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Direct measurement of free radical generation following reperfusion of ischemic myocardium

(free radicals/ischemia/reperfusion/heart/magnetic resonance)

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ABSTRACT **Electron paramagnetic resonance spectroscopy was used to directly measure free radical generation in perfused rabbit hearts. Hearts were freeze-clamped at 77°K during control perfusion, after 10 min of normothermic global ischemia (no coronary flow), or following post-ischemic reperfusion with oxygenated perfusate. The spectra of these hearts exhibited three different signals with different power saturation and temperature stability: signal A was isotropic with g = 2.004; signal B was anisotropic with axial symmetry with $g_{\parallel}$ = 2.033 and $g_{\perp}$ = 2.005; signal C was an isotropic triplet with g = 2.000 and hyperfine splitting a_n = 24 G (1 G = 0.1 mT). The g values, linewidth, power saturation, and temperature stability of signal A are identical to those of a carbon-centered semiquinone, whereas those of signal B are similar to alkyl peroxyl or superoxide oxygen-centered free radicals; signal C is most likely a nitrogen-centered free radical. In the control heart samples signal A predominated, whereas in ischemic hearts signal A decreased in intensity, and signals B and C became more intense; with reperfusion all three signals markedly increased. Free radical concentrations derived from the intensities of the B and C signals peaked 10 sec after initiation of reflow. At this time the oxygen-centered free radical concentration derived from the intensity of signal B was increased over six times the concentration measured in control hearts and over two times the concentration measured in ischemic hearts. Hypoxic reperfusion did not increase any of the free radical signals over the levels observed during ischemia. These experiments directly demonstrate that reactive oxygen-centered free radicals are generated in hearts during ischemia and that a burst of oxygen radical generation occurs within moments of reperfusion.**

Spontaneous thrombosis of a coronary artery produces regional myocardial ischemia and ultimately acute myocardial infarction. Recently, thrombolytic agents including streptokinase, urokinase, and tissue plasminogen activator have been used to dissolve intracoronary thrombus within the early hours of an acute myocardial infarction (1). More recently, acute coronary occlusion has been mechanically reversed by balloon dilatation (2). Both of these procedures result in reperfusion of myocardium at risk for infarction.

There is controversy as to whether reperfusion, while ending ischemia, may actually cause additional damage to the myocardial region at risk. This additional injury, or "reflow injury," is characterized histologically by the formation of contraction bands in the contractile proteins and calcific granules within mitochondria, as well as swelling of the cell and disruption of sarcoplasmic and mitochondrial membranes (3, 4). It has been proposed that a major portion of this reflow injury occurs secondary to the generation of oxygen free radicals (5). During the past decade indirect evidence from studies demonstrating the protective effects of free radical scavengers has suggested that reactive free radicals are formed both during ischemia and, more importantly, during postischemic reperfusion (5–10). Also, reactive oxygen radicals have been shown to produce myocardial damage (6). Unfortunately, all the evidence supporting the free radical hypothesis of ischemic-reperfusion cell damage has been indirect. Thus, a technique that could directly measure the generation of free radicals in heart tissue is greatly needed; ideally, the development of this technique should allow characterization of the radicals formed, as well as clarify the mechanisms of their formation, and finally help determine optimal strategies for the prevention of free radical-induced cellular damage.

Electron paramagnetic resonance (EPR) spectroscopy has been widely used for the past 40 years to identify and characterize free radicals in simple chemical systems. In this

