-centered 1 electron-reduced ubiquinone radical. The second signal has axial symmetry with a $g_\parallel$ of 2.033 and a $g_\perp$ of 2.005, similar to those of the oxygen-centered alkyl peroxy radical [ROO']. The third signal is a triplet with a g value of 2.000 and hyperfine splitting a_N = 24 G, suggestive of a nitrogen-coupled radical. The additional anisotropic signal, which was not as clearly evident in crystalloid perfused rabbit hearts, had a $g_\parallel$ of 2.03 and a $g_\perp$ of 1.94. A $g_\perp$ of <2.000 is unusual, identifying the source of the signal as an iron-sulfur center, such as those found in the iron sulfur class of proteins.

Measurement of free radical concentrations using electron paramagnetic resonance spectroscopy. The concentrations of the free radical species in Groups I to IV are depicted in Figure 3. The oxygen-centered free radical concentration in control rabbits (Group I) was 1.8 ± 0.8 μmol, increasing to 4.4 ± 0.9 μmol after 30 min of ischemia (Group II) and then increasing almost threefold to 13.0 ± 2.5 μmol after 60 s of postischemic reperfusion (Group III) ($p < 0.05$ among the three groups). In contrast, after 60 s of reperfusion, Group IV rabbits treated with recombinant human superoxide dismutase demonstrated an oxygen radical concentration of only 5.9 ± 1.2 μmol ($p < 0.05$ vs. Group III), which was not significantly different from the concentration measured after 30 min ischemia in Group II rabbits.

The carbon-centered semiquinone radical concentration in control rabbits (Group I) was 4.0 ± 0.7 μmol, increasing to 5.6 ± 0.7 μmol after 30 min of ischemia (Group II) (p = NS), and then almost doubling to 10.4 ± 1.7 μmol after 60 s of reperfusion (Group III) ($p < 0.05$ vs. Group II). However, the carbon radical concentration 60 s after reflow in superoxide dismutase-treated rabbits (Group IV) was only 3.4 ± 0.8 μmol (p = NS vs. Group I or II, $p < 0.05$ vs. Group III).

The nitrogen radical concentration in control rabbits (Group I) was 1.0 ± 0.3 μmol, increasing to 3.0 ± 0.6 μmol

Table 1. Regional Myocardial Blood Flow Data

Experimental Group	Nonischemic Region		Ischemic Region	
	Ischemic Period	Reperfusion Period	Ischemic Period	Reperfusion Period
II (occlusion only)	197 ± 12	—	19 ± 6 (10)	—
III (occlusion + reperfusion)	198 ± 24	146 ± 21	20 ± 7 (10)	123 ± 33 (84)
IV (occlusion + reperfusion + superoxide dismutase)	161 ± 13	182 ± 24	26 ± 15 (16)	255 ± 65 (140)
50-min ischemia	168 ± 27	—	8 ± 5 (5)	—
50-min ischemia + reperfusion	209 ± 17	191 ± 10	21 ± 7 (10)	136 ± 29 (71)

Myocardial blood flow for control hearts (Group I, no coronary occlusion) = 137 ± 17 ml/min per 100 g left ventricle. Data presented are mean value ± SEM and, in parentheses, mean percent nonischemic zone blood flow.

after 30 min of ischemia (Group II) ($p < 0.05$) and then increasing to 5.0 ± 1.4 μmol after 60 s of reperfusion (Group III) (p = NS vs. Group II). In animals with reperfusion treated with superoxide dismutase (Group IV), the nitrogen radical concentration 60 s after reflow was only 1.6 ± 0.4 μmol (p = NS vs. Group I or II, $p < 0.05$ vs. Group III).

