Measurement and Characterization of Postischemic Free Radical Generation in the Isolated Perfused Heart*

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Electron paramagnetic resonance spectroscopy has been applied to measure radical generation in the postischemic heart; however, there is controversy regarding the methods used and the conclusion as to whether radicals are generated. In order to resolve this controversy, direct and spin trapping measurements of the time course and mechanisms of radical generation were performed in isolated perfused rabbit hearts. In reperfused tissue, 3 prominent radical signals are observed: A, isotropic $g = 2.004$ suggestive of a semiquinone; B, anisotropic $g = 2.033$ and $g = 2.005$ suggestive of ROO•; and C, a triplet $g = 2.000$ and $a = 24$ G suggestive of a nitrogen centered radical. B and C, however, are highly labile and disappear at temperatures probably encountered in some previous studies. In normally perfused hearts, A is observed with only small amounts of B and C. During ischemia, B and C increase reaching a maximum after 45 min while A decreases. On reflow with oxygenated perfusate all 3 signals increase. With varying duration of ischemia and reflow, peak signal intensities occurred after 15 s of reflow following 30 min of ischemia. Reperfusion with superoxide dismutase, deferoxamine, or mannitol abolished the reperfusion increase of B. Measurements performed with the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) demonstrated a similar time course of radical generation with prominent DMPO-OOH and DMPO-R signals peaking between 10 and 20 s of reflow. Superoxide dismutase and deferoxamine also quenched these signals. Thus, *O2 derived 'OH, R•, and ROO• radicals are generated in postischemic myocardium. While the experimental techniques used can result in loss of intrinsic radicals and generation of extraneous radicals, with proper care and controls valid measurements of free radicals in biological tissues can be performed.

Free radical generation has been hypothesized to be an important mechanism of cellular damage in ischemic and reperfused tissues (1). Studies administering superoxide dismutase or other radical scavengers to the postischemic heart have demonstrated that free radical scavengers can decrease reperfusion injury (2–6). We have demonstrated that there is a burst of free radical generation during the early seconds of postischemic reperfusion (7, 8). The observation of a reperfusion free radical burst has been confirmed by several laboratories (9–15); however, there is controversy regarding the specific methods of measurement, the techniques of sample preparation, and the assignment of the observed signals. Most importantly there is disagreement as to whether the oxygen free radicals which have been proposed to cause cellular injury can be measured. In order to resolve this controversy we have applied a variety of electron paramagnetic resonance, EPR, methodologies to measure the time course of radical generation, to assign the observed signals, and to determine the mechanisms of radical generation.

MATERIALS AND METHODS

Female New Zealand White rabbits (2 kg) were heparinized and anesthetized and their hearts removed. The isolated hearts were perfused by the method of Langendorff at a constant pressure of 80 mm Hg with a Krebs/hydrogen-buffed perfusate consisting of 117 mM NaCl, 24.6 mM NaHCO3, 5.9 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 0.5 mM EDTA, and 16.7 mM glucose, which was bubbled with 95% O2, 5% CO2 gas. For the spin trapping experiments the perfusate was identical except that it contained 2.0 mM CaCl2 with no EDTA and 4 mM glucose. Perfusion solutions were filtered through 0.8-μm filters prior to use. Heart rate and left ventricular developed pressure were measured using a fluid-filled balloon secured within the left ventricle (16).

For direct EPR measurements the hearts were freeze clamped at 77 K using Wollenberger tongs. The tissue was then processed by either grinding or chopping under liquid nitrogen in a ceramic mortar. The tissue particles were then poured along with liquid nitrogen through a funnel into precision 3-mm inner diameter quartz EPR tubes. The tissue, the funnel, and the EPR tube were maintained under liquid nitrogen at all times. For certain experiments frozen tissue was extruded into the shape of a 3-mm core using a stainless steel funnel mounted extrusion cylinder which was partially submerged in liquid nitrogen and the core extruded directly into the EPR tubes. Rapidly frozen core biopsies, freezing time <0.3 s, were obtained using a pneumatic drill freeze biopsy gun (Alko Diagnostic).