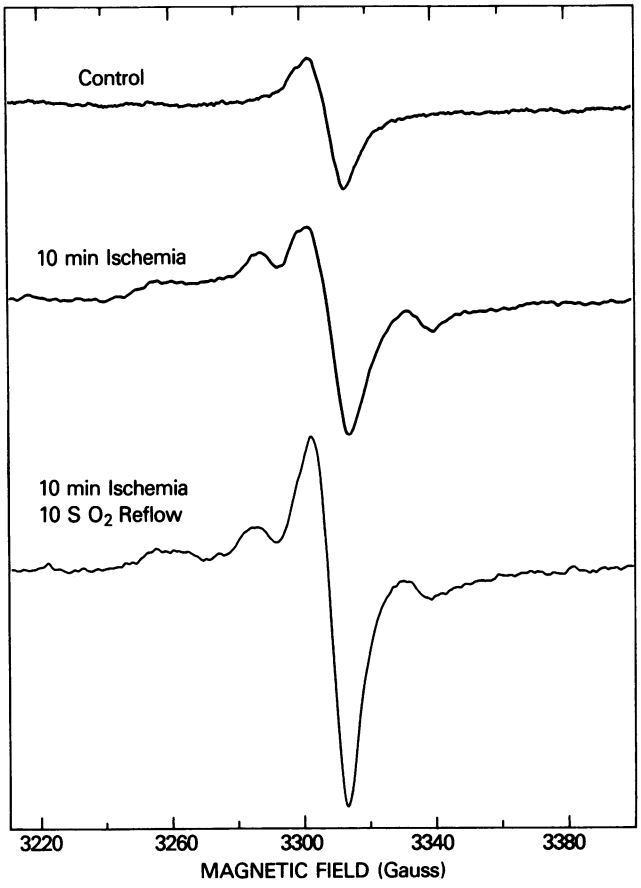

FIG. 1. EPR spectra of control, ischemic, and postischemic reperfused heart samples at 77K. Microwave frequency, 9.278 GHz; microwave power, 1.0 mW; modulation amplitude, 2.5 G.

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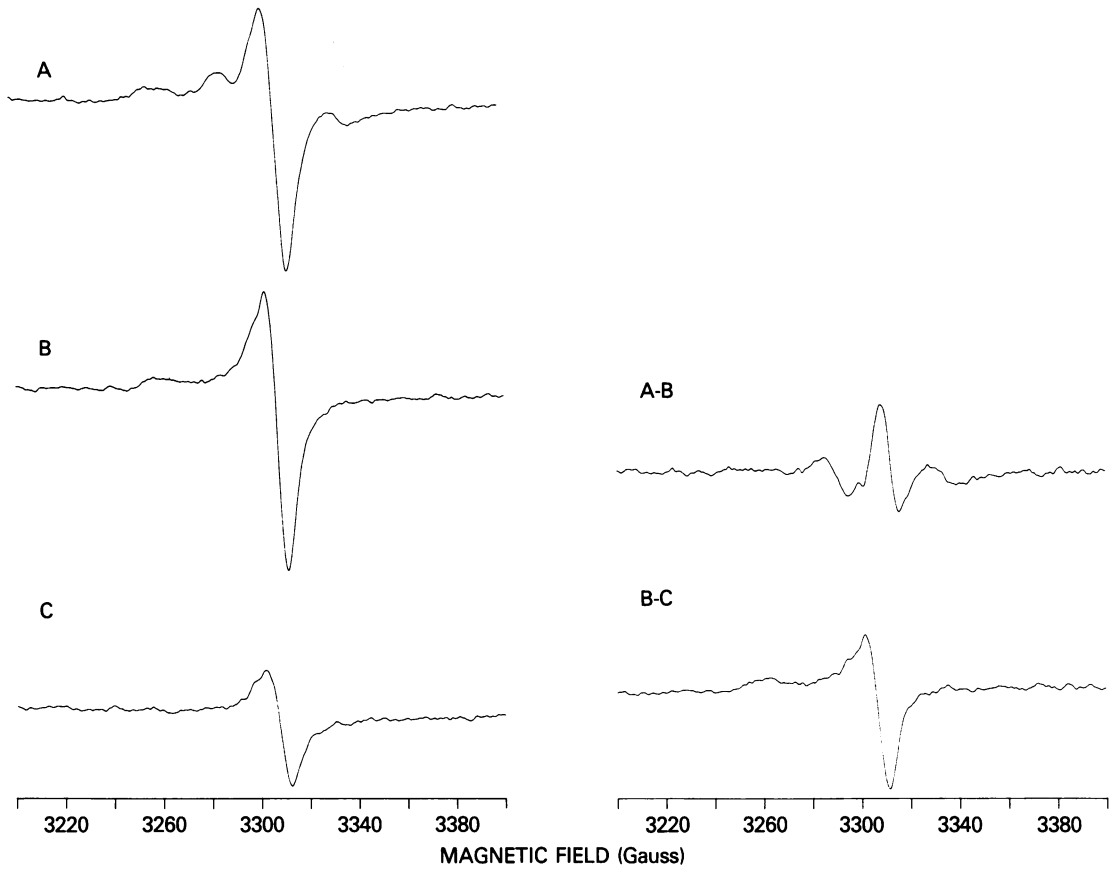

