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ESR spin trapping and NMR spectroscopy of the same heart shows correlation between energy depression and radical formation during postischemic reperfusion

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The relevance of radical formation in disturbances of energy metabolism in the postischemic heart is not clear. This study provides the first evidence of a significant correlation between the amount of oxy-radicals trapped in the effluent of isolated hearts upon reperfusion and the decreased myocardial content of phosphocreatine and ATP. This suggests that the loss of high-energy compounds might contribute to oxy-radical production during reperfusion. The application of ESR spin trapping and of NMR technique to the same heart is a new approach to investigate the pathobiochemical relevance of free radicals for the heart muscle

Postischemic reperfusion, NMR; High-energy phosphate, ESR spin trapping; Oxy-radical

1. INTRODUCTION

^{31}P Nuclear magnetic resonance (NMR) spectroscopy allowed myocardial energy metabolism to be monitored serially and noninvasively, while allowing simultaneous assessment of ventricular function [1–4]. Electron spin resonance (ESR) spectroscopy is specific for detecting free radicals. The steady-state concentration of free radicals in heart-derived samples is low so that ESR spin trapping [5–10] has been used to overcome this difficulty. However, the cardioaction of the spin trap [9,11] has to be considered. It has been postulated that oxy-radicals generated during postischemic reperfusion [6,8,9,12,13] are at least in part responsible for the injury induced [14,15]. If this hypothesis is true, free radical formation has to be a very early event during reperfusion and could be involved in disorders of energy metabolism and contractile function of the heart. To test this idea it is necessary to compare the amount of radicals generated with the energy status of the individual hearts. The aim of this study is to apply the ESR spin trapping procedure and the NMR spectroscopy to the same heart as a means of investigating the pathogenetic role of free radicals during reperfusion injury. To combine both techniques and to avoid interactions of the spin trap with the heart, the spin trap was added to the effluent perfusate.

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2. MATERIALS AND METHODS

Hearts were perfused according to the method of Langendorff in the NMR magnet as described earlier [3]. Female Wistar rats were heparinized (150 IU/kg) and anaesthetized with urethane (2–2.5 g/kg). The hearts ($n = 11$) were perfused with a constant coronary flow of 11.7 ± 0.3 ml/min at perfusion pressure of 8.60 ± 0.44 kPa (65 ± 3 mm Hg) with modified Krebs-Henseleit solution (11 mM glucose, 0.5 mM Na-EDTA without inorganic phosphate) aerated with 95% O_2 and 5% CO_2, pH 7.4 at 37°C. The left ventricular pressure (LVP) and its first derivative (dP/dt) were recorded throughout the experiment using a latex balloon placed into the left ventricle. This balloon was inflated until the developed pressure (LVDP) became maximum to receive maximum of left ventricular work: systolic LVP 24.4 ± 0.8 kPa (183 ± 6 mm Hg), end-diastolic LVP (LVEDP) 3.6 ± 0.4 kPa (27 ± 3 mm Hg), heart rate (HR) 198 ± 10 beats/min. The cardiac work (PRP), product of HR and LVDP (systolic LVP minus LVEDP), was 4044 ± 163 kPa/min ($30\,330 \pm 1224$ mm Hg/min). After perfusion for 40–50 min, total normothermic ischemia was produced by complete cessation of flow during 20 min followed by reperfusion using the initial flow. At the end of the experiment the heart dry weight was determined.

NMR measurements of [^{31}P]compounds of the heart were performed in CXP-200 spectrometer (Bruker, FRG) operating at the frequency of 80.98 MHz for ^{31}P with wide-bore magnet [4]. To monitor changes in the content of phosphorus-containing substances in the heart consequent spectra during each 5 min time interval were accumulated: 90° (27 μs) sampling pulses, 3 s repetition time, 0.512 s acquisition time, number of scans 100, memory size 4/8 K. 20 min prior to and 30 min after ischemia 'quantitative' spectra were accumulated, repetition time 10 s, collecting 90 transients. The areas of the components of the NMR spectra were used to calculate tissue content of phosphocreatine (PC), adenosine triphosphate (ATP), and inorganic phosphate (P_i). The signal of 1-amino-1-phenyl methylene diphosphate (100 mM) sealed in a plastic tube in the vicinity of the heart was used as reference for spectra quantitation.

