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SPIN TRAPPING EVIDENCE FOR RADICAL GENERATION BY ISOLATED HEARTS AND CULTURED HEART CELLS

INGOLF E. BLASIG, BERND EBERT*, GERD WALLUKAT** and HANS LOEWE

*Academy of Sciences of the GDR, Institute of Drug Research, A.-Kowalke-Str. 4, 1136 Berlin, *Central Institute of Molecular Biology, **Central Institute of Cardiovascular Research, 1115 Berlin GDR*

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The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) has been applied to monitor the generation of free oxy-radicals in samples derived from isolated hearts and heart cells. ·OH was trapped in the effluent of isolated hearts in the early phase of reperfusion following an ischemia time of only 10 min. Radical detection was possible even when the cardioactive DMPO was added to the effluent after draining off the heart, demonstrating that the short-lived ·OH was generated by components released from the affected heart. These results support the hypothesis that radicals are of relevance for reperfusion injury.

By omitting antioxidants commonly used for incubation media of cultured cells, it was possible for the first time to demonstrate the formation of ·OH in the incubation solution of cardiac cells.

KEY WORDS: Spin trap technique, pathological conditions of the heart, radical identification, oxy-radicals.

INTRODUCTION

Oxygen-derived free radicals may be of pathogenetic relevance for functional disturbances of the heart.[1-7] Oxy-radicals were detected during myocardial ischemia[1,3,6] and reperfusion.[2-5,7] The steady-state concentration of free radicals is very low in the myocardium, rendering detection difficult. The spin trapping method can overcome this difficulty, because the radicals trapped by this technique accumulate. The detection can be carried out at physiological conditions. Calculation of the hyperfine splitting constant and the g-value of the spin trap adduct may facilitate the identification of the original radical. Therefore, the procedure represents the decisive prerequisite to study which kind of radicals, and in what respect, are of pathogenetic relevance for the myocardium. The aim of this study has been to show modifications in the application of the technique to heart-derived samples.

MATERIAL AND METHODS

The spectra were run on a Varian E-3 spectrometer at 25 °C in the dark using a flat quarts cell. The samples derived from isolated hearts or cultured heart cells were measured and subsequently incubated with a ·OH generating Fenton-system (50 μM $FeSO_4$ and 100 μM H_2O_2) followed by a second measurement. The spin trap DMPO was obtained from the Central Institute of Molecular Biology, Academy of Sciences of the GDR, xanthine oxidase (XO) from Boehringer (Mannheim, FRG).

The reagents were prepared freshly and dissolved in deionized and distilled water. The solutions of DMPO, $FeSO_4$, H_2O_2 were saturated with nitrogen, held on ice in the dark. DMPO or its adducts proved to be stable in liquid N_2 for several h.

Cells from the heart ventricles of 1 to 2 day-old Wistar rats were cultured for 8 days as described previously.[8,9] The cell monolayer was washed with Hank's salt solution and incubated at 37 °C in 1 ml of this solution containing 100 mM DMPO with continued gentle rocking back and forth for 2 h without or with added effectors. Before and immediately after incubation, aliquots of cells and medium were rapidly transferred into liquid N_2. The frozen samples were thawed 3 min before recording the spectra. The pulsation rate of the spontaneously beating myocytes was about 150 min^{-1} under control conditions. DMPO and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) had no influence on the functional state of the cells.

Hearts from 6 male rabbits were used for perfusion experiments. Each heart was rapidly dissected and perfused with Krebs-Henseleit medium pH 7.4 at 37°C in the Langendorff mode as described.[10] After an equilibration interval of 30 min, flow was maintained at 30 ml/min (controls), or total ischemia (no-flow) was induced followed by reperfusion using the perfusion conditions as for the controls. In the control group, the heart rate was near 120 min^{-1}, and the release of lactate dehydrogenase activity was about 10 mU/min · g heart wet weight. The heart frequency slowed down to zero within 5–10 min of ischemia. Upon reperfusion, restoration of the rate was observed within 5 min, accompanied by arrhythmias. One min after onset of reperfusion or during the control, 500 mM DMPO were added to the effluent perfusate which was pooled and rapidly frozen in liquid N_2. The electron spin resonance (ESR) spectra were recorded immediately after thawing the samples.

