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THE DEMONSTRATION OF DMPO SUPEROXIDE ADDUCT UPON REPERFUSION USING A LOW NON-TOXIC CONCENTRATION

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Experiments were conducted in an attempt to circumvent the problem associated with the use of a high concentration of the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), which has been suggested to have serious toxic effects on tissue. A low concentration of DMPO (10 mM) was used to detect the free radical generation in hearts with ischemia/reperfusion insult. In the effluent immediately after reprefusion, DMPO–OOH, a superoxide spin adduct of DMPO, was obtained. DMPO in that concentration range did not interfere at all with the left ventricular (LV) function during the control perfusion period. Even after reperfusion, LV function was not depressed any more than that occurring in hearts without DMPO, whereas DMPO of the conventional concentration (100 mM) markedly depressed the ventricular function. Enzyme leakage from hearts also supported non-toxicity finding of DMPO at 10 mM; confirming that the DMPO superoxide adduct is real evidence of the generation of superoxide upon reperfusion and is not attributed to an artificial generation due to the cytotoxicity of DMPO.

KEY WORDS: ESR, spin-trapping, DMPO, reperfusion, superoxide, enzyme leakage.

INTRODUCTION

A recent review article on the current status of the detection of radical production in the ischemic and reperfused myocardium[1] pointed out that electron spin resonance (ESR) spin trappiong methods have provided evidence of the generation of a variety of radicals during both ischemic and reperfusion phases[2–6]. However careful and critical interpretation of those reports is necessary, particularly, due to the fact that the use of spin traps at high concentrations (up to 100 mM) could produce cytotoxic effects. Therefore, the present study was undertaken to demonstrate the generation of the oxygen radical during reperfusion after an ischemic episode using DMPO at a low concentration (10 mM). Furthermore, hemodynamic effects and enzyme leakage from the hearts were monitored as a supportive means of confirmation of non-toxicity of DMPO.

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MATERIALS AND METHODS

Heart Perfusion System

Male Wistar rats (270–350 g) were anesthetized using sodium pentobarbital and the hearts were excised and perfused with a medium equilibrated with a 95% O_2/5% CO_2 containing (in mM) 5.5 glucose, 5.5 pyruvate, 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 25 $NaHCO_3$. The perfusion system and the temperature jacket surrounding the heart were kept at 37 C. To assess contractile function, a latex balloon was inserted into the LV cavity and was adjusted for an end-diastolic pressure of 5 mmHg.

Experimental Protocols

Rat hearts were stabilized under control perfusion conditions for 25 min prior to the induction of global ischemia. Then hearts were subjected to a 40-min period of ischemia followed by reperfusion. In five hearts, DMPO resolved in buffer was infused at a rate of 1.1 ml/min to achieve a final concentration of 10 mM in the perfusate. The coronary flow utilized in the experiments ranged from 12 to 15 ml/min. The experimental room was dark throughout the study to prevent photolytic degradation of DMPO. Infusion of DMPO was started 30 sec prior to reperfusion and was continued for an additional 4 minutes. For the controls, five hearts were infused with buffer without DMPO; as a conventional high concentration DMPO group, two hearts were infused with buffer in the presence of DMPO at the conventional concentration (100 mM in the perfusate). Coronary effluents were collected at specific intervals for ESR measurements and for enzyme assays. Glutamic-oxaloacetic transaminase (GOT), creatine kinase (CPK) and lactate dehydrogenase (LDH) activities were determined using an autoanalyzer AU550 (Olympus, Tokyo). Cysteine was added to the samples prior to measure activities in order to prevent the inactivation of CPK.[7] At the end of the experiment, the hearts were blotted and weighed. The enzyme activities were expressed in Units/gww (grams wet weight).

ESR Measurements

Approximately 200–500 microlitres of coronary effluents in the presence of DMPO were collected and immediately frozen in liquid N_2 to prevent spin adduct decay. Frozen samples were thawed just before ESR measurements were performed.

ESR measurements were made on a JEOL (JES-FE 2XG, Tokyo). The samples were placed in special flat cells (180 microlitres volume, JEOL). Conditions of ESR spectrometry for measurement were as follows: magnetic field 335 $\pm$ 5 mT, power 8 mW, response 0.3 sec, modulation 0.125 mT, temperature 23 $\pm$ 1 C, amplitude 6.5×10^3, sweep time 2 min and magnetic field modulation frequency 100 KHz. The signal of Mn^{2+} in MgO was used for field calibration and hyperfine coupling constants were obtained by computer simulations.

DMPO was obtained from Shonan Analytic Center (Tokyo) and was purified according to the method of Buettner and Oberley[8] by repeatedly treating the solution with activated charcoal until all free radical impurities were eliminated. To avoid potential artifacts, the buffer used to prepare DMPO solutions was passed through an ion-exchange column of Chelex-100 (Bio-rad, Richmond, CA) and diethylenetria-

minepentaacetic acid (DTPA) (0.1 mM) was added in order to prevent degradation due to metal ion catalysis.