5,5-Dimethyl-1-pyrroline-1-oxide (DMPO) was added to the drained perfusate 30 s after passing the heart, and after passing the magnet

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by a catheter sucking off the effluent, final concentration of 500 mM. The effluent samples were mixed, frozen in liquid N_2 and stored until ESR measurements could be performed. An E-109E ESR spectrometer (Varian, USA) was used to analyse the samples, at room temperature, in a quartz flat cell 5 min after thawing the effluent; spectrometer settings were: 10 mT scan range, 0.2 mT modulation amplitude, 64 ms response time, 30 mW microwave power, 2 min scan time. During reperfusion, the typical 4-line signal of DMPO-hydroxyl radical adduct (DMPO-OH) was monitored, line intensity 1:2:2:1, $a_N = a_\beta^H = 1.48$ mT (inset in Fig. 1B). The signal intensity which is proportional to the concentration of the adduct formed (and hence, the amount of oxy-radicals trapped) in the sample is expressed in arbitrary units adjusted for the gain of the trace and related to the dry weight. To estimate an equivalent of vascular generation of oxy-radicals over a given interval of time, the time course of DMPO-OH intensity per weight was constructed and the corresponding area under the curve was integrated for each heart (cumulative amount). Spin trapping was performed 20 min prior to ischemia and during reflow (immediately after the onset of reperfusion, after 1, 2, 3, 5, 7.5, 10 and then every 5 min), sampling time 5 s.

Results shown are means ± SE. The comparison between cumulative DMPO-OH formation in the effluent (area under the curve) and functional as well as biochemical data was assessed by rank correlation analysis according to Spearman. The estimated rank correlation coefficients (r_s, stochastic independence) were tested against zero (two-sided, $\alpha = 0.05$).

3. RESULTS AND DISCUSSION

The detection of oxy-radicals in reperfused hearts needs millimolar concentrations of DMPO [5–7,10]. However, this trap may protect the heart against reperfusion-induced injuries (up to concentrations of 1 mM) which may disappear when treatment has been extended [11]. Thus, the spin-trapped radicals cannot be related to contractile function and content of high-energy phosphates directly if the trap has been perfused through the heart. Therefore, in the present study DMPO was applied to the drained effluent of heart, after passing the NMR magnet, to avoid interaction with the heart and to perform comparative studies. During control perfusion, no ESR signal could be detected. Thus, the DMPO-OH identified during reperfusion (inset in Fig. 1B) has to be related to the injury induced. Taking into account the very short lifetime of oxy-radicals, it has to be assumed that the radicals trapped are formed by radical-generating systems released from the affected heart. The time course of the intensity of DMPO-OH signal registered (Fig. 1B) is comparable with earlier experiments when the trap passed through the reperfused heart [8,9,16] and with the time course of free radical generation in the myocardium measured by direct ESR spectroscopy [17]. That means the oxy-radicals detected in the effluent reflect radical formation of the injured heart, resulting in the release of radical-generating systems into the perfusate.

Upon reperfusion, control function of the heart is not restored over the period of experiment. Initially the contractile failure is rather enhanced compared to the foregoing ischemic interval; increase of LVEDP. After diminution of LVEDP, functional parameters increase and reach steady levels within 30 min after the onset of reperfusion (Fig. 1A). Also the control values of phosphorus-containing compounds did not recover during reperfusion. After temporary increase and decrease of the content of high-energy phosphates in the initial phase of reflow, constant values appear within 25 min (Fig. 1C). Generation of oxy-radicals in the effluent is observed with the restoration of flow, maximum values after 7.5 min followed by a decrease of the signal intensity (Fig. 1B). The individual data obtained demonstrate a considerable variability in the production of DMPO-OH. However, the mean time course of DMPO-OH shows an opposite characteristic compared to the recovery of contractile function (LVDP, $LVdp/dt_{max}$, PRP) and, especially in the initial phase of reperfusion, to that of PC and ATP used for heart work. That means high levels of high-energy phosphates and function are associated with a low amount of radical-generating systems released into the perfusate resulting, in turn, in low radical trapping and vice versa. This inverse relationship is supported by statistical analysis showing inverse correlation between cumulative amount of radical adduct produced in the effluent and the average myocardial content of high-energy phosphates, when measured over the first 5 min of reflow: for phosphocreatine $r_s = -0.682$ ($P < 0.05$) and for ATP $r_s = -0.782$ ($P < 0.01$). Testing later intervals, the significance of correlation is lost. In a sense, it appears that, in the initial phase of reperfusion, the amount of free radicals formed in the effluent is related to the severity of reperfusion-induced lack of energy and hence, the vascular radicals detected are assumed to be related to the severity of the myocardial injury.