RESULTS

The typical 12-line spectrum of DMPO-perhydroxyl radical adduct (DMPO–OOH) formed in the presence of XO and hypoxanthine, known to generate $\cdot O_2^-$, is shown in Figure 1. Maximum signal intensity was recorded about 60 s after the enzyme reaction was initiated. The lines were superimposed by the 4-line signal of the DMPO-hydroxyl radical adduct (DMPO–OH). The ·OH adduct reached the maximum after 3 min. Without hypoxanthine, no indication of any radical was observed. The addition of 1 mM diethylenetriaminepentaacetic acid (DETAPAC) led to an increase of the signal intensity of DMPO–OOH, the maximum appearing after 90 s. The occurrence of the DMPO–OH signal was delayed, with the maximum after 4 min. When H_2O_2 or Fe^{2+} were added, only DMPO–OH was detected. The signal was intensified and maximum values were reached after 5 and 3 min, respectively.

Figure 2 represents spectra recorded in the incubation solution of cultured heart cells. After 2 h of incubation, ·OH was trapped under control conditions which was almost completely prevented in the presence of HEPES. The decrease of the signal intensity was accompanied by an increase of the antioxidant activity of the solution. This was deduced from the observation that when the standard Fenton-system generating ·OH was added to the solution without incubation or after incubation and ESR spectrometry, a smaller DMPO–OH signal was observed in the solutions containing HEPES compared to those of the control. A decreased trapping of ·OH in the presence of the Fenton-standard is considered to reflect increased antioxidant activity. ESR measurements of the cells did not reveal any radical, even when the Fenton-standard had been added.