FIG. 2. EPR spectra of a heart sample freeze-clamped after 10-min ischemia followed by 10 sec of reflow with oxygenated perfusate; microwave frequency, 9.278 GHz; microwave power, 1.0 mW; modulation amplitude, 2.5 G. Trace A, sample maintained at 77K; trace B, sample raised to −80°C for one min; trace C, sample raised to −80°C for 1 hr—only an isotropic signal at $g = 2.004$ remains. Trace A−B, the subtraction of trace B from trace A, illustrates the triplet signal centered at $g = 2.000$ that disappears on raising the sample to −80°C for just 1 min. Trace B−C, subtraction of trace C from trace B, illustrates the temperature-sensitive anisotropic signal with axial symmetry $g_{\parallel} = 2.033$ and $g_{\perp} = 2.005$, which disappears after raising the sample temperature to −80°C for 1 hr.

study we have applied EPR spectroscopy to directly measure free radical generation in hearts subjected to ischemia and reperfusion.

MATERIALS AND METHODS

Isolated rabbit hearts were perfused by the method of Langendorff at a constant pressure of 80 mm of Hg (1 mm Hg = 133 Pa) with a Krebs/bicarbonate-buffered perfusate consisting of 117 mM NaCl/24.6 mM $NaHCO_3$/5.9 mM KCl/1.2 mM $MgCl_2$/2.5 mM $CaCl_2$/0.5 mM EDTA/16.7 mM glucose that was bubbled with 95% O_2/5% CO_2. Hearts were freeze-clamped using Wollenberger tongs cooled to 77K after either 10 min of control perfusion, 10 min of 37°C global ischemia, or 10 min of ischemia followed by various durations of reflow. The freeze-clamped hearts were then ground to a fine powder under liquid nitrogen, and the powder was transferred to precision EPR tubes. The EPR tubes were 3 mm in inside diameter and were filled to a height of 3 cm. EPR spectra were then recorded at a temperature of 77K using a Varian model E-9 spectrometer. Quantitation of the signals was done by comparison of the integrated signal area with that of the commonly used free radical standard potassium peroxylamine disulfonate (Aldrich) in frozen aqueous solution in identical EPR tubes (11). Care was taken to measure with nonsaturating microwave power.

RESULTS AND DISCUSSION

In the EPR experiment the sample is placed in a microwave-resonant cavity and irradiated with microwaves of fixed frequency, ν. The cavity is placed between the pole pieces of a magnet. The magnetic field, H, is varied, and the absorption of microwave power as a function of magnetic field is recorded. Resonance is defined by the following equation:

$$g = \frac{h}{\beta} \times \frac{\nu\ (\text{MHz})}{\text{H (G)}}$$

where h/β is a constant with a value of 0.714484 and g is a characteristic constant whose value serves to identify any given free radical. The absorption function for an isotropic electron is a lorentzian line. The usual convention in EPR spectroscopy is to show the first derivative absorption function. For a free radical in which the electron is in an anisotropic environment with axial symmetry the spectrum is defined by two g values, $g_{\parallel}$ and $g_{\perp}$. If the electron is coupled to a nucleus with nuclear spin such as a nitrogen nucleus splitting of the signal will be observed. Nitrogen coupling results in a triplet splitting. The magnitude of the splitting is defined by the hyperfine coupling constant a_n. Thus, EPR spectroscopy can provide information about the chemical structure of free radicals—in particular the symmetry of the electron environment and the presence of nuclei in the vicinity of the electron.