The formation of DMPO-OH was found to occur immediately with the initiation of reflow (data not shown). This is coincident with earlier studies [8–10,16] and demonstrates that production of radicals is a very early event of reperfusion injury. On the other hand, it is known that the toxic oxy-radicals may impair myocardial metabolism [18] and function [19] when generated in the vascular system. Therefore, it is possible that the radicals detected in the first minutes of reperfusion correspond, at least in part, to the energetic status and thus, also to the contractile function recovering after reperfusion. To investigate this possibility, correlation analysis between the cumulative amount of oxy-radicals trapped in the effluent over the first 10 min of reflow (including the maximum) and myocardial content of high-energy phosphates recovered was performed. Herein, the amount of oxy-radicals significantly shows inverse correlation. The highest correlation coefficients were obtained using the radical amount cumulated within the first two minutes: for PC $r_s = -0.918$ and for ATP $r_s = -0.936$, $P < 0.001$ (LVDP, $LVdp/dt_{max}$, and PRP -0.693, -0.702, and -0.691, $P < 0.05$). That means the amount of radicals detected in the effluent during the initiation of reperfusion might determine the degree of 'energetic' recovery

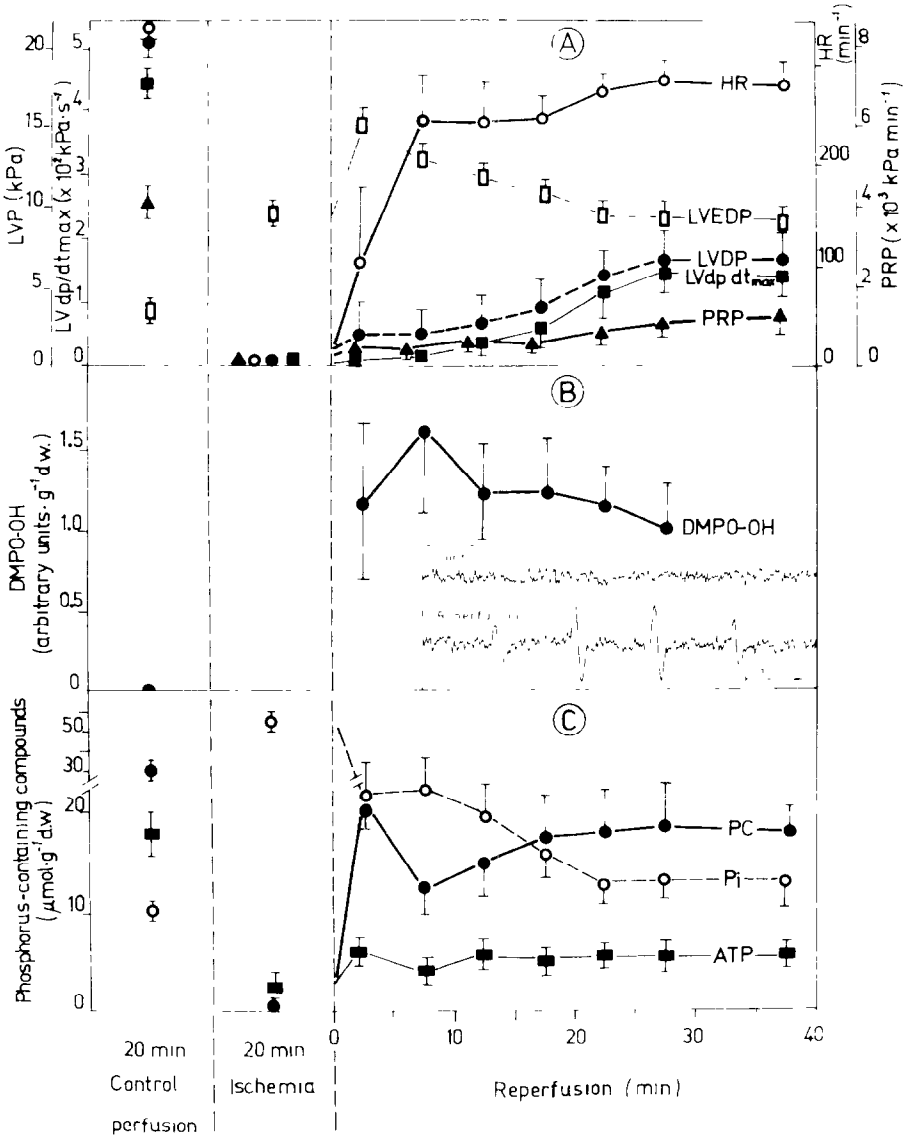

Fig. 1. Time course of changes in physiological parameters (A), in effluent spin trapping of oxy-radicals (B), and in myocardial content of phosphorus-containing compounds (C) during reperfusion of isolated, isovolumetric rat hearts following 20 min of ischemia. The inset in (B) represents the ESR spectra obtained during control perfusion and reperfusion.

of the heart. In general, the relationship found would support our hypothesis that radical formation immediately after the onset of reperfusion is causatively involved in the energy depression occurring during the reperfusion injury of the heart.

By demonstrating the usefulness of applying spin trapping to the effluent of isolated heart perfused in the NMR magnet, our results expand the possible applications of both techniques. The new approach described herein has the potential not only to characterize the time course of generation of oxy-radicals in the perfusate without interaction of the trap with the heart, but simultaneously also to investigate myocardial content of phosphorus-containing compounds. The combination of NMR and ESR spin trapping may be used to quantitatively assess the pathogenetic role of radicals generated in the vascular system for the energy metabolism of the affected heart.

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