EPR spectra were recorded with a Varian E-9 or Bruker ESP-300 spectrometer using a TE104 or TE102 cavity, respectively. Quantitative measurements were performed as described previously (6). EPR tubes were filled to a height of 4 cm which was more than sufficient to achieve complete filling of the critical volume of the cavity. The tissue weight of each tube was identical to within 10%, however, it is the identical filling of the cavity (identical filling factor and Q) with an identical volume and shape of tissue which allows accurate quantitative measurements (17, 18), not just the total mass of tissue (10).

Quantitation of the total radical concentration in the tissue was determined from the ratio of the double integral of the observed signal to that of an aqueous potassium peroxylamine radical standard of known concentration in an identical EPR tube. The relative ratios of the component signals were determined using a computer program designed to provide a best fit of the observed spectrum from a linear combination of the component signals. The component signals used were obtained from computer simulation of the components obtained.
When freeze-clamped tissue from a normally perfused heart was ground for 1 min under liquid nitrogen to a particle size of about 2 mm diameter, the observed EPR signal consisted almost entirely of signal A with only slight components of the other 2 signals (Fig. 1A). When tissue was ground for 10 min to a particle diameter <0.2 mm, the magnitude of signals B and C increased (Fig. 1B). When the tissue was ground for 10 min while exposed to oxygen, with resultant decreased particle size and extent of grinding with resultant decreased particle size and with the concentrations of O$_2$ allowed to come into contact with the tissue surface.

Recently it has been reported that chopping of tissue does not generate mechanical radicals (10), however, when the tissue was chopped to identical particle sizes to those produced with grinding, similar spectra were observed consisting of a combination of signals A, B, and C.

Two other methods of tissue sampling in which mechanical fracturing was minimized were also studied. Tissue fragments were extruded into the shape of a cylindrical core of 3 mm diameter using an extrusion cylinder maintained partially submerged in liquid nitrogen and the observed spectra were identical to those in Fig. 1A. Tissue was also obtained using a rapid freeze liquid nitrogen core biopsy gun and the non-fractured tissue cores again showed identical spectra. Thus, while it is clear that mechanical processing of heart tissue can result in radical generation, this problem can be suitably controlled by minimizing tissue fracturing and handling all samples in an identical manner.

In order to determine if ·O$_2$ or ·OH-derived free radicals could be detected in heart tissue, hearts were infused with an iron redox ·OH-generating system consisting of H$_2$O$_2$ (0.5 mM) and Fe$^{3+}$/nitritotriacetate (10 mM, 1:2) and freeze clamped during infusion. Administration of this ·OH-generating system resulted in an increase in the concentrations of signals B and C (Fig. 2, A and B). When the tissue was transferred and packed directly into the bottom of an EPR Dewar only signal A was observed and signals B and C disappeared (Fig. 2C). These experiments suggest that ·O$_2$ and ·OH-derived free radicals can be measured in rapidly frozen heart tissue; however, the observed signals are temperature labile.

When hearts are subjected to ischemia and reperfusion, alterations are observed in the EPR spectra of the frozen tissue. As we reported previously in tissue allowed to warm up to 190 K, only a single isotropic signal with a g value of 2.004, signal A, is observed (7). This signal decreases during introduced by bubbling O$_2$ gas in the liquid nitrogen containing mortar, the intensities of signals B and C were markedly increased (Fig. 1C). The generation of signals B and C by mechanical processes was found to be proportional to the extent of grinding with resultant decreased particle size and with the concentrations of O$_2$ allowed to come into contact with the tissue surface.

FIG. 1. EPR spectra of frozen tissue from a normally perfused heart. A, ground for 1 min to a particle diameter of approximately 2 mm. B, ground for 10 min to a particle diameter <0.2 mm. C, ground for 10 min while exposed to oxygen. Spectra were recorded at 77 K with microwave frequency of 9.351 GHz using 1.0 milliwatt of microwave power and modulation amplitude of 2.5 G.

FIG. 2. EPR spectra of frozen heart tissue. A, from a normally perfused heart; B, from a heart infused with a ·OH-generating system for 5 min; C, tissue from B packed directly into an EPR Dewar instead of an EPR tube. Spectra were recorded at 77 K with a microwave frequency of 9.301 GHz. Tissue was chopped to a particle size of approximately 2 mm.