Control hearts give rise to a signal with a g value of 2.004 (Fig. 1*A*). In ischemic hearts the signal increases ≈30%, and in hearts reperfused with oxygenated perfusate for 10 sec, the signal increases over 2-fold above control levels (Fig. 1). Measurements were done in seven control, seven ischemic, and seven postischemic reperfused hearts that received oxygenated buffer for 10 sec. The intensity of the free radical signals in the control group (mean ± SEM) corresponded to

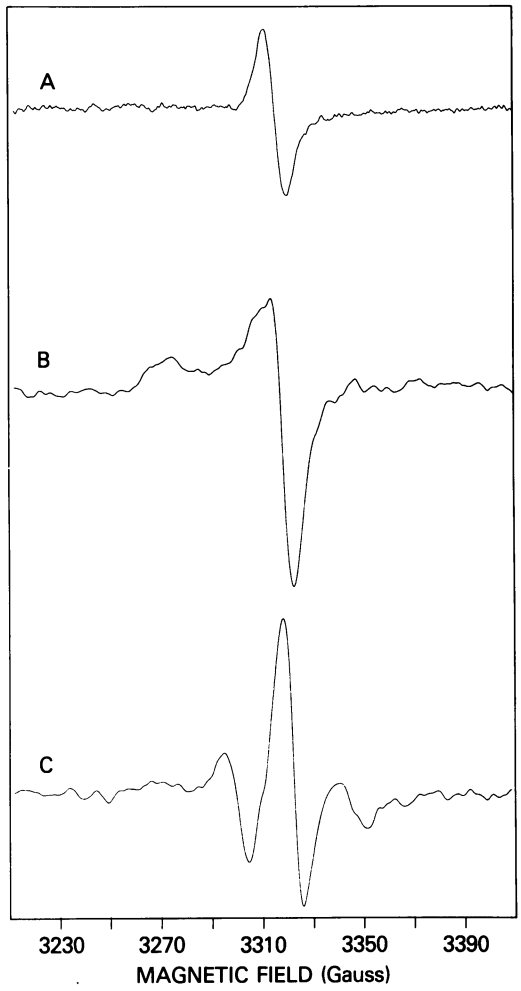

FIG. 3. EPR spectra of the three component signals observed in postischemic reperfused heart samples. These signals were separated using the temperature annealing procedure described in Fig. 2. Each component signal is vertically scaled to clearly show its spectral features. Trace A, signal A of isotropic free radical g = 2.004; trace B, signal B of anisotropic free radical with axial symmetry $g_{\|}$ 2.033 and $g_{\perp}$ 2.005; and trace C, signal C of triplet free radical g = 2.000 and a_n = 24 G. Microwave frequency, 9.290 GHz; microwave power, 1.0 mW; modulation amplitude, 2.5 G.

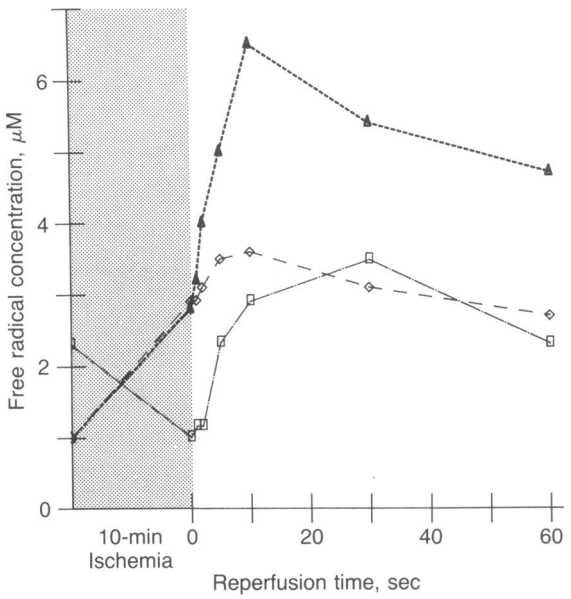

FIG. 4. Time course of free radical generation; each point corresponds to measurements performed on hearts freeze-clamped at different points in time. □, Signal A; △, signal B; ◇, signal C. Quantitation was performed by comparison of the double integral of the signals to that of the known concentration of peroxylamine standard.

