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DIRECT DETECTION OF PARAMAGNETIC SPECIES IN ADRIAMYCIN PERFUSED RAT HEARTS

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Direct detection of paramagnetic species in control and adriamycin-perfused rat hearts has been carried out.Depending on the flow rate of the perfusion solution(8,4,2 and 1 ml/min) different paramagnetic species were observed: Fe(III)(g=2.12)at 4 ml/min;three types of oxygen centered radicals of which two in control hearts($g_{\parallel}^{1}$=2.05 $g_{\parallel}^{2}$=2.038 $g_{\perp}^{1}=g_{\perp}^{2}$=2.008) and the third one ($g_{\parallel}$=2.03 $g_{\perp}$=2.005) in adriamycin perfused hearts,at 2 ml/min.The latter radical was the only one observed at perfusion rate of 1 ml/min both in control and adriamycin treated systems.A relationship between the intracellular enzymatic reductive activation of the anthracycline and the occurrence of ischemic conditions(4,2 and 1 ml/min) in myocardial tissues is proposed basing on the relative amounts of the paramagnetic species above mentioned.

The involvement of oxygen-centered free radicals,superoxide and OH·,in the mechanism of adriamycin(ADR)-induced cardiotoxicity has been postulated on the basis of experiments performed in isolated subcellular preparations and cell-free systems(1).In these experimental conditions,radical formation was demonstrated either as the indirect result of different chemical reactions (2,3),or by e.s.r. studies with suitable spin traps(4).

Recent reports indicate that e.s.r. spectroscopy can be successfully used to detect free radicals in tissues,either by means of spin traps(5),or in freeze-clamped preparations(6,7).Experiments performed on "in vitro" surviving organs demonstrated that oxygen-centered radicals can be directly detected in frozen samples of hearts subjected to reperfusion following a period of global ischemia.However,up to now e.s.r. spectroscopy has not been applied to the detection of ADR-induced free radical formation in working hearts.

We present here the results of an e.s.r. investigation aimed to the direct detection of paramagnetic species in rat hearts perfused with ADR.

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MATERIALS AND METHODS

Female Spreague Dawley rats,with an average weight of 120-140 g,were used in this study.The hearts were isolated and perfused by the Langendorff method with a modified Tyrode solution of the following composition (mM): glucose 11,NaCl 137,KCl 5.37,$MgCl_2$ 0.51,$NaHCO_3$ 12,$CaCl_2$ 1.8,Tris-HCl 1.0, pH 7.4.The solution was gassed with a CO_2(5%)-O_2(95%) mixture;the hearts were electrically driven at 3 Hz throughout the whole experimental period. The organs were allowed to equilibrate for 30',during which the coronary flow was set at 8 ml/min by means of a peristaltic pump.This value corresponds to the average spontaneous flow rate (range:6.3-10.3 ml/min), and therefore it was chosen in order to ensure baseline normoxic conditions for the hearts. The equilibration period was followed by a 15' period during which the organs were perfused at different flow rates:8 ml/min(normoxic),4,2 and 1 ml/min, in order to produce increasing degrees of ischemia.In parallel sets of experiments,after the equilibration period,ADR was added to the perfusion medium to a final concentration of 10^{-4} M.At the end of the experiments,the hearts were freeze-clamped and ground to a powder under liquid nitrogen; the powder was subsequentely transferred to an e.s.r. tube and stored at 77 K. Spectra were recorded at 123 K on a Varian E-109 spectrometer equipped with a Varian automatic temperature control.Quantitation of the paramagnetic species was performed by double integration of signals taking as reference area that of Varian weak pitch(10^{13} spin/cm).

RESULTS AND DISCUSSION

The experimental conditions adopted in these experiments are based on the use of pump-perfused Langendorff heart preparations.This method was selected in order to avoid the spontaneous decrease in coronary flow occurring when hearts are perfused at constant pressure;this would in fact produce an individually variable degree of anoxia during the experiment.

E.s.r. spectra recorded on frozen samples of control normoxic hearts did not display any signals in the resonance field of 500-5000 G.This finding is in contrast to the observation,reported by Zweier and coworkers(6,7),that oxygen- and carbon-centered radicals can be detected in preischemic conditions;differences in the perfusion technique might possibly account for this discrepancy.As observed in control preparations ADR-treated normoxic hearts did not show the presence of e.s.r. signals.These results suggest that in

intact myocardial tissue under normoxic conditions the radical producing reactions do not occur.

