

Evidence of cytosolic iron release during post-ischaemic reperfusion of isolated rat hearts

Influence on spin-trapping experiments with DMPO

François Boucher, Sylvie Pucheu, Charles Coudray, Alain Favier and Joël de Leiris

Groupe de Physiopathologie Cellulaire Cardiaque, URA CNRS 1287, Université Joseph Fourier, BP 53X, 38041 Grenoble Cedex, France

Received 1 April 1992

Previous studies of oxygen-derived free radical generation based on spin-trapping methods have shown a signal formed of six bands (sextet) using electron spin resonance spectrometry (ESR) of coronary effluents collected during post-ischaemic reperfusion of isolated hearts perfused with 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The origin of this signal has recently become controversial. In the present study we show that, in the rat, this sextet and cytosolic iron release occur simultaneously, and that this signal can be inhibited by the iron chelator desferrioxamine. It also appears that the iron release is not protein bound, and could therefore have a marked catalytic activity. This may be responsible for the production of an artefactual signal observed as the sextet.

ESR spin-trapping; Ischaemia; Reperfusion; Cytosolic iron; Desferrioxamine; 5,5-dimethyl-1-pyrroline N-oxide (DMPO)

1. INTRODUCTION

Since the description by Hearse et al. in 1973 [1] of the 'oxygen paradox' it has been suggested [2–7] that oxygen free radical production (mainly hydroxyl radicals (OH^\bullet), superoxide anions ($\text{O}_2^{\bullet-}$) and lipid peroxydes (R^\bullet)) could increase at the moment of post-ischaemic reperfusion of the heart. Formation of free radicals can have adverse effects on myocardial function. There is as yet, however, no conclusive evidence as to the real involvement of these radical species in myocardial injury associated with reperfusion. Until now, only indirect methods of detecting oxygen free radicals, such as spin-trapping and electron spin resonance spectroscopy (ESR) have shown a sudden burst of formation of these labile species at the moment of reperfusion in experimental models of isolated hearts subjected to various ischaemic conditions [8–12]. Most of the spin-trapping agents used in these experiments have a nitron-type group which is able to form a nitroxide (spin-adduct) during the trapping of a free radical, making it detectable in ESR spectroscopy. Furthermore, the ultra-fine splitting between the adduct and the spin-trap can sometimes give precise information on the trapped radical [13]. Nitrones are, however, extremely reactive mol-

ecules which, in addition to their ability to trap radicals, can enter numerous reactions. Furthermore, it should be noted that nitroxides can be produced from nitron through processes other than the trapping of radicals, for example through the catalytic action of certain metals such as copper or iron [13]. Thus, ferric iron (Fe^{3+}) in an aqueous chloride solution can catalyse the oxidation of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) into hydroxamic acid and then into the corresponding nitroxide. Similarly it has been suggested [13] that the iron present in trace amounts in buffers can produce the dimerization of DMPO, generating an artefactual ESR signal of 6 bands with splitting constants of $a_N = 15.31$ G, and $a_H = 22.0$ G. Several spin-trapping studies using DMPO in isolated rat [8,9] or rabbit [12] hearts have described the occurrence of a sextet signal in coronary effluents collected during post-ischaemic reperfusion. This signal was attributed to the trapping of alkyl or alkoxyl radicals formed during reperfusion. However, when considering the splitting constants of this signal ($a_N = 15.3$ G and $a_H = 22.0$ G) Pou et al. [14] suggested that it could in fact be linked to a catalytic action of iron on DMPO [13] in the perfusion liquid. In that case this ESR signal would be entirely artefactual.

To assess the validity of this last hypothesis we have used ESR spectroscopy to study the kinetics of appearance of the various spin-adducts of DMPO in coronary effluents of isolated rat hearts during post-ischaemic reperfusion. In this same experimental model we also followed the time-course of changes in iron concentra-

Correspondence address: F. Boucher, Groupe de Physiopathologie Cellulaire Cardiaque, URA CNRS 1287, Université Joseph Fourier, BP 53X, 38041 Grenoble Cedex, France. Fax: (33) 76 46 74 63.

tion. Lactate dehydrogenase (LDH) activity was measured as an index of protein release.