a total free radical concentration of 4.7 ± 0.5 μM; the total free radical concentration of the ischemic group was 6.1 ± 0.4 μM, and that of the reperfused group was 11.4 ± 0.6 μM. Analysis of variance indicated that the differences among the three groups were statistically significant, $P < 0.001$. Careful examination of these spectra reveals that the spectrum from control heart samples consists of one component, a symmetric gaussian line at g = 2.004, whereas ischemia and reperfused samples exhibit spectra with three components (Fig. 1).

Temperature stability studies were performed in an effort to separate these three signals. If each of the three component signals has different temperature stability, it should be possible to isolate each signal by gradual warming of the sample. The most unstable temperature-sensitive free radicals will disappear first. Fig. 2, A shows the spectrum of a heart freeze-clamped after 10 min of ischemia followed by 10 sec of reflow with oxygenated perfusate. On warming to −80°C for 60 sec the spectrum shown in Fig. 2, B is obtained. One of the original spectral components has disappeared. The difference spectrum, A − B, shows this component to be a triplet signal. On further warming to −80°C for 1 hr, a second component disappears leaving only one remaining component—the symmetric gaussian line shown in Fig. 2, spectrum C. The difference spectrum, B − C, reveals a signal that exhibits the characteristic absorption function of an electron in an environment with axial symmetry. Fig. 3 shows the three component signals: A, a symmetric gaussian line at g = 2.004 indicative of an isotropic free radical; B, a signal with axial symmetry $g_{\|}$ = 2.033, $g_{\perp}$ = 2.005; and C, a triplet signal with g = 2.000 and hyperfine splitting a_n = 24 G.

When samples were thawed at room temperature for 10 min and then refrozen, the magnitude of the paramagnetic signals in the g = 2 region greatly decreased; signals B and C totally disappeared, and signal A decreased by 70%, confirming that all three observed signals are due to free radicals. Even when spectra were recorded with increased modulation amplitudes of 5 G and microwave power of 20 mW, no superimposed signals from paramagnetic metals ions were seen.

Signal A exhibits an identical g value, linewidth, power saturation, and temperature stability to that of a semiquinone free radical (12, 13). This signal, which is observed in control hearts, may correspond to the signal arising from the one electron-reduced ubiquinone free radical that has been previously noted in cells and mitochondrial suspensions (14). Power saturation studies were performed in control heart samples that exhibit the A signal, and the saturation behavior was found to be similar to that of semiquinone free radicals such as the doxorubicin (adriamycin) semiquinone free radical (12). At microwave power greater than 1.0 mW, saturation was observed. The intensity of this signal was stable at −80°C.

Signal B, which exhibits axial symmetry, is similar to signals previously reported for oxygen-centered free radicals. The observed g values are identical to those of alkyl peroxyl free radicals but also fall within the range of values described for superoxide (15, 16). The power saturation behavior of this signal was investigated in samples from post-ischemic reperfused hearts, and saturation was observed only at high microwave power (>20 mW). This signal was seen to be stable only at very low temperatures (<100K). As shown above, on warming to temperatures much higher

than that of liquid nitrogen, this signal disappears. Similar power saturation and temperature instability have been observed for the superoxide anion free radical and the alkyl peroxyl free radical (15, 16).

Signal C is a triplet that is suggestive of a nitrogen-centered free radical. However, the identity of this free radical remains uncertain.