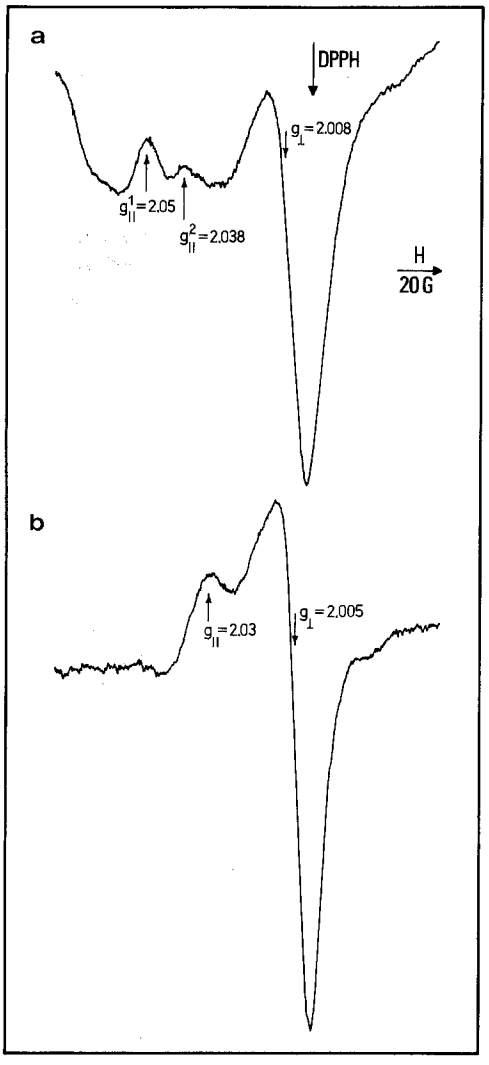
The resonance lines of a radical species become evident in hearts working in hypoxic conditions induced by a coronary flow rate of 2 ml/min (Fig. 1).The asymmetrical shape of the signals and their resonances fields are similar to those observed by Zweier et al. in ischemic rabbit hearts(6). Signals disappear by raising the temperature from 123 K to 193 K and saturation occurs at high microwave power(>20 mW).In control hearts (Fig. 1a) two different components of the magnetic g tensor are well distinguishable,the parallel one being split,$g_{\parallel}^{1}$=2.05 $g_{\parallel}^{2}$=2.038 $g_{\perp}^{1}$=$g_{\perp}^{2}$=2.008;these data are consistent with the presence of oxygen-centered radicals(probably O_2^-) and suggest that two different species of this radical occur.Although at this time we cannot offer an explanation,it is not surprising that oxygen radicals with different ligand field strenghts may occur in a complex chemical system,such as the intact cell.

Under the same perfusion conditions,ADR-treated hearts show the presence of a single superoxide radical species($g_{\parallel}$=2.03;$g_{\perp}$=2.005),with a 40% increase in signal intensity as compared to control hearts(Fig. 1b).

No other radical components were detected from total resonance by the temperature annealing procedure(6,7).

The use of an intermediate flow rate of perfusion (4 ml/min) produced intermediated effects both in control and in ADR-treated hearts (Fig. 2). In 50% of the organs the e.s.r. spectrum was silent throughout the entire resonance field.A possible explanation is based on the large individual differences existing in spontaneous coronary flow,so that hearts from animals ranging in the lower tail of the coronary flow distribution would not be affected by an experimental flow rate of 4 ml/min.In the remaining 50% of the preparations,a radical signal of very low intensity was detected in both controls and ADR-treated hearts.

Strong resonances due to a transition metal ion were also detected;this effect is enhanced in ADR-perfused organs.The shape of the resonances and the values of "g" component(g=2.12) are reminiscent of those of Fe(III) centers;the notion that iron is the most abundant transition metal in myocardial tissue seems to support this hypothesis.



