Volume 244, number 2, 311–314 FEB 06820 February 1989

Ischemia-induced changes in myocardial paramagnetic metabolites: implications for intracellular oxy-radical generation

John E. Baker and B. Kalyanaraman*

Department of Cardiothoracic Surgery, Medical College of Wisconsin, Milwaukee, WI 53226 and **National Biomedical Electron Spin Resonance Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*

Received 21 December 1988

Evidence to identify the cellular sources of oxy-radical generation in myocardium has been of an indirect nature. We have used low-temperature ESR spectroscopy to identify and characterize ischemia-induced changes in myocardial paramagnetic metabolites. Iron-sulfur proteins associated with the NADH or succinate dehydrogenases of the mitochondrial electron-transport chain were progressively reduced with the onset and development of ischemia. This study provides direct evidence for ischemia-induced changes in an intracellular source of superoxide radical generation that may contribute to oxy-radical production during reperfusion.

Ischemia; Iron-sulfur center; Ubisemiquinone; ESR; Oxy-radical; (Rat heart)

1. INTRODUCTION

Abundant data indicate that free radicals derived from molecular oxygen contribute to myocardial cell injury induced during the cycle of ischemia and reperfusion [1,2]. However, evidence to identify the cellular sources of oxy-radical generation has been of an indirect nature. Recently, we [3] and others [4] have applied low-temperature ESR spectroscopy to detect directly paramagnetic metabolites in myocardial tissue which can act as a potential source for oxy-radical generation. These studies [3,4] identified the iron-sulfur centers and the ubisemiquinone associated with the mitochondrial electron-transport chain. The molecular changes that occur in these paramagnetic metabolites during ischemia, and how they impact upon oxy-radical production are unknown. The objectives of this study were (i) to characterize ischemia-induced changes in the iron-sulfur center and ubisemiquinone as they relate to oxy-radical production, and (ii) to demonstrate that the identity of the iron-sulfur centers is distinct from the peroxy radical artifactually generated by grinding of frozen myocardial tissue [5].

Correspondence address: J.E. Baker, Department of Cardiothoracic Surgery, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

2. MATERIALS AND METHODS

2.1. *Isolated heart model*

Adult Sprague-Dawley rats maintained on a standard diet were used for this study. Anesthesia was induced and maintained with halothane (4 and 1–2%, respectively), after which the right femoral vein was exposed and heparin (150 IU/kg) was administered intravenously. After 1 min, the heart was rapidly excised and placed in ice-cold perfusion medium. After 30 s, the aorta was attached to a stainless-steel cannula, the pulmonary artery was incised to permit adequate coronary drainage, and the heart was perfused normothermically at 37°C by the method of Langendorff [6] at a perfusion pressure equivalent to 12 kPa (90 mmHg). A three-way tap located immediately above the site of cannulation allowed the entire perfusate to be diverted away from the heart to produce global, no-flow ischemia. The heart and perfusion fluids were kept in temperature-controlled chambers to maintain the myocardial temperature at 37°C.

2.2. *Perfusion medium*

The standard perfusion fluid was Krebs-Henseleit bicarbonate buffer (pH 7.4 when gassed with 95% O_2/5% CO_2) in which the calcium content was reduced to 1.8 mM [7]. To this

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

was added glucose (11.1 mM). During the preparation of all calcium-containing solutions, precautions were taken to prevent the precipitation of calcium by gassing the solution with 5% CO_2. Before use, all perfusion fluids were filtered through cellulose acetate membranes (pore size, 5.0 μm).

2.3. *Perfusion sequence*

Immediately after mounting on the cannula, hearts ($n = 8$ per group) were perfused aerobically at 37°C for a 20 min equilibration period. Hearts ($n = 8$ per group) were then subjected to increasing durations of normothermic, global, no-flow ischemia at 37°C (10, 30, and 60 min). At the stated times in the perfusion sequence, hearts were freeze-clamped between aluminium tongs (Biomedix, Elm Grove, WI) previously cooled to liquid N_2 temperature.