The intensity of the iron-sulfur signal increased from that corresponding to a radical standard concentration of 0.7 ± 0.1 μmol in control patients to 1.1 ± 0.1 μmol during ischemia ($p < 0.05$) and then decreased to 1.0 ± 0.1 μmol after 60 s of reperfusion. In reperfused animals treated with superoxide dismutase (Group IV), the intensity of the iron-sulfur signal 60 s after reflow was only 0.6 ± 0.1 μmol ($p < 0.05$ vs. Group II or III).

The peak free radical concentrations measured in rabbits subjected to 50 min of ischemia with and without reperfusion are shown in Figure 4. The oxygen free radical concentration at end-ischemia was 4.6 ± 0.9 μmol and then increased to only 7.5 ± 0.9 μmol after 60 s of reperfusion. The carbon radical concentration increased to 6.4 ± 0.2 μmol at end-ischemia and to 9.5 ± 0.5 μmol after reperfusion. Finally, the nitrogen-centered radical concentration was only 1.5 ± 0.5 μmol at end-ischemia and 1.9 ± 0.8 μmol after reperfusion. In each instance there was no difference between the free radical concentration at end-ischemia versus control or reperfusion values; however, for the carbon and oxygen radicals there was a significant difference ($p < 0.05$) between the reperfusion and control values.

There was no significant difference in the peak oxygen free radical concentration measured at the end of 30 versus 50 min of ischemia (p = NS). In contrast, the oxygen free radical concentration measured after 60 s of postischemic reperfusion was significantly higher after 30 min (13.0 ± 2.5 μmol), than after 50 min of ischemia (7.5 ± 0.9 μmol) ($p < 0.05$).

Discussion

Evidence for free radical generation in isolated heart models. The initial evidence that oxygen free radicals might play a role in reperfusion injury was obtained indirectly. Superoxide dismutase given at the moment of postischemic reperfusion was able in animal models to decrease myocardial infarct size (14–16) and to enhance both functional and metabolic recovery after an ischemic insult (17–21). Because the only known beneficial action of superoxide dismutase is to catalyze the conversion of superoxide radical to hydrogen peroxide and water, it was presumed that the enzyme's beneficial effect was related to its ability to detoxify super-

Figure 3. Oxygen-, carbon- and nitrogen-centered free radical concentrations in rabbits in Group I (control, no coronary occlusion), II (occlusion only), III (occlusion + reperfusion), IV (occlusion + reperfusion + superoxide dismutase). For the oxygen-centered radical, $p < 0.05$ among Groups I, II and III and for Group III versus Group IV. p = NS for Group II versus Group IV.

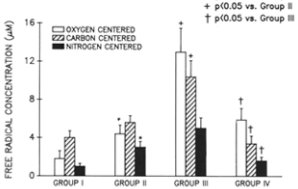
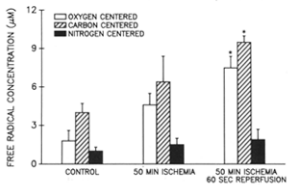


Figure 4. Oxygen-, carbon-, and nitrogen-centered free radical concentrations in rabbits subjected to 50 min of ischemia with and without 60 s of reperfusion. For the oxygen-centered radical, $p < 0.05$ for control versus reperfusion; for other comparisons, p = NS.



oxide radicals generated after reperfusion. Administration of the enzyme during the ischemic period before reperfusion provided no measurable additional benefit, suggesting that the majority of free radical generation occurred at the time of restoration of blood flow (14,21). In more recent studies the administration of exogenous free radical–generating systems to isolated hearts produced myocardial tissue damage, providing evidence that free radicals are themselves deleterious and not merely a marker for other injurious processes (30). However, the role of free radicals in in vivo reperfusion injury remains controversial, with several recent animal studies failing to demonstrate benefit from treatment with free radical scavengers (22–24).