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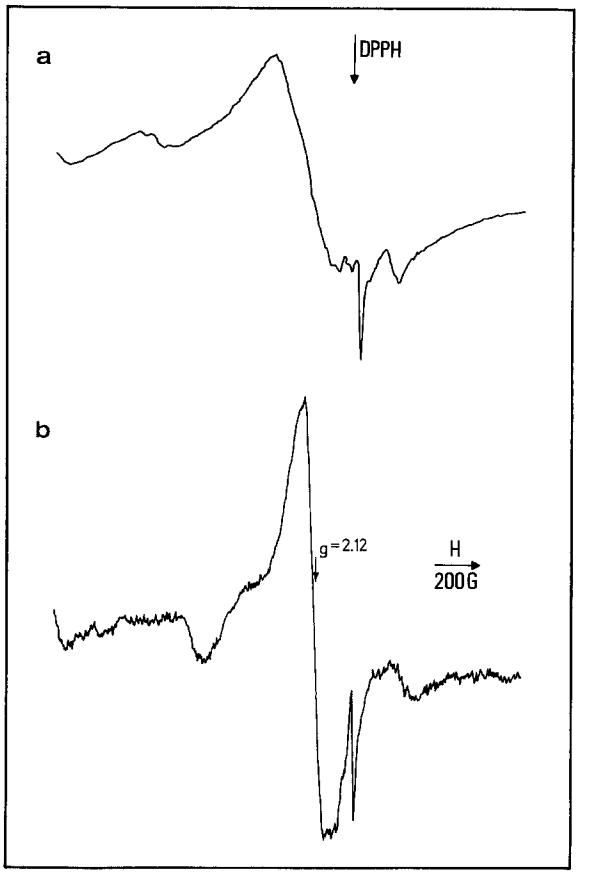

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Fig.1- E.s.r. spectra at 123 K of a) control rat hearts b) adriamycin perfused rat hearts, both at 2 ml/min flow rate

Fig.2- E.s.r. spectra at 123 K of a) control rat hearts b) adriamycin perfused rat hearts, both at 4 ml/min flow rate

Hearts perfused at a lower flow rate(1 ml/min) showed the presence of a small amount of radicals and no Fe(III),while no substantial difference was found between controls and ADR-perfused hearts.

In order to suggest a rationale for understanding the origin of the paramagnetic species observed in the present experiments,we recall that formation of carbon-centered radicals is a pathway for the production of oxygen-centered radicals;this "initiator" radical was tentatively identified as ubisemiquinone in normal tissues(6).

In a similar way the enzymatic reduction of ADR to the corresponding semiquinone radical and its subsequent reaction with molecular oxygen to produce the active oxygen species(superoxide,hydrogen peroxide)are thought to be a prerequisite for the cardiotoxicity of this drug(1).

Our results show that in normoxic hearts(flow rate 8 ml/min) no oxygen-centered radicals are detectable;this observation suggests that neither the reduced form of ubiquinone in control hearts,nor the semiquinone radical of ADR in treated organs can be produced under these conditions.
It is tempting to speculate that the carbon-centered radicals mentioned above are more easily produced at lower values of intracellular oxygen tension,such as those induced in preparations perfused at a flow rate of 4 ml/min and even more at 2 ml/min.As expected,the amount of oxygen radicals detected in ADR-perfused hearts is higher than in controls,possibly because the effects of ADR semiquinone radicals and ubiquinone-derived radicals in the electron transfer to molecular oxygen are additive.

When more marked hypoxic working conditions are adopted(1 ml/min) only a slight oxygen radical production was detected;the low availability of oxygen in the cells might account for the almost complete block of the electron transfer.Therefore,the formation of O_2^- radicals require that two apparently opposite conditions are met:hypoxia,which promotes the formation of carbon-centered radicals,and the availability in cells of oxygen that ultimately accepts electrons from the mentioned carbon radicals.

The mechanism seems to be further complicated by the involvement of iron.We note that Fe(III) signals we observe at 4 ml/min flow rate require the iron coordination to be different from that found in ferritin,(g=4.3),the suggested main source of Fe(III) in heart tissue (120 nmoles ferritin-bound iron/g of tissue)(8). The release of Fe(III) from ferritin entails the reduction to Fe(II) and this step can be mediated by O_2 (9.10). It is reasonable to expect that Fe(II) becomes subsequently engaged in redox cycles, such as the Fenton or Haber-Weiss reactions.

The lack of Fe(III) signal under normoxic conditions implies that,in the absence of O_2^-,the Fe(II) release referred to above does not occur.The results we obtain at 4 ml/min seem to indicate that under these conditions sufficient O_2^- is produced both to release Fe(II) from ferritin and to induce its reoxidation via H_2O_2.At 2 ml/min flow rate,the apparent increase of the O_2^- e.s.r. signal,

and the absence of the Fe(III) resonance may be due to an inefficient dismutation of O_2^-,resulting from the leakage of cellular enzymes that occurs in ischemic organs.The net result would be the lack of formation of H_2O_2,the required oxidizing agent of Fe(II) to Fe(III).

The results of the present investigations show that the production of the semiquinone derivatives(ADR-derived or of physiological origin),the subsequent formation of oxy-radicals and their interactions with transition metals,are strictly dependent upon the reducing properties of the environment and upon the availability of oxygen;therefore these events do not occur in myocardial tissue working in normoxic conditions,or in severe hypoxia.

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