Finally, we added desferrioxamine to the perfusate of isolated rat hearts in order to verify whether iron chelation is capable of inhibiting the appearance of the sextet signal of coronary effluent sampled during post-ischaemic reperfusion with DMPO.

2. MATERIALS AND METHODS

Male Wistar rats, weighing between 300 and 340 g, kept in standard laboratory conditions, were used.

The spin-trap used was 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Aldrich-Chimie, France), purified through distillation, stored at -20°C and protected from light in an argon atmosphere to prevent its spontaneous decomposition [15].

The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p., 1 ml/kg) and heparinized (100 IU/100 g in femoral vein). Following thoracotomy the heart was excised and perfused *ex vivo* via the aorta. The heart was first perfused for 10 min at constant flow (11 ml/min) using a modified Krebs-Henseleit buffer [16] (Ca^{2+} 2.4 mM; K^{+} 5.6 mM; glucose 5.0 mM; pyruvate 5.0 mM; pyruvate 5.0 mM) equilibrated with a gas mixture of 95% O_2 and 5% CO_2 at 37°C , pH 7.4. The iron concentration in the perfusate, measured with flame atomic absorption spectroscopy, was 357 ± 50 mM. After the control normoxic perfusion period the heart was perfused for 1 min with a similar buffer containing, in addition, DMPO at a final concentration of 80 mM to obtain baseline values for spin-adduct formation under standard conditions (iron concentration in the perfusate after DMPO addition: 410 ± 62 mM). To prevent the degradation of the spin-trap it was introduced continuously into the perfusion buffer via the aortic cannula using an infusion syringe (1 ml/min), and the heart was kept in the dark. At the end of this period 1 ml of coronary effluent was collected and immediately frozen in the dark using liquid nitrogen. The heart was then subjected to a further 10 min of perfusion under control conditions (without DMPO), followed by 30 min of total global ischaemia in normothermia. After this, reperfusion for 3 min in the presence of DMPO 80 mM was initiated, during which time period coronary effluent samples were collected for each 15 s period and frozen. Iron concentration in coronary effluents was determined using flame atomic absorption spectrometry and LDH activity was assessed using the method of Wroblewski and La Due [17].

A second set of experiments was performed using the above protocol with the addition of desferrioxamine (Ciba-Geigy, France) ($n = 5$) at a concentration of 0.15 mM to the perfusate used throughout.

Immediately after thawing in the dark the coronary effluent samples were analyzed in ESR spectroscopy using a Bruker ER 100D spectrometer with parameters set as follows: modulation frequency 100 kHz; modulation amplitude 2.5 G; microwave frequency 9.4 GHz; microwave power 10 mW.

Relative concentrations of spin-adducts in coronary effluent were evaluated by measuring the amplitude of ESR signals.

Results are expressed as the mean \pm standard error of the mean (SEM).

3. RESULTS

The ESR spectrum recorded at the end of the control normoxic perfusion period (Fig. 1a) in coronary effluent enriched with DMPO (80 mM) did not exhibit any signal. However, three signals appeared during post-ischaemic reperfusion: (i) a signal showing as a triplet (signal C, Fig. 1b), which cannot correspond with any

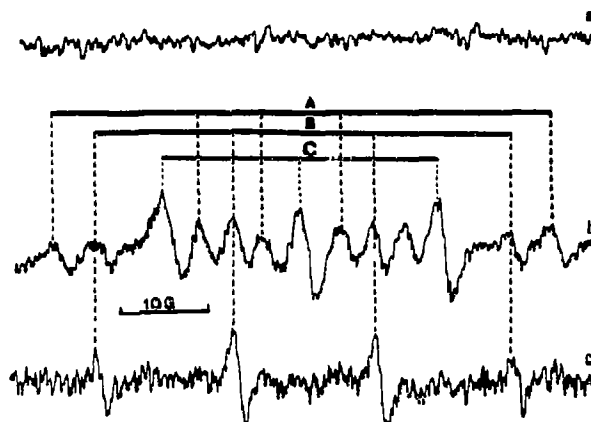


Fig. 1. ESR spectra of coronary effluent collected from isolated rat hearts perfused with DMPO (80 mM). (a) At the end of the control normoxic perfusion period. (b) At 15 s of reperfusion. (c) At 2 min of reperfusion.