RESULTS

In order to perform direct EPR measurements of labile free radicals in the heart, the tissue must be rapidly frozen and then mechanically processed to fit within the sample tube, Dewar, and microwave cavity. Since the whole frozen heart, will not fit within a standard X-band cavity and Dewar, some mechanical processing of the tissue must be performed. A variety of different techniques including grinding, chopping, core extrusion, and core biopsy were studied to characterize the appearance or disappearance of radical signals as a function of tissue processing. Three different radical signals have been previously measured in heart tissue: signal A, isotropic with $g = 2.004$ suggestive of a semiquinone radical; signal B, with axial symmetry $g_\perp = 2.033$ and $g_\parallel = 2.005$ identical to ROO$^-$; signal C, a triplet with $g = 2.000$ and $\alpha = 24$ G suggestive of a radical with nitrogen coupling such as a peroxylamine (8). There is also an additional signal with $g$ values of 2.027 and 1.936 attributable to an iron-sulfur protein with a Fe$_2$S$_2$ cluster.

When freeze-clamped tissue from a normally perfused heart was ground for 1 min under liquid nitrogen to a particle size of about 2 mm diameter, the observed EPR signal consisted almost entirely of signal A with only slight components of the other 2 signals (Fig. 1A). When tissue was ground for 10 min to a particle diameter <0.2 mm, the magnitude of signals B and C increased (Fig. 1B). When the tissue was ground for 10 min while exposed to oxygen, which was deliberately

$^1$ The abbreviation used is: DMPO, 5,5'-dimethyl-1-pyrroline-N-oxide.
ischemia and then increases during reflow (Fig. 3). In later studies we observed that if the tissue is meticulously maintained at 77 K, signals B and C are observed and increase during ischemia and postischemic reflow (8). As shown in Fig. 4, tissue from a normally perfused heart exhibits a spectrum consisting of mostly signal A, however, after 30 min of ischemia, signals B and C increase while signal A decreases, and on postischemic reflow with oxygenated perfusate all 3 signals markedly increase. Hearts were frozen after either normal perfusion, 30 min ischemia, or 30 min ischemia followed by a 15-s reflow, \( n = 7 \) in each group. Significant differences \( p < 0.01 \) in the intensities of signals A and B were noted in all 3 groups. For signal A, radical concentrations were calculated to be 2.1 \( \pm 0.2 \), 0.5 \( \pm 0.2 \), and 3.9 \( \pm 0.5 \) \( \mu \text{M} \) for the control, ischemic, and reflow groups while for signal B, radical concentrations were calculated to be 1.0 \( \pm 0.4 \), 4.0 \( \pm 1.0 \), and 8.1 \( \pm 1.4 \) \( \mu \text{M} \), respectively.

Experiments were performed to measure the time course of radical generation as a function of the duration of ischemia and reflow. Hearts were subjected to 10, 30, or 60 min of ischemia and then freeze clamped after 2-s to 30-min periods of reflow. A maximum increase in signals A and B was observed on reperfusion after 30 min ischemia (Fig. 5). With only 10 min of ischemia a lesser increase was observed upon reflow, and after 60 min ischemia no clear increase was observed. Therefore, reperfusion associated radical generation is maximum after approximately 30 min of global ischemia with peak radical concentrations observed after only 15 s of reflow.

In order to further measure radical generation in the postischemic heart by an independent technique where there was no processing of the heart tissue and thus no potential for mechanical radical generation, spin trapping EPR experiments were performed. Hearts were perfused with 40 mM DMPO and the effluent collected for EPR measurements. On infusion of DMPO during normal perfusion there was no detectable signal in the effluent (Fig. 6). Upon reflow after 30 min ischemia, however, there was a prominent EPR spectrum consisting of two components, a quartet 1:2:2:1 signal with \( a_u = 14.9 \) G indicative of DMPO-OH and a 1:1:1:1:1 six-peaked spectrum with \( a_u = 15.8 \) G and \( a_H = 22.5 \) G indicative of DMPO-R. The intensities of both signals peaked after 10-20 s of reflow, then gradually decreased until after 5 min there was almost no detectable signal (Fig. 6).

