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Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO

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The spin trapping ESR technique was applied to investigate oxygen-derived radicals in ischemic and post-ischemic rat hearts. Using 5,5'-dimethyl-1-pyrroline-*N*-oxide, carbon-centered radicals were identified during ischemia and oxy-radical adducts (superoxide anion radical, $O_2^{\cdot-}$ and hydroxyl radicals, $^{\cdot}OH$) in post-ischemic rat heart. The formation of these spin adducts was inhibited by superoxide dismutase, suggesting that superoxide plays a role in the adducts' formation. The results demonstrate that oxygen derived free radicals are important byproducts of abnormal oxidative metabolism during myocardial ischemic and reperfusion injuries.

Free radical; Ischemia; Reperfusion; Oxy-radical adduct; ESR spin trapping; Carbon-centered radical

1. INTRODUCTION

It has been suggested that during myocardial ischemia short-lived free radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), or lipid peroxide ($RO^{\cdot}$) are produced. The evidence, however, for the participation of such radicals in cardiovascular injury has been based upon activation of lipid peroxidation and on myocardial protection provided by scavengers [1,2]. Direct detection of free radicals by electron spin resonance (ESR) spectroscopy has not provided conclusive identification of radicals produced during these injuries [3]. However, spin trapping and ESR offers an indirect technique that provides the necessary evidence to confirm the involvement of these labile intermediates. Although spin trapping has been applied very successfully to studies of free radical mechanisms in a variety of chemical and photochemical reactions, it has only recently been applied to biological systems [4]. The majority of the spin traps used are molecules containing a nitroso or nitrone group which, upon addition of a labile free radical, form a stable nitroxide that is easily detected by ESR. We applied spin trapping to study biologically generated free radicals in ischemic and post-ischemic rat hearts. We detected a 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO)-carbon radical and DMPO-OH adducts during ischemia. Furthermore, during post-ischemic myocardial reperfusion we observed oxy-radical adducts (superoxide anion and hydroxyl radical). The formation of these three different spin adducts was inhibited by superoxide dismutase (SOD), suggesting that formation of the adducts (DMPO-OH and DMPO-carbon) involves superoxide as a precursor.

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2. MATERIALS AND METHODS

DMPO was obtained from Aldrich and was pu-

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rified according to the method of Buettner and Oberley [5] by repeatedly treating the solution with activated charcoal until all the free radical impurities were eliminated. The concentrations of the aqueous DMPO solutions were determined spectrophotometrically at 227 nm ($\epsilon = 8 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [6]. The experimental room was kept in darkness throughout the study to prevent photolytic degradation of DMPO.

Male Sprague-Dawley rats (200–224 g) were decapitated and their hearts were cannulated within 1 min after excision. The perfusion apparatus used has been described [7]. The hearts were perfused (non-recirculating) at 37°C with physiological Krebs-Henseleit buffer containing 5 mM glucose and 5 mM sodium acetate in the presence or absence of DMPO. The perfusate was aerated with a 95% O_2/5% CO_2 gas mixture to maintain a pH of 7.4. Rat hearts were stabilized under control perfusion conditions for 20 min prior to induction of global ischemia.

Global ischemia was carried out by perfusing hearts with buffer containing DMPO at a low-coronary flow rate (0.1 ml/min) for 40 min. Coronary effluents containing the spin trap were immediately frozen in liquid N_2 to prevent spin adduct decay. In post-ischemic reperfusion experiments, hearts were exposed to DMPO during the last 5 min of ischemia and subsequent reperfusion; again coronary effluents containing DMPO were immediately frozen in liquid N_2.

Frozen samples were thawed just before their ESR spectra were obtained. Control experiments showed that when solutions containing DMPO adducts were frozen and stored for 5 h at liquid nitrogen temperature, there was no significant loss in the ESR signal of these spin adducts if the ESR spectra were obtained immediately after thawing.

ESR measurements were made on a Bruker-ER 100 series, X-band spectrometer using a magnetic field modulation frequency of 100 kHz. The ESR spectra were obtained using a quartz flat cell ($60 \times 10 \times 0.25$ mm).

The microwave power was maintained at 10 mW to avoid saturation, and the scan was traced with a modulation equal to 1 G. Hyperfine coupling constants were measured directly from the field scan using a 10 G marker signal for calibration.

In some cases superoxide dismutase (EC 1.15.1.1; Sigma) was administered in the perfusion medium containing DMPO during ischemia and reperfusion.