of the DMPO spin-adducts as it comprises only the three bands of the nitrogen of the spin-trap; (ii) a signal showing as a quartet (signal A, Fig. 1b and c) with relative amplitudes of 1:2:2:1 and splitting constants of $a_N = a_H = 15.02 \pm 0.15$ G. This signal appeared progressively in coronary effluent from 15 s of reperfusion, and its amplitude decreased after 2 min (Fig. 2); finally, (iii) a signal showing as a sextet (signal B, Fig. 1b) with relative amplitudes of 1:1:1:1:1:1, $a_N = 15.53 \pm 0.11$ G and $a_H = 22.12 \pm 0.16$ G, present in coronary effluents as soon as reperfusion was initiated and whose intensity decreased exponentially during reperfusion (Fig. 3) was observed.

Iron concentration in coronary effluent collected during reperfusion peaked in the first samples, then decreased very quickly to reach a plateau by 45 s of reperfusion. The mean value of the plateau was not significantly different from the initial concentration of iron in

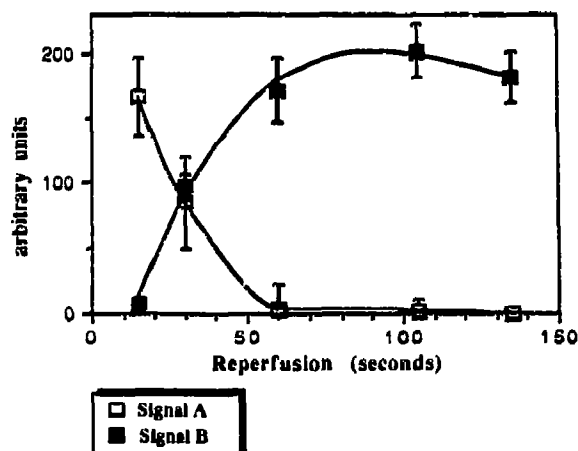


Fig. 2. Evolution of the amplitude of signals A and B of coronary effluent (see Fig. 1) during post-ischaemic reperfusion of isolated rat hearts (perfused with DMPO 80 mM).

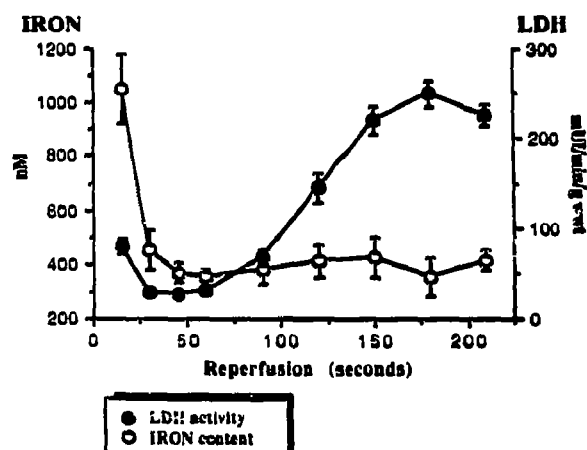


Fig. 3. Evolution of iron concentration and LDH activity in coronary effluents during post-ischaemic reperfusion of isolated rat hearts.

the perfusion buffer (respectively, 398 ± 12 vs. 410 ± 62 nM; ns).

LDH activity increased rapidly during reperfusion to peak at 3 min.

The addition of desferrioxamine to the perfusate inhibited the production of all paramagnetic species in coronary effluents during the first seconds of post-ischaemic reperfusion (Fig. 4b). However, the quartet signal (A), appearing within the first minute of reperfusion persisted (Fig. 4c).

4. DISCUSSION

Within the last 10 years experimental evidence has accumulated to suggest that oxygen-derived free radicals are involved in myocardial injury occurring upon post-ischaemic reperfusion. Spin-trapping experiments have a prominent place in these studies because of the information they give on the nature of the radical species produced. However, because of the high chemical reactivity of the spin-trapping agents and the complexity of the biological systems in which they are used, artefacts can be created which must be taken into account.