Zweier et al. (25,27) used electron paramagnetic resonance spectroscopy to provide direct evidence that reperfusion induces a burst of oxygen-centered free radicals in crystalloid perfused rabbit hearts made globally ischemic and then reperfused. Three distinct electron paramagnetic resonance signals were resolved and quantitated. A signal with axial symmetry, $g_{\parallel} = 2.033$ and $g_{\perp} = 2.005$, consistent with an oxygen-centered alkyl-peroxy radical formed as a result of membrane lipid peroxidation, was observed. An isotropic signal with $g = 2.004$ consistent with a carbon-centered ubi-semiquinone radical, a major component in the mitochondrial electron transport chain, was also observed. The third signal was a triplet with $g = 2.000$ and $a_N = 24$ G, suggestive of a nitrogen-centered radical; however, the identity of this radical was uncertain. Reperfusion after a 30-min period of global ischemia resulted in an approximately twofold increase in oxygen radical concentration, from 4.0 ± 1.0 μmol at the end of the ischemic period to a peak concentration of 8.1 ± 1.4 μmol after only 30 s of reperfusion.

Differences between isolated heart and in vivo models. Although the present in vivo study documented the same three free radical signals previously identified in the buffer-perfused preparation and a similar burst of oxygen radical generation with postischemic reperfusion, several important differences were observed. In the globally ischemic isolated heart model the magnitude of the carbon-centered semiquinone-like radical signal decreased during ischemia, whereas in the regionally ischemic in vivo model it remained unchanged. One possible explanation for this result is that global ischemia entails total cessation of coronary flow. The resulting tissue hypoxia is more severe than in the in vivo model and is reflected as an even greater decrease in the concentration of the carbon radical, which is thought to represent the oxidized form of ubi-semiquinone, a major component in the mitochondrial electron transport chain. In the in vivo, regionally ischemic model, a small quantity of collateral flow might allow sufficient delivery of oxygen to maintain a low level of mitochondrial electron transport, thereby preventing the carbon radical concentration from decreasing. Alternatively, blood perfusion in the in vivo model might result in the generation of an additional isotropic free radical, such as the ascorbate radical, with a g value identical with that of ubi-semiquinone. Increasing concentrations of such a radical could mask an underlying decrease in the concentration of oxidized ubi-semiquinone. In samples of whole rabbit blood, an ascorbate radical signal can be identified.

In the isolated heart preparation, superoxide dismutase treatment was associated with an increase in the carbon-semiquinone radical concentration after reperfusion. Presumably, the protection afforded by the enzyme improved metabolic recovery, resulting in increased electron transport. However, this finding was not replicated in the in vivo model where treatment decreased the carbon radical concentration measured 60 s after reperfusion.

In addition to the same three signals seen in the isolated heart model, a fourth signal, which was considerably less evident in buffer-perfused hearts, was also identified. This signal with a g value of 1.94 is characteristic of a 1 electron-reduced iron-sulfur protein. The magnitude of this signal significantly increased during ischemia. This increase is similar to that previously observed in the isolated, crystalloid perfused heart. It probably arises from mitochondrial iron-sulfur proteins, including nicotine adenine dinucleotide, reduced form (NADH) dehydrogenase at site 1 or the cytochrome b,c_1 complex at site 2 and may increase during ischemia because of the presence of a more highly reduced state, which would occur in the absence of oxygen.

Validation of the frozen tissue electron paramagnetic resonance technique. Mechanical processing, such as excessive grinding or pulverization of tissue, can generate oxygen-centered free radicals, presumably from the reaction of molecular oxygen with $R^{\cdot}$ generated by the mechanical breakage of covalent bonds. The generation of such radicals might be expected to confound our electron paramagnetic resonance measurements. However, recent experiments (27) have demonstrated that the magnitude of mechanically induced free radical generation can be minimized by proper tissue handling techniques. Special care was taken to minimize tissue grinding, compatible with the 3-mm internal diameter of the quartz electron paramagnetic resonance tubes. Grinding for <1 min produced a particle size of approximately 2 mm. This method of grinding frozen, blood-perfused rabbit heart tissue resulted in only a 1.8 ± 0.8 μmol oxygen radical concentration in normally perfused control hearts. This "control" concentration represents both "naturally occurring" oxygen radicals and those mechanically induced by tissue processing.

