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A NEW APPROACH TO THE DIRECT DETECTION OF FREE RADICALS IN THE INTACT MYOCARDIUM

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A new method for the direct ESR detection of free radicals in rat myocardial tissue is described. Isolated rat atria are continuously monitored for heart rate and contractile force; at the end of the experimental period the beating organs are inserted into quartz ESR tubes and immediately frozen in liquid nitrogen. Spectra obtained from these preparations show the presence of very weak radical signals. When ESR spectra are recorded on samples obtained from pools of rat atria pulverized under liquid nitrogen, the radical lines are markedly stronger than those observed for intact organs; contaminating metals are also frequently detected. These findings indicate that crushing or grinding procedures carried out under liquid nitrogen produce artifactual ESR active species. The new method described in the present paper does not involve mechanical interventions and therefore should yield reliable artifact-free results.

KEY WORDS: ESR spectroscopy, radicals, rat heart.

INTRODUCTION

In recent years considerable attention has been devoted to the study of free radical generation in the myocardium and to its possible role as a mediator of a variety of spontaneous and drug-induced pathologic conditions. However, due to difficulties in detecting free radicals in living tissues, only indirect evidences that these species initiate cellular injury were reported. The determination of free radicals in intact myocardium was mainly performed by spin trapping techniques: nitrone or nitroso compounds interact with the radicals, yielding comparatively stable adducts which are more easily detected by conventional ESR spectroscopy at room temperature.[1–8] Unfortunately, the application of this method to living tissues has some significant drawbacks, recently reviewed by Pou *et al.*[9] First, the rate of the spin trap reaction with some important oxygen-centered radicals is slow; therefore, high concentrations of the spin trap are required, which may lead to the development of severe cellular toxicity.[8,11,12] Second, identification of the free radicals is impaired by a possible decomposition of the adducts, which is accelerated in the presence of peroxidating enzymes;[10] moreover, spin traps themselves are chemically and metabolically unstable.[8,9] Third, different traps have largely different partition coefficients, leading to different affinities for the hydro- and lipohilic cellular compartments: the experimental results depend upon the specific spin trapping compound used.[1]

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The direct detection of free radicals in tissues might yield better results, provided that suitable methods to stabilize the short-living radicals were available. This stabilization has been attempted in myocardial tissues by performing low temperature measurements on frozen tissues. Zweier *et al.*[13] carried out experiments on Langendorff perfused rabbit hearts, which were freeze-clamped under liquid nitrogen, ground to a powder and transferred to ESR tubes. This procedure was criticized by Baker *et al.*,[14,15] who demonstrated that grinding of myocardial tissues generates artifactual signals. Another recent attempt to detect free radicals *in vivo* was performed by Zweier *et al.*,[16] by means of a modified ESR apparatus equipped with a loop gap resonator; in principle, this approach allows free radical detection in Langendorff perfused beating rat hearts and enables serial measurements of both free radicals and functional parameters of cardiac activity. However, with this method free radicals were not observed in normal tissues, probably because hearts operate at 37°C and at this temperature the amount of free radicals is too low to be detected by ESR.

The present investigation describes a new procedure based on the freezing of the intact beating myocardial preparations (isolated rat atria). Monitoring of the heart functional parameters (contractility, heart rate) can be individually performed throughout the experiment; spectra are subsequently recorded at low temperature (85°K) with a conventional ESR spectrometer. The method is designed to avoid generation of mechanical artifacts and contamination by metal devices commonly used in perfusion experiments (cannulae, hooks) or by foreign substances (e.g. liquid nitrogen); these characteristics might be exploited in the study of myocardial pathologic conditions and of drug effects on heart muscle.