In the present work two principal ESR signals (signals A and B) were observed in coronary effluents collected during the post-ischaemic reperfusion of isolated rat hearts. Blasig et al. in 1986 [9] and Zweier et al. in 1988 [12] described these signals using isolated rabbit or rat hearts. Analysis of these signals led to the conclusion that hydroxyl (OH^\bullet) or superoxide ($\text{O}_2^{\bullet-}$) radicals were produced generating the quartet signal (signal A) characteristic of the spin-adduct DMPO-OH , and radicals of lipid nature (R^\bullet) derived from hydroxyl radicals were responsible for the occurrence of a sextet signal (signal B). However, the simple measurement of the splitting constants of the sextet signal does not allow the identification of the specific spin-adduct produced. Indeed, it

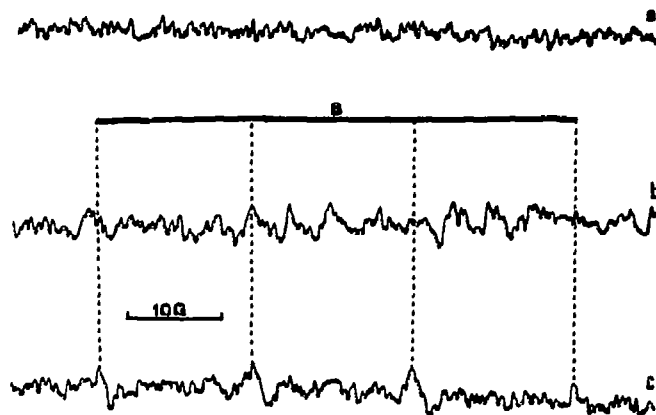


Fig. 4. ESR spectra of coronary effluent collected from isolated rat hearts perfused with DMPO (80 mM) and desferrioxamine (0.15 mM). (a) At the end of the control normoxic perfusion period, (b) At 15 s of reperfusion, (c) At 2 min of reperfusion.

has been shown [13] that DMPO can be subjected to an oxidation process in aqueous media in the presence of EDTA-iron complexes (Fe^{3+} as well as Fe^{2+}), generating a similar sextet signal in ESR in the absence of lipid radicals. More recently, Pou et al. [14] suggested that the sextet signal observed by Zweier [12] during reperfusion of isolated rabbit hearts could either be linked to the presence of EDTA (0.5 mM) which is able to complex the residual iron contained in the perfusion buffer, or else could be observed because of a direct consequence of ischaemia, that is, a possible increase in the availability of iron for the catalysis of DMPO oxidation. Moreover, it should be noted that the possible release of iron upon post-ischaemic reperfusion has been suggested to explain ischaemia reperfusion-induced injury because of the ability of this metal ion to catalyse the Haber-Weiss reaction, whereby $\text{O}_2^{\bullet-}$ and H_2O_2 give rise to the highly reactive OH^\bullet radical [18]. In the present study we have demonstrated that cytosolic iron is released into the vasculature either during ischaemia, or during the early phase of post-ischaemic reperfusion, and the release of iron and the occurrence of the sextet signal appear as two simultaneous phenomena (Figs. 2 and 3). Furthermore, iron release precedes the release of cellular proteins, as demonstrated by the leakage of LDH (Fig. 3). These results suggest that the iron present in coronary effluent at this time is not complexed to proteins, but rather to small biological chelators such as ATP or citrate [19]. Non-protein-bound iron, also called 'free' iron, is highly reactive [20] and could therefore catalyse DMPO oxidation.

Finally, our ESR analysis indicates that desferrioxamine inhibits the appearance of the sextet signal from coronary effluent collected at the onset of reperfusion (Fig. 4). Desferrioxamine, a trihydroxamic acid, is the most potent and the most specific chelator of iron. This molecule chelates 'free' iron to form a chemically

inert complex and thus can inhibit the catalytic properties of this metal [21]. Desferrioxamine has recently been shown to be cardioprotective when administered to dogs at the time of post-ischaemic reperfusion [22]. This observation supports the concept that iron-catalysed radical reactions occurring in the initial seconds of reperfusion may play a major role in reperfusion injury. However, according to our results, the release of 'free' iron may be a source of artefact in ESR studies with the spin-trap DMPO.