In order to study the mechanism of radical generation, experiments were performed reperfusing hearts \( (n = 7 \) in each group) after 30 min of global ischemia with superoxide dismutase (2,000 units/ml), deferoxamine (1 mM), or mannitol (50 mM). Free radical concentrations were measured from tissue frozen after 15 s of reflow, the time of peak radical generation. Additional experiments were performed determining the effect of each agent on radical generation measured with the spin trap DMPO. As shown in Table I, superoxide dismutase, deferoxamine, and mannitol all significantly decreased the concentration of signal B, \( \cdot \text{O}_2^- \), as well as the concentration of DMPO-OH observed in the spin trap measurements. The semiquinone signal A, however, was not decreased. In similar experiments infusion of denatured superoxide dismutase or Fe**-saturated deferoxamine did not decrease the observed signals. These experiments suggest that \( \cdot \text{O}_2^- \) is generated in reperfused myocardium and that
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**FIG. 6.** EPR spectra of the effluent perfusate of a heart perfused with 40 mM DMPO. Measurements were performed on effluent collected in 10-s samples prior to ischemia and upon reflow after 30 min ischemia. Spectra were recorded at room temperature with a microwave frequency of 9.774 GHz using 10 milliwatts of microwave power and 0.5 G modulation amplitude.

\[ \cdot \text{OH}, \cdot \text{R}^{'}, \text{and ROO}^* \text{are generated from } \cdot \text{O}_2^- \text{via the iron-mediated Fenton reaction.} \]

It has been suggested that superoxide dismutase may alter the observed concentrations of tissue radicals solely due to the scavenging of mechanically generated radicals (21). Therefore, experiments were performed in which normally perfused hearts were infused with superoxide dismutase, deferoxamine, or mannitol and then freeze clamped and mechanically processed as described above. The radicals generated by mechanical processes did not appear to be altered since a similar extent of grinding or chopping again produced spectra identical to those in Fig. 1 with similar proportions of signals A, B, and C.

While the above experiments suggest that \( \cdot \text{O}_2^- \) is generated in reperfused myocardium, a signal with \( g \) values characteristic of \( \cdot \text{O}_2^- \) has not been previously measured. In an effort to determine if a weak \( \cdot \text{O}_2^- \) signal is observable in rapidly frozen tissue, spectra were acquired from tissue frozen at 15 s of reflow after 30 min of ischemia. A broader sweep width of 400 G was used along with an increased microwave power of 5.0 milliwatts and 2 h of time averaging performed. In these spectra a small peak was observed with a \( g \) value of 2.092 (Fig. 7). This peak occurs within the range of \( g_1 \) values previously noted for \( \cdot \text{O}_2^- \)-cation complexes in frozen aqueous solutions (22). When identical acquisitions were performed on heart tissue from a heart infused with superoxide dismutase (2000 units/ml), this signal was absent. When these samples were warmed the peak at \( g = 2.092 \) disappeared after 1 min at 190 K along with the triplet signal C. In the absence of superoxide dismutase a signal was observed superimposed on the center peak of the triplet at \( g = 2.001 \) while in the presence of superoxide dismutase the 3 peaks had closer to 1:1:1 intensities. Therefore, the \( g_1 \) features which accompany the signal

### Table I

| Semi-quinone* | ROO* | DMPO-OH* | DMPO-R* |
|--------------|------|----------|---------|
| Control      | 3.9 ± 0.5 | 8.1 ± 1.4 | 0.20    | 0.40    |
| Superoxide dismutase | 4.2 ± 0.4 | 3.8 ± 0.4 | 0.03    | 0.12    |
| Deferoxamine | 3.6 ± 0.5 | 3.2 ± 0.3 | 0.02    | 0.04    |
| Mannitol     | 3.4 ± 0.6 | 4.3 ± 0.4 | 0.02    | 0.60    |

* Determined from a series of 7 hearts in each group which were freeze clamped after 15 s of reflow, the time of peak radical concentration. EPR spectra were recorded at 77 K and analyzed as described above.

* Determined from spin trapping experiments in hearts exposed to 30 min ischemia and then reperfused with 40 mM DMPO. The reported concentrations correspond to measurements of coronary effluent collected at 10-20 s of reflow.

Fig. 7. Time averaged wider field EPR spectrum of a heart subjected to 30 min ischemia followed by 15 s of reflow. The spectrum was recorded at 77 K using 5 milliwatts of microwave power, 2.5 G modulation amplitude, and a microwave frequency of 9.214 GHz. The inset shows the \( g = 2.092 \) peak at 20-fold increased gain.
at \( g = 2.092 \) appear to be centered at \( g = 2.001 \). While the intensity of this latter signal cannot be exactly determined measurements of the \( g_i \) peak and estimates of the \( g_i \) peak suggest a radical concentration on the order of \( 10^{-8} \) to \( 10^{-7} \) M.