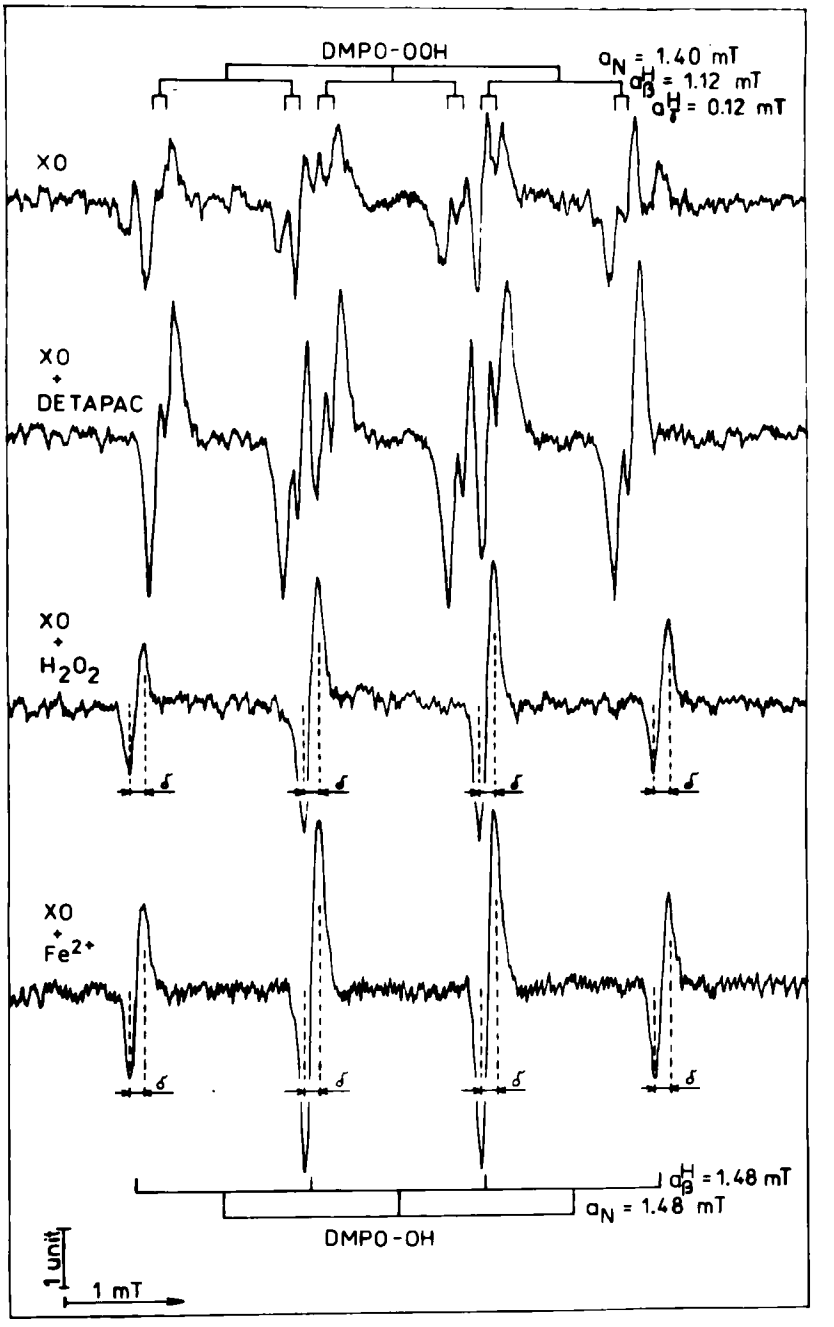

FIGURE 1 *ESR spectra of oxygen radicals trapped in the presence of 100 mM DMPO using the XO/hypoxanthine system.* Additions of 1 mM DETAPAC, 100 μM H_2O_2 or 50 μM Fe^{2+}, each representing 3 experiments. Recording of traces followed 1 min after initiation of the enzyme reaction. The medium consisted of 0.4 U/ml XO, 400 μM hypoxanthine and 10 mM potassium phosphate buffer pH 7.4 at 25°C. Spectrometer settings: 20 mW microwave power, 0.1 mT modulation amplitude, 50 ms response time, scan time 1 min. Line width, δ = 0.15 mT, of DMPO-hydroxyl radical adduct (DMPO-OH) was calculated at 0.05 mT modulation amplitude. Units represent arbitrary units of the signal intensity. DMPO-OOH: DMPO–perhydroxyl radical adduct.