3. RESULTS

Despite numerous experimental artifacts associated with the use of DMPO as a 'universal' spin trap [5], DMPO appears to be an optimal choice in view of its low cytotoxicity, accessibility to the cell, and a high rate constant of reaction with ·OH radicals [8–15]. Purified DMPO solutions were used to test ischemic rat hearts for their capacity to generate $O_2^{\cdot -}$ or ·OH radicals. After 40 min of pre-ischemic perfusion in the presence of DMPO, no signal was observed.

When global ischemia was carried out in the presence of 100 mM DMPO, the ESR spectra of the coronary effluent indicated that two free radical adducts were formed (fig.1). One of the radicals appears to be ·OH and its generation is dependent on the presence of O_2 (DMPO-OH, $A_N = 1.49$ mT; $A_H^\beta = 1.49$ mT). The other spin-trapped radical is a derivative of an unknown carbon compound and its formation is decreased by the presence of oxygen. The hyperfine constants are $A_N = 1.55$ mT and $A_H^\beta = 2.26$ mT for the

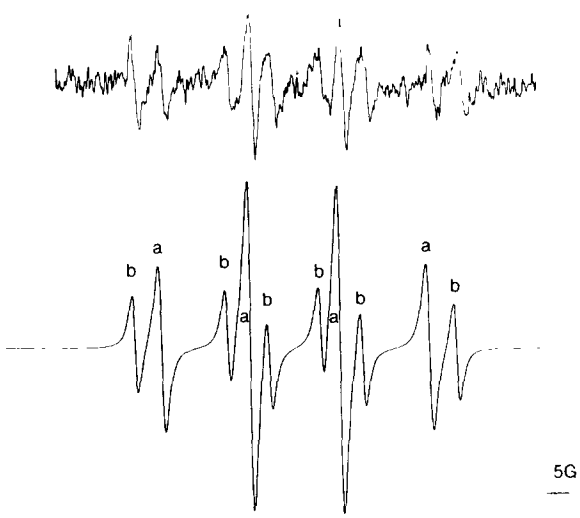

Fig.1. ESR spectra of DMPO adducts in ischemia rat heart. Hearts were subjected to 40 min of global ischemia in the presence of 100 mM DMPO. Scan time 4 min; receiver gain 1.25 ± 10^5; modulation amplitude 0.5 G.

DMPO-carbon radical adduct. Similar coupling constants have been reported for carbon-centered radical adducts from lipid peroxidation [16–18] varying from 1.5 to 1.6 mT for A_N and 2.26 to 2.32 for A_H^β. Besides the possibility that DMPO itself could lead to a similar signal [18], our results support the assumption that the carbon-centered radicals might be derived from lipid peroxidation. Fig.2 shows that the carbon radical could be inhibited by administration of SOD during global ischemia. The inhibition of the ESR signal suggests that the superoxide anion is the precursor of this carbon-centered radical adduct. Furthermore, no ESR signals were observed during the first 15 min of global ischemia when SOD was added to the system (fig.2B); however, after 15 min a very weak signal started to appear at a g value of 2.0052. The ESR signal observed was identified as ascorbyl free radical with a g value of 2.0052 and splitting of 0.17 mT as shown in fig.2C.

Post-ischemic, reperfused rat hearts in the presence of DMPO resulted in the formation of different paramagnetic species (fig.3). In the absence of DMPO, no ESR signal spectrum was observed. Spectrum A, obtained 20 s after reperfusion was initiated, can be attributed to DMPO-OOH adducts (A_N = 1.425 mT; A_H^β = 1.13 mT, A_H^γ = 0.14 mT). 1 min after reperfusion was initiated, the initial adduct appeared to decompose to DMPO-OH species (fig.3B). SOD was found to interfere with this process by inhibiting the formation of the DMPO-OOH adduct. This observation agrees with the work of others [19] and indicates another source of generation of DMPO-OH adducts without the obligatory formation of free ·OH radicals. Therefore, the data suggest that $O_2^{\cdot-}$ (and $HO_2^{\cdot}$) are initially trapped by DMPO. Half-life values of 80 s at pH 6 and approx. 35 s at pH 8 [5] have been reported for the DMPO-OOH adduct.