METHODS

Spontaneously beating atria were isolated from 20 Sprague Dawley rats of 180 g average body weight; the organs were suspended with glass hooks in a suitable bath, incubated in Tyrode solution (mM composition: NaCl 137; KCl 5.37; $MgCl_2$ 0.51; $NaHCO_3$ 12; $CaCl_2$ 1.8; glucose 11, Tris-HCl 1, pH 7.4 at 37°C; saturated with O_2–CO_2 95:5). Chemicals were of the highest available degree of purity. Heart rate and contractile force were continuously monitored during a 45 min equilibration period and up to 60 min after equilibration. Hearts showing arrhythmias or abnormalities in contractile force or beating rate were discarded. Finally, the intact beating organs were inserted into a 3 mm diameter quartz ESR tube open on both sides, in appropriate position to cover the active part of the resonant ESR cavity; in some of the samples the bottom of the tube was sealed with polyethylene prior to dipping in liquid nitrogen, so as to prevent direct contact between organs and liquid nitrogen itself. The length (usually 15–16 mm) and dry weight of the organ were measured in order to calculate the amount of tissue in the active part of the cavity (10 mm length). For a comparison, groups of 7–8 beating atria were selected on the basis of the presence of normal and stable heart rate and contractility: they were pooled and pulverized under liquid nitrogen and the powder was subsequently transferred to an ESR tube so that the active part of the cavity was covered. Again the amount of tissue in the cavity (10 mm length) was determined from the dry weight of the sample.

Spectra were recorded at 85°K by means of a Varian E-109 spectrometer equipped with a Varian automatic temperature control. Spectrometer operating conditions are reported in Figures 1 and 2.

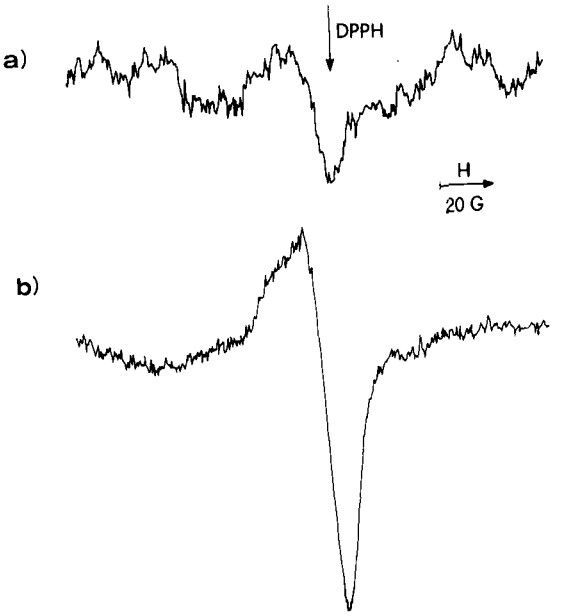

FIGURE 1 a) ESR spectrum of rat atrium frozen in liquid nitrogen, recorded at 85°K. b) ESR spectrum of pulverized rat atria, recorded at 85°K. Spectrometer conditions: microwave frequency 8.98 GHz; microwave power 5 mW; modulation amplitude 16 G; receiver gain 2×10^4; scan range 400 G; scan time 4 min; time constant 0.064 sec.

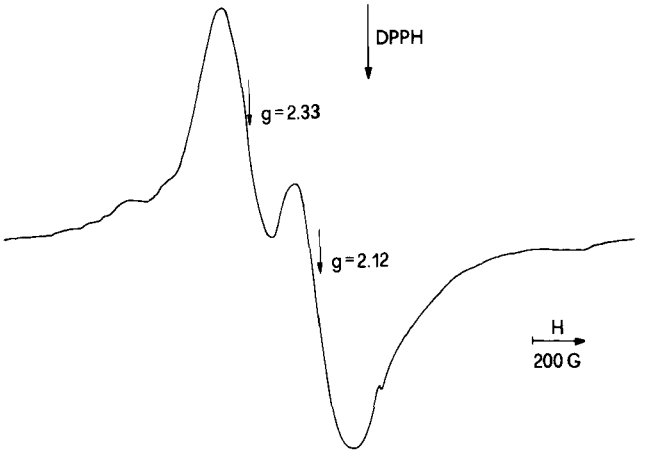

FIGURE 2 ESR spectrum of pulverized rat atria, recorded at 85°K. Spectrometer conditions: microwave frequency 8.98 GHz; microwave power 5 mW; modulation amplitude 16 G; receiver gain 1.25×10^3; scan range 4000 G; scan time 4 min; time constant 0.064 sec.