RESULTS

No ESR signal was observed in the fresh oxygenated buffer containing DMPO with 0.1 mM DTPA (identical preparation as that infused) or in effluents during control perfusion prior to the ischemia/reperfusion episode. Upon reperfusion, complex ESR signals appeared. Figure 1 shows a representative ESR spectrum obtained from effluents collected immediately after reperfusion. This poorly resolved spectrum can be identified as at least three different ESR signals which overlap. The best-resembling spectral simulation was obtained from the superposition of three components: DMPO-OOH adduct with hyperfine coupling constants (hfcc's) of $a_N = 1.43$, $a_H^\beta = 1.15$, $a_H^\gamma = 0.13$ mT, a 1:2:2:1 quartet with hfcc's of $a_N = a_H^\beta = 1.49$ mT attributed to DMPO-OH adduct, and a triplet with a hfcc of $a_N = 1.9$ mT attributed to an unknown nitroxide. This nitroxide could be generated by a deductive decomposition of DMPO. One minute after reperfusion, DMPO-OOH was no longer observed but the quartet of DMPO-OH alone was observed. To determine the origin of DMPO-OH, superoxide dismutase was added to the system which supressed both DMPO–OOH and DMPO–OH adducts, indicating indirectly that the DMPO–OH adduct did originate from superoxide.

Regarding the cytotoxicity of DMPO, the time courses of the LV developed pressure in the absence of DMPO (control), in the presence of 10 mM DMPO and in the presence of 100 mM DMPO are shown in Figure 2. DMPO at concentrations of 10 mM and 100 mM did not depress LV pressure nor heart rate when the test infusions were applied for one min during the pre-ischemic perfusion period. During the ischemic period, the decreases in LV pressure were almost identical and the characteristics of arrhythmias which occur at the time of reperfusion were also similar among the three groups. However the time course of LV function during the early and

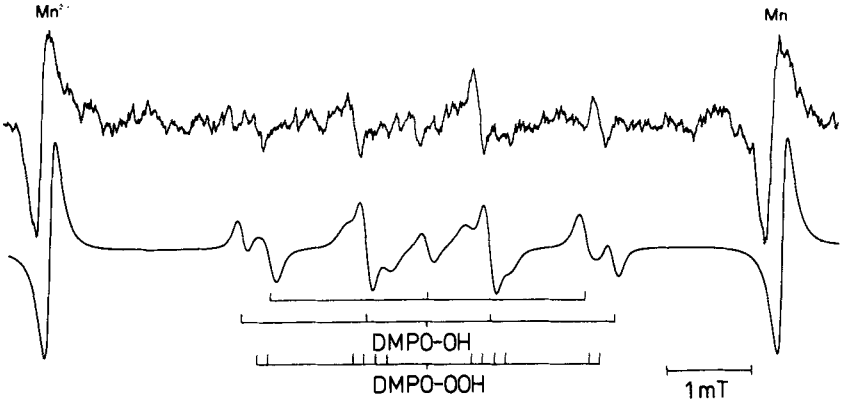

FIGURE 1. ESR spectrum of DMPO adducts in the effluent immediately reperfusion Mn: an internal standard of MnO

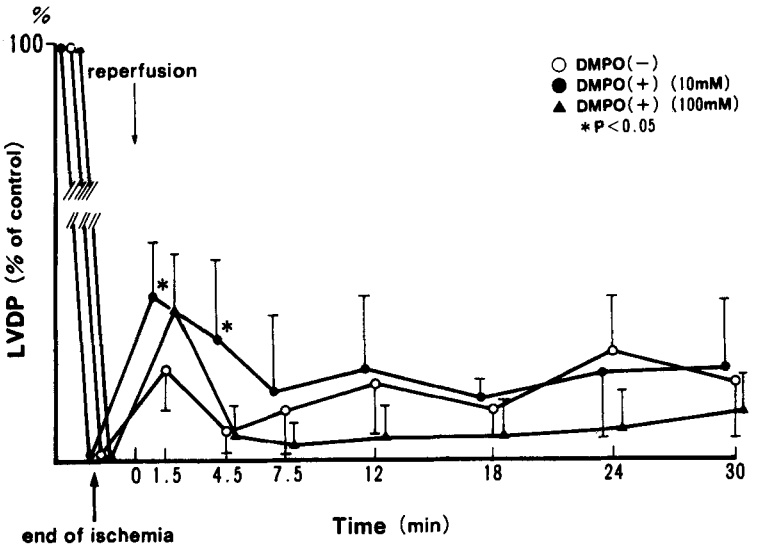

FIGURE 2. The time course of the LV developed pressure during ischemia and reperfusion LVDP: LV developed pressure

late reperfusion periods differed among the three groups; LV pressure in the 100 mM DMPO group was markedly depressed compare with that in the control group. In contrast, LV pressure was better preserved in the 10 mM DMPO group than in the group without DMPO and also maintained a tendency toward higher values until 4.5