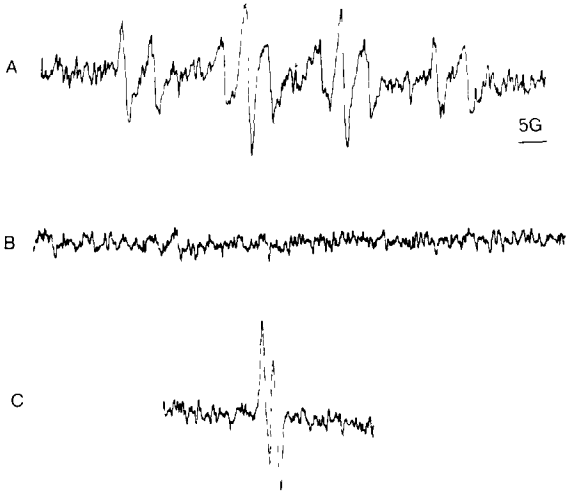

Fig.2. ESR spectra obtained: (A) 10 min of ischemia without SOD; (B) 10 min of ischemia in the presence of SOD (93 μg/ml); (C) 30 min of ischemia in the presence of SOD (ESR signal of the ascorbyl free radical). Scan time 4 min; receiver gain 2×10^5; modulation amplitude 0.5 G.

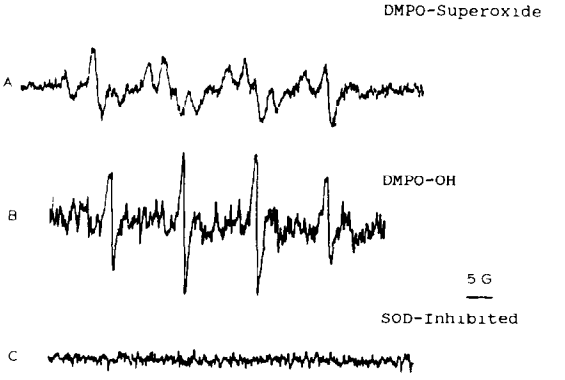

Fig.3. Transition of the ESR spectra from the DMPO-OOH adduct to the DMPO-OH adduct as a function of time. Samples were taken (A) 20 s, (B) 1 min and (C) 3 min in the presence of SOD (93 μg/ml) after reperfusion was initiated. Scan time 4 min; receiver gain 1.25×10^5; modulation amplitude 0.5 G.

4. DISCUSSION

A number of mechanisms have been proposed to generate $O_2^{\cdot-}$ in post-ischemic myocardium. During ischemia, high concentrations of hypoxanthine accumulate due to the degradation of ATP [19]. Metabolism of this nucleotide is normally mediated by xanthine dehydrogenase; during ischemia, however, this enzyme is converted to the oxidase form which reduces O_2 forming $O_2^{\cdot-}$ [20]. Other possible sources of $O_2^{\cdot-}$ include mitochondrial oxication [21], oxidation of catecholamines released locally during ischemia [22], and generation by polymorphonuclear leukocytes which migrate to the ischemic tissue [23].

In our blood-free perfused heart model it is unlikely that sufficient numbers of polymorpho-

nuclear leukocytes would remain to account for the observed oxygen radical formation. Nevertheless, we have no evidence indicating the cellular or subcellular source of the superoxide anion. Clearly, further work is needed to identify the actual mechanisms of O_2^- generation in reperfused myocardium and quantitate the contributions of each mechanism.

One consequence of enhanced radical generation is an increase in peroxidation of lipids. Free radical production is involved in lipid peroxidation, this has been indicated by accumulation of products of this pathway in the ischemic heart [24]. Therefore, peroxidizing lipids can be assumed to be an additional radical source during ischemia. The appearance of the DMPO carbon-centered adduct during global ischemia suggests that lipid radicals can be released from the ischemic myocardium. The absence of a detectable DMPO-OOH ESR signal during global ischemia does not negate the presence of a small amount of superoxide in the process, but merely define the stability of the spin adduct under the experimental conditions used. This was supported by the observation that SOD completely inhibited the spin adducts' formation during ischemia and reperfusion. This observation suggests that oxygen-radicals induce peroxidation of lipids, and may lead to membrane dysfunctions during tissue injury. Direct evidence of tissue injury was suggested by the presence of ascorbyl free radical during global ischemia.

Our results demonstrate that short-lived free radicals, superoxide anion, hydroxyl, and carbon-centered radicals, are formed during myocardial ischemia and post-ischemic reperfusion. We definitely demonstrate that superoxide anion is produced during the early phase of reperfusion. These studies provide direct evidence of the validity of the free radical hypothesis of ischemia-reperfusion cell injury.

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