In conclusion, the release of iron during ischaemia or during the early phase of reperfusion reinforces the hypothesis that the ESR signal originally attributed to the trapping of lipid radicals is, in fact, produced by an oxidation process of the spin-trap under the catalytic action of iron. Further studies are required to assess the physiological and/or pathophysiological role that this transition metal with a high catalytic activity could play in the development of myocardial injury during ischaemia and reperfusion.

Acknowledgements: We are greatly indebted to Miss Linda King for helpful discussions and for her careful reading of the manuscript. This work was supported in part by the Ministère de la Recherche et de la Technologie (France) and the Fondation de France.

REFERENCES

- [1] Hearse, D.J., Humphrey, S.M. and Chain, E.B. (1973) *J. Mol. Cell. Cardiol.* 5, 395-407.
- [2] Burton, K.P., McCord, J.M. and Ghai, G. (1984) *Am. J. Physiol.* 246, H776-H783.
- [3] Hess, M.L., Manson, N.H. and Okabe, E. (1981) *Can. J. Physiol. Pharmacol.* 60, 1382-1389.
- [4] Hess, M.L. and Manson, N.H. (1984) *J. Mol. Cell. Cardiol.* 16, 969-985.
- [5] Jamieson, D. (1989) *Free Rad. Biol. Med.* 7, 87-108.
- [6] Myers, C.L., Weiss, S.J., Kirsh, M.M. and Schlafer, M. (1985) *J. Mol. Cell. Cardiol.* 17, 675-684.
- [7] Schlafer, M., Kane, P.F., Wiggins, V.Y. and Kirsh, M.M. (1982) *Circulation* 66 (suppl 1), 85-92.
- [8] Arroyo, C.M., Kramer, J.H., Dickens, B.F. and Weglicki, W.B. (1987) *FEBS Lett.* 221, 101-104.
- [9] Blasig, I.E., Ebert, B. and Löwe, H. (1986) *Stud. Biophys.* 116, 35-42.
- [10] Charlton, V. and de Leiris, J. (1988) *Basic Res. Cardiol.* 83, 306-313.
- [11] Kramer, J.H., Arroyo, C.M., Dickens, B.F. and Weglicki, W.B. (1987) *Free Rad. Biol. Med.* 1, 153-159.
- [12] Zweier, J.L. (1988) *J. Biol. Chem.* 263, 1353-1357.
- [13] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) *Arch. Biochem. Biophys.* 200, 1-16.
- [14] Hamer, J. and Maculuso, A. (1964) *Chem. Rev.* 64, 473-495.
- [14] Pou, S., Hassel, D.J., Britigan, B.E., Cohen, M.S. and Rosen, G.M. (1989) *Anal. Biochem.* 177, 1-6.
- [15] Janzen, E.G. (1984) *Methods Enzymol.* 105, 188-198.
- [16] Krebs, H.A. and Henseleit, K. (1932) *Hoppe Seylers Z.* 210, 33-36.
- [17] Wroblewski, F. and La Due, J.S. (1955) *Proc. Soc. Exp. Biol. Med.* 90, 210-213.
- [18] Halliwell, B. (1989) *Free Rad. Res. Commun.* 5, 315-318.
- [19] Halliwell, B. and Gutteridge, J.M.C. (1984) *Biochem. J.* 219, 1-14.
- [20] Baker, M.S. and Gebicki, J.M. (1986) *Arch. Biochem. Biophys.* 246, 581-588.
- [21] Keberle, H. (1964) *Ann. N.Y. Acad. Sci.* 119, 758-768.
- [22] Bolli, R., Patel, B.S., Jeroudi, M.O., Li, X.Y., Triana, J.F., Lai, E.K. and McCay, P.B. (1990) *Am. J. Physiol.* 259, H1901-H1911.