RESULTS AND DISCUSSION

In the present investigations, spontaneously beating rat atria were continuously monitored for heart rate and contractile force; preparations showing arrhythmias, abnormalities of contractility or modifications of the heart rate were discarded. In conditions of oxygen saturation, both the heart rate and the contractile force remained stable (240 ± 12 beats/min initial and final rate; 10.7 ± 0.7 g/sec initial contractile force; 10.6 ± 0.7 g/sec final value), thus showing that the organs were in physiological conditions throughout the experiment. Spectra obtained at 85°K simply by inserting the beating organs into ESR tubes and freezing the samples, without any previous mechanical manipulations (crushing, grinding or chopping), showed the presence of very weak resonance signals at g = 2.00 (Figure 1a), barely detectable over the background noise. Spectra obtained from atria pooled and pulverized under liquid nitrogen also showed the presence of a signal at g = 2.00, but significantly higher in intensity than to those observed in intact atria (Figure 1b). Considering that in our experiments the amount of tissue in the active part of the ESR cavity is lower in the samples obtained from pulverized atria (5.84 ± 0.28 mg dry weight/cm) than in those obtained from intact preparations (9.14 ± 0.34 mg dry weight/cm), it can be concluded that the presence of paramagnetic centers is artifactually enhanced by grinding.

These results are in line with those obtained by Baker *et al.*[14,15] in ventricular myocardium and by Symons[17] in organic and inorganic samples as well as in tissues. In the attempt to avoid such mechanical artifacts, Baker *et al.*[14,15] proposed to chop the myocardium into small pieces under liquid nitrogen after crushing the organs with a pre-cooled Wollenberger clamp. Spectra recorded on these samples showed only one out of all the signals previously reported by Zweier *et al.*;[13] the authors suggested that the remaining signals were artifactually generated by grinding and tentatively identified the "true" line as the ubisemiquinone radical.[14,15] However, in our experiments, the signal described by Baker *et al.* was never observed in intact beating atria. Since a mechanical process is also involved in the crushing-and-chopping procedure recommended by Baker *et al.*, it may be argued that the radical they observed is a mechanical artifact. It should be recalled that electron transfer from ubisemiquinone radicals to molecular oxygen is strongly accelerated in normal cells, where superoxide dismutase and oxygen are present; therefore the presence of the ubisemiquinone signal would suggest that the organs used for ESR spectroscopy were anoxic. Although functional conditions of the hearts were not fully documented, we assume that the hypothesis of a mechanical artifact is more likely. Similarly, the coupled signals described by Baker and Kalyanaraman[15] at g = 2.025 and g = 1.94, and assigned by the authors to iron-sulfur centers, were never observed in the series of 20 preparations examined in the present study.

Besides free radicals, other paramagnetic species were detected in the present investigations, consisting in broad lines ranging from g = 2.13 to g = 2.33 (Figure 2). These signals can be attributed to a transition metal, probably iron; in our experiments they were frequently present in samples ground under liquid nitrogen, but were seldom observed in intact atria dipped in liquid nitrogen before ESR experiment and completely absent in intact atria frozen in liquid nitrogen after sealing the ESR tube. Given the experimental procedure adopted for the preparation of these last samples (absence of contact with metal devices and/or contaminants in liquid nitrogen), it is reasonable to suggest that these metal-dependent signals, when present,

are probably due to some form of contamination. However, this does not seem to be the case for similar signals observed in other tissues. Benedetto *et al.*[18] described the presence of spectral lines (from g = 2.13 to g = 2.20) in both ground and intact samples of uterine tissue frozen in liquid nitrogen. The Authors suggested that they could be related to the high levels of iron-dependent enzymes, such as cyclooxygenase and lipoxygenase, which are typical of the uterine tissue.

The evidence reported in the present paper suggests that myocardial samples obtained by mechanical procedures show artifactual ESR signals. The method described here avoids some of the pitfalls connected with mechanical manipulation and contamination and therefore should yield reliable results, which in principle might allow direct correlations between the observed radicals, the functional properties of the heart and the effect of drug administration. However, the technique shares with other free radical detection methods the common problem of a very low sensitivity, which in this case might be explained by the slow freezing rate of the tissues. As the temperature approaches 0°C, the free radical-producing enzymatic reactions are dramatically slowed down, while chemical reactions are less temperature-sensitive, so that some decomposition of previously formed radicals can occur during the freezing-time of the organs. As a consequence, radicals might be detectable by ESR spectroscopy only when present in very large amounts; it has been shown that this does not occur in normal hearts, but the method might be usefully employed for the study of pathologic and/or drug-related conditions involving an enhanced free radical production.

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