2.4. *Tissue processing*

Frozen ventricular tissue was then processed for spectroscopic analysis by chopping the wafer of frozen tissue under liquid N_2 with a stainless-steel spatula to produce small fragments (≈ 2 mm cubes). These fragments were immediately transferred to the lumen of a Dewar flask previously cooled to liquid N_2 temperature. Sample sizes were approximately equal. All flasks containing the processed heart tissue were then placed in the resonance chamber of the spectrometer. We have previously demonstrated that this processing technique is less likely to produce paramagnetic artifacts in comparison to processing tissue by grinding [3]. At the end of each experiment, heart tissue was removed from the Dewar flasks and kept in a dessicator containing phosphorus pentoxide until constant dry weight was achieved.

2.5. *ESR spectroscopy*

ESR spectra from all samples were recorded at liquid N_2 temperature with a Varian E-109 spectrometer at 9.5 GHz and 100 kHz field modulation. Magnetic field measurements were determined by using a Radiopan MJ-110 gaussmeter, and microwave frequency was measured by using an EiP 200 frequency counter. Hyperfine splitting was directly measured to 0.05 G from the magnetic field separations. All g value derivations were obtained from measurements of magnetic field and microwave frequency after correcting for the position of the gaussmeter probe.

All experimental procedures used in this study were in keeping with the established guidelines prepared by the National Academy of Sciences for the humane care of laboratory animals.

3. RESULTS

The ESR spectrum of aerobically perfused rat heart, freeze-clamped with the frozen tissue chopped prior to recording of spectrum is shown in fig.1A. This spectrum has previously been assigned to a ubisemiquinone ($g = 2.004$) [3–5]. Note also the presence of a weak signal at $g = 1.94$, possibly due to an iron-sulfur center. The ESR spectrum of rat heart tissue, freeze-clamped after increasing

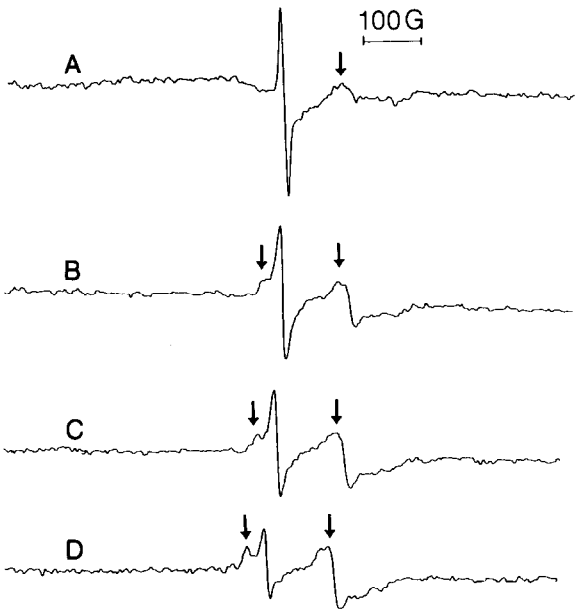

Fig.1. ESR spectra of (A) control, aerobically perfused, and (B) after 10 min ischemia, (C) 30 min ischemia, and (D) 60 min ischemia with heart freeze-clamped and the frozen tissue chopped under liquid N_2 prior to recording the spectra at −190°C. Spectrometer conditions: microwave frequency, 9.17 GHz; microwave power, 2 mW; modulation amplitude, 2.0 G; gain, 3.2×10^4; scan range, 1000 G; scan time, 8 min; time constant, 0.5 s. The absorption positions that correspond to the reduced iron-sulfur center are marked ↓.

durations of ischemia, is shown in fig.1B–D. The intensity of the ESR absorption peaks at $g = 2.025$ and 1.94 (marked ↓) increased with increasing ischemic duration. In contrast, the intensity of the peak corresponding to a secondary ubisemiquinone ($g = 2.0045$) [3] decreased over this time period.