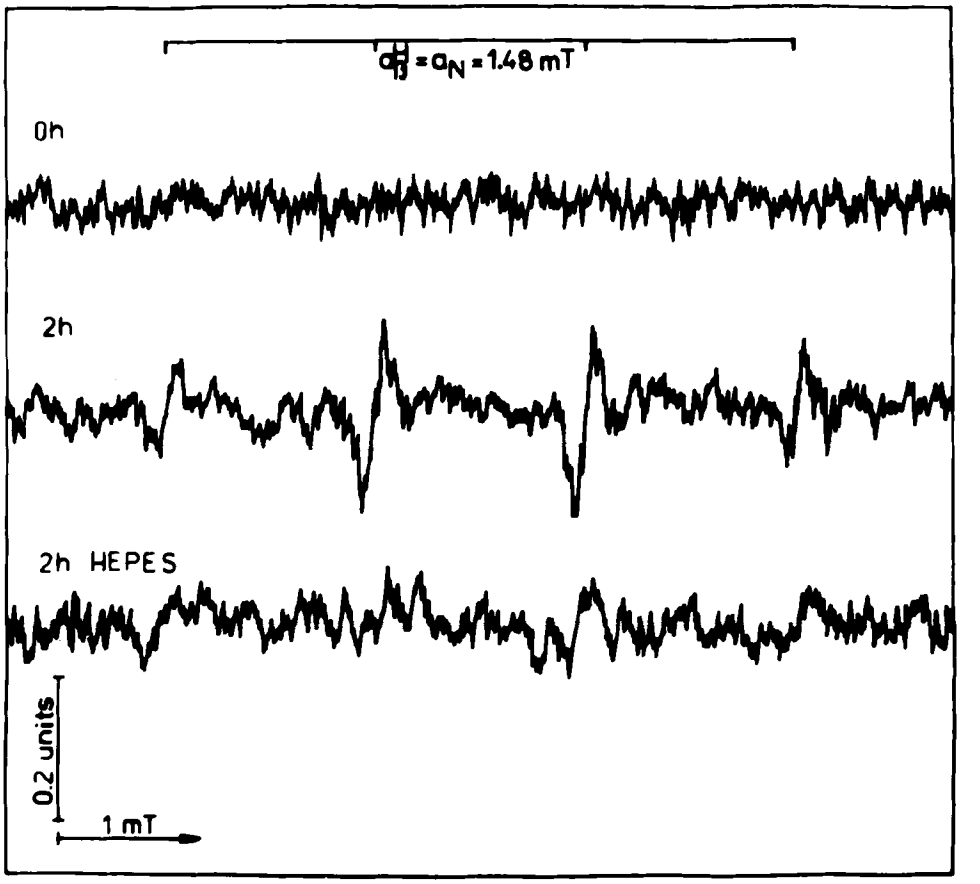

FIGURE 2 *ESR spectra monitored in the incubation solution of cultured heart cells.* 100 mM DMPO was present before incubation (0 h) or 2 h after incubation without and with 20 mM HEPES; each spectrum represents 5 independent cultivations. Settings as in Figure 1, except scan time 2 min, modulation amplitude 0.2 mT and response time 150 ms.

Representative spectra recorded in coronary effluent of isolated perfused hearts are shown in Figure 3. Neither within the control perfusion nor 1 min after initiation of reperfusion, a considerable ESR signal was detected if the preceding ischemia had not exceeded 5 min. After at least 10 min of ischemia followed by 1 min of reperfusion, the ·OH adduct was identified. The adduct is superimposed by an additional signal as indicated by increased line width and asymmetric lines.

DISCUSSION

DMPO is the most commonly used spin trap as it allows to discriminate different oxy-radicals.[11] As model of radical formation, the xanthine oxidase/hypoxanthine