**DISCUSSION**

Free radical generation has been hypothesized to be an important mechanism of postischemic injury in cells and tissues, however, this hypothesis is still very controversial. In recent years EPR spectroscopy has been applied in an effort to directly measure and characterize free radical generation in the heart. Studies from our laboratory demonstrated that there is a burst of radical generation on reperfusion of the ischemic heart. In our early studies only one radical signal was observed in frozen tissue, an isotropic signal with \( g \) value of 2.004, later named signal A (7, 8). This signal was observed in normally perfused hearts, it decreased during ischemia, and increased on reflow. Subsequently two additional signals were observed when tissue was meticulously maintained at 77 K: an axially symmetric signal with \( g_1 = 2.034 \) and \( g_2 = 2.005 \), signal B; and a triplet with \( g = 2.000 \) and \( a_g = 24 \) G, signal C. These additional temperature labile signals were observed to increase during ischemia and further increase after reperfusion. The three signals were resolved based on their different temperature stability which allowed quantitation of each component signal within the tissue (8). In addition to these 3 radical signals there is an additional nonradical signal with the unusual \( g \) values of 2.027 and 1.936 uniquely attributable to an iron-sulfur protein with a Fe\(_2\)S\(_2\) cluster (23). With nonsaturating power, the magnitude of the \( g = 2.027 \) peak of this signal is negligible while the larger \( g = 1.936 \) peak is considerably upfield and does not overlap with the observed radical signals.

Over the last year several laboratories have performed similar experiments and confirmed that there is a measurable increase in radical concentrations during postischemic reperfusion (9–11). Controversy has arisen, however, regarding the methods of tissue processing as well as the assignment of the observed signals. Maupoil and Rochette (11) and Limm et al. (9) have reported similar multicomponent spectra to those observed in our laboratory, while Baker et al. (10) have reported observing only signal A and suggested that signals B and C arise only from the mechanical processing of the tissue. Baker et al. (10) reported a decrease in radical concentration during ischemia and an increase on reperfusion, while Maupoil and Rochette (11) reported increases during both ischemia and reperfusion. Nakazawa et al. (21), however, have reported measuring radical generation only during ischemia and not on reperfusion.

The experiments performed in this study explain the differences observed by the different laboratories. First it was determined that mechanical processing of tissue can result in the generation of signals B and C while not altering the intensity of signal A. It was determined that the magnitude of mechanical radical generation was proportional to the extent of mechanical fracturing of the tissue and the concentration of \( O_2 \) in contact with the tissue regardless of the specific technique used. It was shown that the technique of tissue chopping also produces mechanical radical generation identical to that with grinding. Generation of the ROO\(^*\) radical, signal B, appears to occur from the reaction of \( O_2 \) with \( R' \), generated by mechanical bond breakage. Signal C which is similar to that of a peroxyxylamine radical could similarly arise from the generation of amine radicals on breakage of peptide bonds. While oxygen radical generation can thus be caused by mechanical processing, this does not mean that oxygen radical generation occurs solely from mechanical processing. Infusion of a 'OH-generating system into a heart increased concentrations of ROO\(^*\), signal B, demonstrating that ROO\(^*\) can be generated from 'OH via hydrogen atom abstraction as previously proposed (24). On warming of the sample, however, this signal is not observed. The technique of loading tissue directly into an EPR Dewar without an EPR tube can result in the disappearance of this signal due to warming of the sample and this could account for the results reported by Baker et al. (10) in which only signal A was observed. In their experiments the changes in signal A were similar to those which we previously reported for that signal (7, 8).