Nakazawa et al. (31) have suggested that 1) tissue from ischemic hearts might be more susceptible to such artifactual oxygen-centered free radical production, and 2) because the presence of oxygen is necessary to produce such radicals, their generation is increased in hearts that have been reperfused. However, the finding of a *lower* peak concentration of oxygen free radicals measured after reperfusion in rabbits subjected to 30 min compared with 30 min of ischemia suggests that it is unlikely that ischemia or ischemia followed by reperfusion somehow "primes" the tissue for the me-

chanical production of free radicals. If such were the case, increasing the duration of ischemia from 30 to 50 min might be expected to result in an increase in the peak oxygen radical concentration measured either at end-ischemia or after reperfusion. In addition, if the presence of oxygen favors the artifactual production of oxygen-centered free radicals, one would expect an equally elevated oxygen-centered radical concentration in all reperfused tissue samples and not the rapid rise and fall in oxygen radical concentration observed during the first several minutes of reperfusion in our pilot studies.

To further investigate the role, if any, that our method of tissue processing might play in generating artifactual oxygen-centered free radicals, we employed a rapid freeze core biopsy gun and an extrusion cylinder to obtain and process frozen tissue samples. Both techniques allow direct tissue free radical measurements to be made without any grinding. The electron paramagnetic resonance spectra obtained from these samples were not significantly different from those obtained from tissue that had been ground for 1 min (27).

Finally, the spin trapping technique allows electron paramagnetic resonance measurement of free radical concentrations without tissue sampling. When spin trap experiments were performed in isolated, crystalloid perfused rabbit hearts, the free radical concentrations measured in room temperature coronary effluent after ischemia and reperfusion were similar in time course with those measured in frozen tissue specimens processed with our grinding technique (27).

Study limitations. Although this in vivo study documented both a burst of oxygen free radical generation with postischemic reperfusion and its attenuation with superoxide dismutase, it did not demonstrate that this attenuation decreased tissue injury. To conclusively document such a beneficial effect of free radical scavenging, infarct size would need to be measured. Because of the relatively small size of the rabbit heart and the need to remove a large portion of the ischemic zone for free radical and regional blood flow measurements, a separate study would have been required for such measurements.

To document that the biopsy sample was not contaminated with tissue from outside the ischemic zone, regional myocardial blood flow was measured in the rim of tissue surrounding the biopsy specimen. In all rabbits myocardial blood flow within the ischemic zone was ≤16% of that in the nonischemic zone. Moreover, because flow was measured in the rim of tissue surrounding the biopsy site, measurements of flow would overestimate flow in the biopsy specimen that was even more remote from the surrounding nonischemic myocardium. Finally, in the event of contamination of the biopsy specimen with normally perfused tissue lying outside the ischemic zone, the "true" oxygen radical concentration would only have been higher because nonischemic tissue had the lowest oxygen radical concentrations.

Conclusions. Oxygen free radicals are generated in even higher concentration after reperfusion in the regionally ischemic, in vivo rabbit heart than in the globally ischemic, crystalloid perfused rabbit heart. This finding suggests that in the blood-perfused heart there may be additional mechanisms of oxygen free radical generation that amplify the burst observed in the crystalloid perfused heart. Possible mechanisms could include adhesion of activated leukocytes to damaged vascular endothelium with resultant superoxide radical generation, enhanced generation of hydroxyl radicals through superoxide-derived Fenton chemistry catalyzed by serum bound iron or the ability of hemoglobin in red blood cells to increase oxygen delivery at the time of reperfusion. However, the results of the present study also demonstrate that the oxygen radical scavenging ability of superoxide dismutase was not overwhelmed by the increased concentration of oxygen radicals generated in the in vivo model.

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