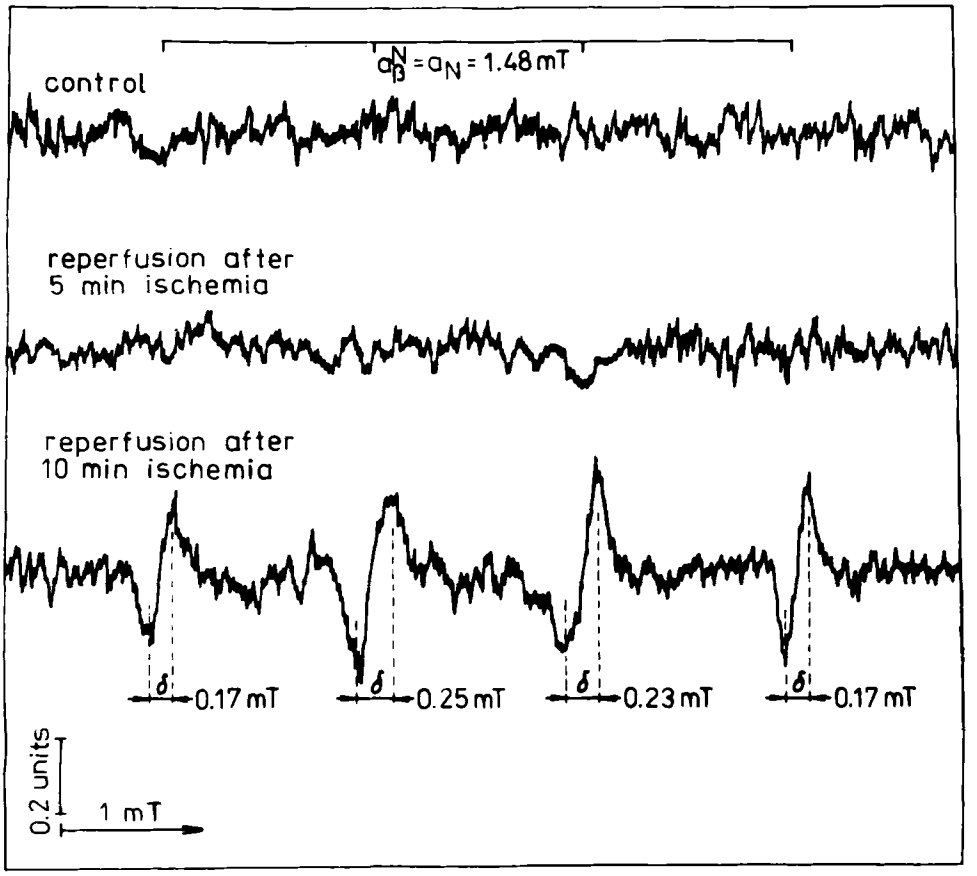

FIGURE 3 *ESR spectra of DMPO adducts formed in the perfusion effluent of isolated hearts.* Spectra were taken at 10 min of control perfusion (control) or 1 min after initiation of reperfusion following 5 or 10 min of total ischemia. δ indicates the line width of the signals (modulation amplitude 0.1 mT for the calculation). Spectrometer settings and units as in Figure 2.

system and its interaction with H_2O_2 and Fe^{2+}, constituents of the Fenton-system, have been studied. Both systems are thought to be involved in the production of radicals during heart injury.[7,12,13] In the model experiments with xanthine oxidase, known to generate superoxide, the 12-line spectrum of DMPO–OOH is recorded. DMPO–OOH is the adduct of $HO_2\cdot$ originating from $\cdot O_2^-$ which is protonated under physiological conditions.[14] Investigation of the enzyme reaction also demonstrates that the first two min after initiation of the trapping have to be used, and a trap concentration of at least 50 mM is necessary to detect $\cdot O_2$ by DMPO. Lower DMPO concentrations result in the detection of DMPO–OH only.[15] The lifetime of DMPO–OOH is shorter and, thus, its maximum intensity is reached earlier than those of DMPO–OH.[16] In addition, DMPO–OOH is decomposed to yield the $\cdot$OH adduct.[17] Therefore, initially the determination of $\cdot O_2^-$ predominates which is followed by conversion to the $\cdot$OH adduct, dominating after 3 min. The detection of superoxide

may be increased and, thus, prolonged by metal-chelating agents like DETAPAC.[18] The assumption of metal ions being involved in the XO system is indirectly supported by the addition of H_2O_2 which completely prevents the formation of DMPO–OOH in favour of the ·OH adduct. The Fenton-type reaction seems to be of importance in this case. Fe^{2+} incubated with the XO system also yields the formation of the ·OH adduct. Transition metal ions present in trace amounts can react with HO_2·[14,16] or DMPO–OOH to form ·OH and DMPO–OH, respectively. In general, the model investigation shows that in the presence of both ·OH and $\cdot O_2^-$, DMPO–OH is preferentially detected. Therefore, the sole identification of ·OH does not generally exclude the primary existence of $\cdot O_2^-$. Furthermore, the reaction kinetics of trapped radicals depends on the initial radical concentration as well as on the spin trap concentration. At higher amounts of ·OH generated by the Fenton-reagent, the maximum of adduct formation and, hence, its decay is reached earlier than at lower ones.[1] Conversely, the rate of adduct decay decreases with increasing spin trap concentration when 1 mM[19] and 100 mM DMPO[1] are compared.