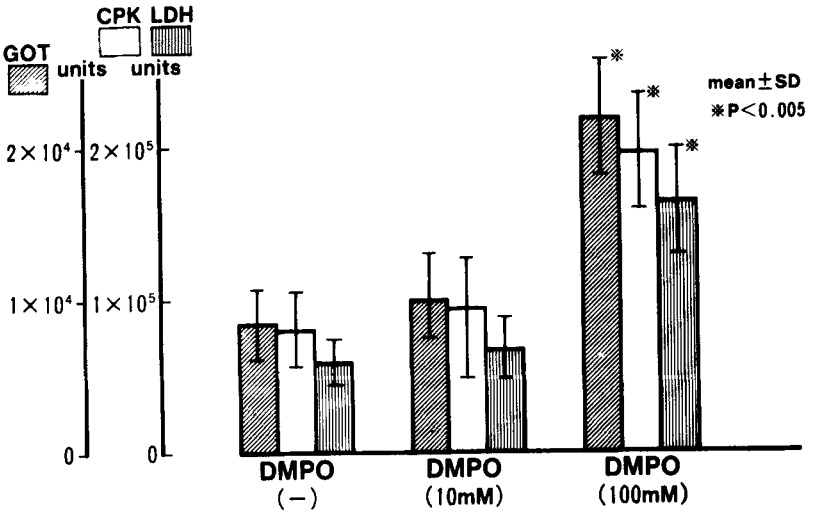

FIGURE 3 Influence of DMPO infusion on enzyme release GOT: glutamic-oxaloacetic transaminase, CPK: creatine kinase LDH: lactate dehydrogenase. The left columns represent hearts in which no DMPO was infused, the middle columns represent hearts with 10 mM DMPO and the right shows hearts with 100 mM DMPO.

min of reperfusion which coincided with the duration of DMPO infusion. After that period, LV pressure in the 10 mM DMPO group became similar to that in the control group.

As shown in Figure 3, infusion of 100 mM DMPO significantly augmented enzyme leakage, indicating cytotoxicity of DMPO but infusion of 10 mM DMPO neither decreased nor increased the enzyme leakage.

DISCUSSION

In spin trapping experiments using biological materials and in the presence of oxygen such as in reperfusion experiments, ESR signals are broad, and are poorly resolved. Thus, ESR spectra observed under these conditions often require simulation to obtain the hyperfine coupling constants. This is especially true in the spectrum shown in Figure 1, which consists of at least three different overlapping adducts, DMPO–OOH, DMPO–OH, and a nitroxide, a reduced product of DMPO. DMPO–OOH was detectable for only one minute after reperfusion; the signal of DMPO–OH remained longer, which was shown as a secondary adduct and did not originate from a primary OH radical. The numerous ambiguities concerning the origin of DMPO–OH in the biological system have been extensively discussed elsewhere.[9–12] The signal of DMPO–OOH in contrast, is considered to be proof of the presence of superoxide since DMPO–OOH adduct is a primary adduct and other radical species do not yield the DMPO–OOH adduct, and thereby indicating the generation of superoxide upon reperfusion.

In 1987, Arroyo *et al.* reported that a high concentration of DMPO (100 mM) is essential to clearly demonstrate superoxide in ischemia/reperfusion model.[3] However, that concentration range was reported by Davies[1] in 1989 to be cytotoxic. That criticism was supported by a study done by Hearse and Tosaki in which DMPO at a concentration of 0.5 mM was found to be the optimal concentration to decrease the incidence of reperfusion arryhythmias; also a higher dose was found to produce serious damage to hearts.[13] Very recently Tosaki *et al.*[14] showed that DMPO at a concentration as low as 0.1 mM reduced reperfusion arrhythmias, but for the demonstration of the DMPO radical adduct use of a higher concentration of DMPO (20–200 mM) was necessary. With a high concentration of DMPO, they showed only the DMPO–OH adduct and the DMPO–OOH adduct could not be demonstrated; suggesting the potential impractability of trapping superoxide in ischemia/reperfusion experiments. Their results support the significance of the demonstration of the DMPO–OOH spectrum in this study, even though the spectrum was very weak.

Regarding cytotoxicity in the present study, we found no significant functional damage nor enzyme leakage in the DMPO concentration range of 10 mM. An explanation of the inability to show the protective effect of DMPO against reperfusion injury might be that the protective effect may have been compromised by the toxic effect of DMPO even at this low concentration range. The beneficial effect of DMPO in the 10 mM DMPO group can be confirmed by the evidence of better LV function during the initial 4 min of the reperfusion period since the time of DMPO infusion coincided with the period of improved LV function.

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