The ESR peaks at $g = 1.94$ and 2.025 are assigned to a reduced iron-sulfur center (center 1) associated with mitochondrial dehydrogenases such as NADH or succinate dehydrogenase. Whereas the ESR spectra of most other iron-sulfur centers in the mitochondrial respiratory chain can be detected only at liquid helium temperatures [4,8,9], spectra of center 1 of NADH and succinate dehydrogenases have been observed at liquid N_2 temperatures [8]. At liquid N_2 temperatures, signals due to centers 2 and 3 were not detected. However, evidence of broad underlying lines from these centers can be seen in fig.1B–D. Ledenev et al. [4] have also observed ESR signals at $g = 2.10$, 2.05,

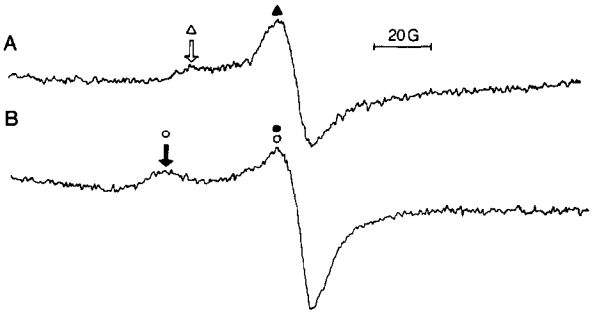

Fig.2. ESR spectra of (A) chopped myocardium after 10 min of ischemia and (B) ground myocardium after 10 min of ischemia that has been subsequently exposed to air for 60 s. (●) Ubisemiquinone radical; (○) lipid peroxy radical; (△) iron-sulfur center; (▲) secondary ubisemiquinone. The absorption position marked (⇩) indicates $g = 2.025$ and (↓) denotes $g = 2.04$. Spectrometer conditions: microwave frequency, 9.17 GHz; microwave power, 2 mW; modulation amplitude, 2.0 G; gain, 8.0×10^4; scan range, 200 G; scan time, 8 min; time constant, 0.5 s; sample temperature, −190°C.

1.92, 1.89, and 1.86 corresponding to centers 2 and 3 of NADH dehydrogenase in ischemic rat myocardium.

Ischemia-induced lipid peroxidation has been shown to occur in myocardial tissues [10–12]. Peroxy radicals produced during lipid peroxidation exhibit characteristic ESR absorptions in the region $g = 2.03$–2.04 [3,13]. Thus, we need to make a clear distinction between the spectra of the peroxy radical and that of the iron-sulfur center. We have previously shown that peroxy radicals can be artifactually induced upon grinding of freeze-clamped ischemic rat and rabbit myocardium [3,14]. The ESR spectrum of ground ischemic myocardium as shown in fig.2B, after exposure to room air for 60 s, should be compared with that of chopped tissue as shown in fig.2A. Note the absorption region of the iron-sulfur center (marked △) at $g = 2.025$ (⇩) is clearly different from that of the peroxy radical (○) at $g = 2.04$ (↓).

4. DISCUSSION

This study characterizes ischemia-induced changes in an intracellular source of oxy-radical generation in the rat heart. Low-temperature ESR spectroscopy has been used to characterize ischemia-induced changes in the spectra of the iron-sulfur proteins associated with NADH or succinate dehydrogenase, in addition to the ubisemiquinone of the cardiac mitochondrial electron-transport chain.

During aerobic mitochondrial respiration, approx. 1% of electron transport results in the production of the superoxide radical [15,16]. The site of production of superoxide occurs at the site between NADH dehydrogenase and ubiquinone [17]. Our study demonstrates that with the onset and progression of ischemia, these components are progressively reduced, resulting in the accumulation of reduced metabolites. Ischemia also causes the degradation of the adenine nucleotide pool, which can result in the additional reduction of the mitochondrial electron carriers [15]. Upon aerobic reperfusion of the ischemic myocardium, the immediate reoxidation of these reduced components may be responsible for the increase in production of the superoxide radical from this intracellular source. In addition, when molecular oxygen is restored to the mitochondria during reperfusion, electron transport across cytochrome oxidase may be impaired due to the depletion of ADP during ischemia [15]. This would result in an increase in the percentage of electron transferred directly to molecular oxygen to generate the superoxide radical. Thus, the intracellular production of superoxide upon reperfusion may be related to the redox state of the iron-sulfur centers and the ubisemiquinone during ischemia, with the extent of superoxide generation determined by the severity of the ischemic episode. In support of this, Kramer et al. [18], using ESR spin trapping, have shown that the extent of oxy-radical production during reperfusion is directly related to the duration of the preceding ischemic period. Thus, it is possible that oxy-radicals spin trapped during reperfusion [19–21] may be related to the auto-oxidation of the mitochondrial ubiquinone/ubiquinol redox couple.