In the experiments of isolated perfused hearts subjected to ischemia and reperfusion, ·OH has been identified in the coronary effluent during reperfusion following an ischemia of only 10 min. Ten min of ischemia is the shortest ischemic interval known to result in the reperfusion damage. The spin trapping evidence of radicals in the effluent during the initial phase of reperfusion already after 10 min of ischemia supports the hypothesis that radicals generated in the vascular system may be involved in the induction of the postischemic reperfusion damage. In this study DMPO has been added to aliquots of the effluent perfusate to avoid the cardioprotective action[22] of the trap. The results obtained are in agreement with our earlier investigations[1-3] and recent reports,[4-7] which show the generation of ·OH after prolonged ischemia and reperfusion. In these latter experiments DMPO was perfused through the heart. The possibility to demonstrate the radicals also in the effluent, after passing the heart, suggests the application of the method to comparative studies. Using this modification, the radical formation may be compared to the myocardial function without interaction of the cardioactive spin trap with the heart. Thereby, the spin trapping technique as used here becomes a decisive prerequisite to further clarification of the pathogenetic role of free radicals for functional disturbances of the heart.

Although the trapping of ·OH is preferred compared to that of $\cdot O_2^-$, there are indications that DMPO–O_2^- or its protonated form are possibly formed in addition to DMPO–OH in the reperfusion effluent. DMPO–OOH is observed when the samples have been taken 1 min after onset of reperfusion. The early reperfusion time has been studied because superoxide was suggested to be the primary oxy-radical[4,5,7] and because maximum of production of DMPO–OH was reported to occur 3 min after the initiation of reperfusion.[4] The appearance of DMPO–OOH could be suggested by the DMPO–OH signal asymmetry, increase of line width and loss of intensity of its middle lines. In model investigations, such spectra may be obtained when DMPO–OOH is simultaneously present showing signal lines at similar positions as those of DMPO–OH. The results give hints for the generation of superoxide radicals in the reperfusion perfusate and support the notion that the latter species is primarily generated and could be the source of the ·OH adduct. According to that conclusion the formation of the DMPO–OH signal in the reperfusate has been found to be prevented by additional perfusion with superoxide dismutase.[4,5,7] This seems to be possible because DMPO–OH can be formed from DMPO–O_2^- via DMPO–OOH.

The determination of radicals in the effluent after passage of the heart demonstrates

that constituents in the effluent released from the heart are the source of the short-lived radicals. The radicals are attributed to the reperfusion injury as no radical signal was detected under control perfusion conditions. Therefore, the radicals detected reflect radical processes which take place in the injured myocardium as found by direct ESR measurements under comparable experimental conditions.[23] If the perfusion medium was supplemented with 1% washed erythrocytes, the antioxidant activity of the medium appeared to be increased. Thus, a distinctly lower amount of radicals was trapped in the reperfusion effluent in the presence of erythrocytes. Moreover, there are no indications of trapped radicals in heart tissue even after infusion of DMPO or within the cultured heart cells incubated with the trap. This suggests that DMPO does not penetrate into the cell in an amount which can be monitored. Also all other attempts to show radical formation by cultured heart cells were unsuccessful. However, under certain conditions generation of radicals may be demonstrated in the incubation solution of cultured heart cells even under control conditions without remarkable disturbances of cell function. This becomes possible when antioxidants commonly used for incubation media of cultured cells were omitted from the medium or Hank's salt medium was used containing glucose only as radical scavenger. That is, radicals may also be generated under control conditions, and they may not only be a sign of disturbed cell function. Cellular components with high antioxidant activity or high radical scavenger activity compete with the spin trapping agent for the radicals. Thus, the possibility of permeation of the nitrone into the cell as reported for erythrocytes is not fully excluded. However, in the latter case the spin adducts were not detected because of their rapid decay.[20] To monitor free radicals intracellularly, the application of more lipophilic spin traps[21] appears to be more efficient. In conclusion, the use of DMPO as spin trap is especially useful in systems showing relatively low content of radical scavengers competing for the radical studied.

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