There are seven observations which suggest that the increase in the ROO\(^*\), signal B, in reperfused tissue is truly due to intrinsic generation of this radical. 1) Mechanical radical generation was found to be proportional to the extent of tissue fracturing and this was identical in all groups. 2) Different processing techniques yielded similar results. 3) Infusion of the heart with a 'OH-generating system gave rise to this signal. 4) A distinct time course of radical generation was observed with elevated concentrations observed only early after reflow. 5) Spin trapping measurements which involved no processing of tissue demonstrated oxygen radical generation with an identical time course. 6) Superoxide dismutase, deferoxamine, and mannitol all decreased the reperfusion associated increase in this signal while they did not alter mechanical radical generation. 7) Spin trapping measurements confirmed that these substances prevent the reperfusion radical burst. Thus, multiple lines of evidence suggest that intrinsic ROO\(^*\) generation can be measured and that the increased concentrations of this radical in reperfused tissue are due to endogenous generation.

The effect of the duration of ischemia on the magnitude and time course of radical generation on reperfusion was studied. It was determined that the concentration of the semiquinone radical, signal A, decreased as a function of the duration of ischemia while ROO\(^*\), signal B, increased. On reperfusion after 10 or 30 min ischemia, both signals A and B increased peaking after 10–15 s of reflow, while with prolonged duration of ischemia of 60 min no increase in radical concentration was noted. After 10–30 min of reflow, radical concentrations returned to control values. The increases in radical generation are observed only during the early seconds of reflow, studies performed on tissue at later times would miss this increase. This may explain the failure of Nakazawa et al. (21) to measure an increase in radical concentrations in reperfused tissue.

Independent measurements of free radical generation performed using the spin trap DMPO demonstrated a similar time course of radical generation with DMPO-OH and DMPO-R signals observed peaking at 10–20 s of reflow with no observable radical generation before ischemia or after >5 min of reflow. The observed DMPO-OH signal could arise from direct trapping of 'OH or secondarily from the breakdown of the trapped superoxide adduct, DMPO-OOH. The observation of a DMPO-R signal suggests that 'OH is generated and results in hydrogen abstraction from alkyl groups on lipids or proteins generating \( R' \) radicals.

Experiments performed on reperfusing hearts with the superoxide scavenging enzyme superoxide dismutase showed significant reductions in the concentrations of the ROO\(^*\), as well as DMPO-OH and DMPO-R demonstrating that these radicals are derived from superoxide. The concentrations of the semiquinone radical, however, were not decreased. Similar
results were obtained on reperfusion with the high affinity Fe\(^{3+}\) chelator, deferoxamine, suggesting that these radicals are derived from superoxide via iron-mediated Fenton chemistry. Reperfusion with the 'OH scavenger mannitol resulted in a decrease in DMPO-OH with an increase in DMPO-R, suggesting that 'OH was being scavenged.

A fourth weak radical signal was observed in reperfused heart tissue with axial symmetry with \( g_1 = 2.092 \) and \( g_1 = 2.001 \), values within the range of those previously reported for O\(_2^-\) or O\(_2^-\)-cation complexes in frozen neutral aqueous solutions (22). The fact that the concentrations of this signal are on the order of 10\(^{-8}\)-10\(^{-7}\) M and that this signal disappears on reflow with superoxide dismutase further suggest that it may be O\(_2^-\). It is known that O\(_2^-\) readily forms complexes with alkaline earth cations such as Ca\(^{2+}\) and Na\(^+\) and that these ions drastically reduce the rate of superoxide dismutation (25). Since the cations Ca\(^{2+}\) and Na\(^+\) are abundant in heart tissue, the observed signal may actually be from a cation-superoxide complex.

The source of superoxide generation in these experiments is uncertain. Superoxide generation could occur secondary to mitochondrial O\(_2^-\) reduction. It is known that the one-electron reduced ubiquinone can donate an electron to O\(_2^-\)-generating O\(_2^-\) (26). A marked increase in the semiquinone signal A which appears to be the one-electron reduced ubiquinone radical is observed in reperfused myocardium suggesting that this mechanism may occur. It is of particular interest that the rise in the concentration of this radical follows an identical time course to that of the measured reactive oxygen radicals, suggesting that it could be responsible for the burst in O\(_2^-\) generation on reperfusion. There are other possible mechanisms of radical generation, however, which may also contribute to superoxide generation on reperfusion including: iron-redox cycling, endothelial radical generation, and the xanthine oxidase reaction (27).

Thus, we conclude that EPR spectroscopy can be applied to measure free radical generation in the reperfused heart. While the specific techniques used can result in the disappearance of intrinsic radicals or the production of extraneous radicals, valid measurements can be performed. These measurements demonstrate that there is a burst of superoxide-derived 'OH, R', and ROO radicals via iron-mediated Fenton chemistry as well as a marked rise in a cellular semiquinone radical which could cause superoxide generation.