To determine the time course of free radical generation during postischemic reperfusion, a series of hearts were freeze-clamped after varying periods of reflow ranging from 1 to 60 sec. The temperature annealing procedure described above was used to quantitate each of the three signals in each sample. Fig. 4 shows the time course of each of the three signals. In control hearts signal A was predominant with only small quantities of the other two signals. During ischemia, signal B and signal C increased, while signal A decreased. After postischemic reperfusion with oxygenated perfusate, a dramatic increase in signal B, and to a lesser extent signal C, was observed with levels peaking at 10 sec of reflow. Thus on reperfusion with oxygenated perfusate, there is a burst of free radical generation. As mentioned above, the properties of the B signal suggest an oxygen-centered free radical, such as an alkyl peroxyl or superoxide. Quantitation of the B signal was done in five control, five ischemic, and five postischemic hearts, which were reperfused with oxygenated buffer for 10 sec. The intensity of this signal in the control hearts (mean ± SEM) corresponded to a free radical concentration of 0.9 ± 0.2 μM, in the ischemic hearts the concentration was 2.8 ± 0.4 μM, and in the reperfused hearts the concentration was 6.8 ± 0.3 μM. Analysis of variance indicated that differences among the three groups were statistically significant ($P < 0.001$). Thus, after 10 sec of reperfusion with oxygenated perfusate, the concentration indicated by the B signal increased more than 6-fold above the concentration in control hearts and more than 2-fold above the concentration measured at the end of ischemia. On similar reperfusion with anoxic perfusate equilibrated with 95% N_2/5% CO_2, the levels of free radicals did not increase above the levels measured at the end of ischemia.

In summary, we have demonstrated that EPR spectroscopy can be used to directly measure the generation of free radicals in the heart. Our studies indicate that reactive oxygen-derived free radicals are generated in hearts subjected to global ischemia and reperfusion. We definitively demonstrate that there is a burst of oxygen free radical generation in the early moments of reperfusion. These studies provide direct evidence for the validity of the free radical hypothesis of ischemia-reperfusion cell injury. The technique that we have presented offers great promise for the measurement and characterization of free radical generation in all biological tissues, as well as for the determination of optimal strategies for the prevention of free radical-induced cellular injury.

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1. The TIMI Study Group (1985) *N. Engl. J. Med.* **312,** 932–936.
2. Pepine, C. J., Prida, X., Hill, J. A., Feldman, R. L. & Conti, C. R. (1984) *Am. Heart J.* **107,** 820–822.
3. Whalen, D. A., Hamilton, D. G., Ganote, C. E. & Jennings, R. B. (1974) *Am. J. Pathol.* **74,** 381–398.
4. Kloner, R. A., Ganote, C. E., Whalen, D. A. & Jennings, R. B. (1974) *Am. J. Pathol.* **74,** 399–422.
5. Hess, M. S. & Manson, N. H. (1984) *J. Mol. Cell. Cardiol.* **16,** 969–985.
6. Burton, K. P., McCord, J. M. & Ghai, G. (1984) *Am. J. Physiol.* **246,** H776–H783.
7. Shlafer, M., Kane, P. F. & Kirsch, M. M. (1982) *J. Thorac. Cardiovasc. Surg.* **83,** 830–839.
8. Myers, M. A., Bolli, R., Lekich, R. F., Hartley, C. J. & Roberts, R. (1985) *Circulation* **72,** 915–921.
9. Ambrosio, G., Weisfeldt, M. L., Jacobus, W. E. & Flaherty, J. T. (1987) *Circulation* **75,** in press.
10. Ambrosio, G., Becker, L. C., Hutchins, G. M., Weisman, H. F. & Weisfeldt, M. L. (1986) *Circulation* **74,** 1424–1433.
11. Wertz, J. E. & Bolton, J. R. (1980) *Electron Spin Resonance* (McGraw-Hill, New York), pp. 192–221.
12. Zweier, J. L. (1985) *Biochim. Biophys. Acta* **839,** 209–213.
13. Gianni, L., Zweier, J. L., Levy, A. & Myers, C. E. (1985) *J. Biol. Chem.* **260,** 6820–6826.
14. Onishi, T. & Trumpower, B. (1980) *J. Biol. Chem.* **255,** 3278–3284.
15. Knowles, P. F., Gibson, J. F., Pick, F. M. & Bray, R. C. (1969) *Biochem. J.* **111,** 53–58.
16. Copeland, E. S. (1975) *J. Magn. Reson.* **20,** 124–129.