Evidence for peroxy radical generation during ischemia using direct ESR has not been demonstrated. The peroxy radical species reported by Zweier et al. [5] has been shown to be an artifact generated during tissue processing [3]. Our study demonstrates that the identity of the iron-sulfur centers, detected and characterized by ESR during ischemia, is distinct from that of the artifactually generated peroxy radical.

In conclusion, this study characterizes ischemia-induced changes in an intracellular source of oxy-radical generation that may play an important role in superoxide generation during reperfusion of the ischemic myocardium.

Acknowledgements: This work was supported by National Institute of Health Grants GM-29035 and RR-01008 and by the Wisconsin Affiliate of the American Heart Association 87-GA-85.

REFERENCES

[1] McCord, J.M. (1985) N. Engl. J. Med. 312, 159-163.
[2] Hess, M.S. and Manson, N.H. (1984) J. Mol. Cell. Cardiol. 16, 969-985.
[3] Baker, J.E., Felix, C.C., Olinger, G.N. and Kalyanaraman, B. (1988) Proc. Natl. Acad. Sci. USA 85, 2786-2789.
[4] Ledenev, A.N., Lakomkin, V.L. and Ruuge, E.K. (1986) Biophysics 31, 748-751.
[5] Zweier, J.L., Flaherty, J.L. and Wesfeldt, M.L. (1987) Proc. Natl. Acad. Sci. USA 84, 1404-1407.
[6] Langendorff, O. (1895) Pflügers Arch. Ges. Physiol. 61, 291-332.
[7] Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33-66.
[8] Beinert, H. (1978) Methods Enzymol. 54, 133-150.
[9] Orme-Johnson, N.R., Orme-Johnson, W.G., Hansen, R.E., Beinert, H. and Hatefi, Y. (1973) in: Oxidases and Related Redox Systems (King, T.E. et al. eds), pp. 769-797, University Park Press, IL.
[10] Rao, P.S., Cohen, M.V. and Mueller, H.S. (1983) J. Mol. Cell. Cardiol. 15, 713-716.
[11] Rao, P.S. and Mueller, H.S. (1983) Adv. Exp. Biol. Med. 161, 347-363.
[12] Romaschin, A.D., Rebeyka, I., Wilson, G.J. and Mickle, D.A.G. (1987) J. Mol. Cell. Cardiol. 19, 289-302.
[13] Yanez, J., Sevilla, C.L., Becker, D. and Sevilla, M.D. (1987) J. Phys. Chem. 91, 487-491.
[14] Baker, J.E., Felix, C.C. and Kalyanaraman, B. (1988) in: Oxy-Radicals in Molecular Biology and Pathology (Cerutti, P.A. et al. eds) pp. 343-351, Alan R. Liss, Inc.
[15] McCord, J.M. (1988) Free Rad. Biol. Med. 4, 9-14.
[16] Fridovich, I. (1974) Adv. Enzymol. 41, 36-97.
[17] Turrens, J.F. and Boveris, A. (1980) Biochem. J. 191, 421-427.
[18] Kramer, J.H., Arroyo, C.M., Dickens, B.F. and Weglicki, W.B. (1987) Free Rad. Biol. Med. 3, 153-159.
[19] Blasig, I.E., Lowe, H. and Ebert, B. (1987) Biomed. Biochim. Acta 46, 539-544.
[20] Zweier, J.L. (1988) J. Biol. Chem. 263, 1353-1357.
[21] Arroyo, C.M., Kramer, J.H., Dickens, B.F. and Weglicki, W.B. (1987) FEBS Lett. 221, 101-104.