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REFERENCES
1. Korthuis, R. J., and Granger, D. N. (1986) in Physiology of Oxygen Radicals (Taylor, A. E., Matalon, S., and Ward, P. A., eds) pp. 217-249, Williams & Wilkins, Baltimore
2. Shlafer, M., Kane, P. F., and Kirah, M. M. (1982) J. Thorac. Cardiovasc. Surg. 83, 830-839
3. Jolly, S. R., Kane, W. J., Baille, M. B., Abrams, G. D., and Lucchesi, B. R. (1984) Circ. Res. 54, 277-285
4. Zweier, J. L., Shea, M. J., Driscoll, E. M., Cohen, C., Abrams, G. D., Pitt, B., and Lucchesi, B. R. (1985) Circ. Res. 56, 885-898
5. Chambers, D. E., Parks, D. A., Patterson, G., Roy, R., McCord, J. M., Yoshida, S., Parmley, L. P., and Downey, J. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 874-878
6. Ambrosio, G., Weisfeldt, M. L., Jacobs, W. E., and Flaherty, J. T. (1987) Circulation 75, 282-291
7. Zweier, J. L., and Weisfeldt, M. L. (1985) Clin. Res. 33, 240A
8. Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1404-1407
9. Limm, W., Mugishimi, M., and Piette, L. H. (1987) Fourth International Congress on Oxygen Radicals, June 27-July 3, La Jolla, CA, Vol. 2 (July 2), pp. 123-126, National Bureau of Standards, Washington, D. C.
10. Baker, J. E., Felix, C. C., Olinger, G. N., and Kalyanaraman, B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2786-2789
11. Maupoil, V., and Rochette, L. (1988) Cardiovasc. Drugs Ther. 2, 615-621
12. Alpert, C. M., Kramer, J. H., Dickens, B. F., and Weglicki, W. B. (1987) FERS Lett. 221, 101-104
13. Bolli, R., Patel, B. S., Jeroudi, M. O., Lai, E. K., and McCoy, P. B. (1988) J. Clin. Invest. 82, 476-485
14. Blasig, I. E., Ebert, B., and Lowe, H. (1986) Studia Biophys. 116, 21-32
15. Blasig, I. E., Lowe, H., and Ebert, B. (1987) Biomed. Biochem. Acta 46, S39-544
16. Zweier, J. L., and Jacobs, W. E. (1987) J. Biol. Chem. 262, 8015-8021
17. Feher, G. (1987) Bell Syst. Tech. J. 36, 449-460
18. Zweier, J. L., and Kuppusamy, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5703-5707
19. Nettar, D., and Vialafancre, J. J. (1988) J. Magn. Res. 64, 61-65
20. Zweier, J. L. (1988) J. Biol. Chem. 263, 1352-1357
21. Nakazawa, H., Ichimiya, K., Shimozaki, Y., Okuno, H., and Hori, S. (1988) Am. J. Physiol. 255, H213-215
22. Bray, R. C., Mautner, G. N., Fielden, E. M., and Carle, C. I. (1977) in Superoxide and Superoxide Dismutases (Michelson, A. M., McCord, J. M., and Fridovich, I., eds) pp. 61-75, Academic Press, Orlando, FL
23. Hall, D. O., Cammack, R., and Rao, K. K. (1974) in Iron in Biochemistry and Medicine (Jacobs, A., and Worwood, M., eds) pp. 279-354, Academic Press, Orlando, FL
24. Zweier, J. L., Rayburn, B. K., Flaherty, J. T., and Weisfeldt, M. L. (1987) J. Clin. Invest. 80, 1728-1734
25. Fee, J. A., and Valentine, S. W., Shea, M. J., Driscoll, E. M., Cohen, C., Abrams, G. D., Pitt, B., and Lucchesi, B. R. (1985) Circ. Res. 56, 885-898
26. Cadenas, E., Boveris, A., Ragan, C. L., and Stoppani, A. O. M. (1977) Arch. Biochem. Biophys. 180, 248-257
27. Zweier, J. L., Kuppusamy, P., and Lutty, G. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4046-4050