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COMPARISONS OF ESR AND HPLC METHODS FOR THE DETECTION OF OH RADICALS IN ISCHEMIC/REPERFUSED HEARTS

A RELATIONSHIP BETWEEN THE GENESIS OF FREE RADICALS AND REPERFUSION ARRHYTHMIAS

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Abstract—In this study we compared two methods, electron spin resonance (ESR) spectroscopy and high performance liquid chromatography (HPLC), which are currently used to detect directly hydroxyl radical ($OH^{\cdot}$) formation in the ischemic and reperfused heart. Isolated buffer-perfused rat hearts were subjected to 30 min of normothermic global ischemia followed by 30 min of reperfusion. 5,5-Dimethyl-pyrroline-*N*-oxide (DMPO) was used as a spin-trap agent to detect $OH^{\cdot}$ radicals by ESR and HPLC. In additional HPLC studies, salicylic acid was infused into the heart for the detection of $OH^{\cdot}$ radicals. In all studies, the effects of superoxide dismutase (SOD) and catalase (CAT) on the $OH^{\cdot}$ generation were examined. The results of our studies indicate that, irrespective of the method, $OH^{\cdot}$ was always detected when an ischemic heart was reperfused and showed ventricular fibrillation. The $OH^{\cdot}$ concentration increased dramatically between 60 and 90 sec of reperfusion, peaked between 180 and 210 sec, and then progressively decreased. In all cases, both SOD and CAT were able to reduce the formation of $OH^{\cdot}$ radicals, with SOD being relatively more effective. Our results indicate that $OH^{\cdot}$ was produced only in the fibrillating hearts that peaked between 180 and 210 sec (1.64 ± 0.09 nmol/mL measured by ESR), but not in the non-fibrillating hearts. Although SOD or CAT reduced the $OH^{\cdot}$ formation, they had no effects on the incidence of reperfusion-induced ventricular fibrillation (VF) and ventricular tachycardia (VT). However, when SOD (5×10^4 IU/L) was coadministered with CAT (5×10^4 IU±L), the incidence of reperfusion-induced VF (total) and VT was reduced from their control value of 92 and 100 to 33 ($P < 0.05$) and 50% ($P < 0.05$), respectively. The results of this study indicate that the HPLC method, as well as ESR, can be used to detect $OH^{\cdot}$ formation in ischemic/reperfused hearts. Because of the convenience, reproducibility and greater sensitivity, the HPLC technique may be more suitable for $OH^{\cdot}$ detection. Our results further suggest the potential therapeutic value of the combination therapy of SOD and CAT for the reduction of reperfusion-induced VF and VT.

A growing body of evidence supports the hypothesis that a large quantity of oxygen free radicals are generated in the post-ischemic heart upon reperfusion [1–3], and that this phenomenon may be responsible for the occurrence of many deleterious effects including life-threatening arrhythmias associated with reperfusion [4, 5]. These oxygen-derived free radicals are highly reactive, extremely short-lived, and very difficult to detect by conventional assay techniques. In biological systems, electron spin resonance (ESR) spectroscopy remains the method of choice for identification of hydroxyl radicals ($OH^{\cdot}$)§ in ischemic and reperfused hearts [6–8]. The success of the ESR technique depends on the conversion of unstable free radicals into a relatively more stable form using specific spin-trapping agents [7, 9]. Among the various spin traps, 5,5-dimethyl-pyrroline-*N*-oxide (DMPO) has been used most extensively [9]. DMPO reacts with superoxide anion forming a highly unstable DMPO-$\dot{O}OH$ adduct, which decomposes rapidly into more the stable DMPO-$OH^{\cdot}$ adduct, which can also be formed if DMPO reacts with $OH^{\cdot}$ directly. Despite the popularity of ESR, this method has certain disadvantages, viz. (i) it is very expensive, and is not readily available to many scientists; (ii) chemical interconversions between spin-trap radical adducts occasionally lead to confusion in properly identifying a radical species; (iii) the proper interpretation of the data is extremely difficult; (iv) in biological systems, oxygen free radicals may be generated in such a small quantity that they may be overpowered by other free radical signals; and, finally, (v) ESR cannot detect the oxygen free radical signal unless it is generated in sufficient quantities.

During recent years, the high performance liquid

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§ Abbreviations: $OH^{\cdot}$, hydroxyl radical; SOD, superoxide dismutase; CAT, catalase; DMPO, 5,5-dimethyl-pyrroline-*N*-oxide; VF, ventricular fibrillation; VT, ventricular tachycardia; SR, sinus rhythm; and 2,3- and 2,5-DHBA, 2,3- and 2,5-dihydroxybenzoic acid.

chromatography (HPLC) method has been used in a few laboratories to estimate directly the $OH^{\cdot}$ in ischemic and reperfused hearts [10–13]. This method is easily adaptable, extremely sensitive, and can detect very small amounts of $OH^{\cdot}$ radicals. However, compared to ESR, HPLC for the *in vivo* detection of $OH^{\cdot}$ is only in its infancy, and therefore, despite the many advantages of HPLC, ESR is still considered as the most reliable method.

This study was designed to explore the suitability of HPLC for the detection of $OH^{\cdot}$ in isolated rat hearts. Two different HPLC techniques were used, one of them using DMPO as the spin trap, and the other using salicylate as a chemical trap for $OH^{\cdot}$. The results were compared with those obtained by ESR. Our results indicate that the HPLC technique is a highly reliable method for the detection of $OH^{\cdot}$ in the effluents of isolated hearts. Furthermore, our data show that the $OH^{\cdot}$ radical was formed only in fibrillating hearts, and not in non-fibrillating hearts, and the combination of superoxide dismutase (SOD) with catalase (CAT) attenuated the ischemia/reperfusion-induced injury.

MATERIALS AND METHODS

Perfusion technique

Male Sprague–Dawley rats (250–300 g body weight) were used for all studies. Animals were anesthetized with intraperitoneal pentobarbital (85 mg/kg), and heparin (200 IU/kg) was injected intravenously. After 20 sec, the hearts were excised and placed in cold (4°) perfusion buffer until contraction had ceased (approximately 20 sec). Each heart was then cannulated via the aorta and perfused by the Langendorff method at a constant perfusion pressure equivalent to 100 cm of water (10 kPa) [14]. The perfusion buffer consisted of a modified Krebs–Henseleit bicarbonate buffer (millimolar concentrations: NaCl, 118; KCl, 5.8; $CaCl_2$, 1.85; $NaHCO_3$, 25; NaH_2PO_4, 0.36; $MgSO_4$, 1.2; and glucose, 5.0). A reduced concentration of NaH_2PO_4 and glucose in the buffer was necessary because they can disturb the electron spin resonance spectra [15]. During preparation of the perfusion buffer, precautions were taken to prevent the precipitation of calcium; and, prior to use, all solutions were filtered through a 5 μm pore filter to remove any particulate contaminants. The perfusate fluid was gassed with 95% oxygen and 5% carbon dioxide (pH 7.3 to 7.5 at 37°).

ESR spin-trapping studies

DMPO was obtained from the Central Institute of Molecular Biology (Academy of Sciences, Berlin, Germany) and Aldrich (Milwaukee, WI, U.S.A.), and was vacuum distilled prior to use. The spin-trapping studies were performed by infusing the spin trap, DMPO, through a side arm located just proximal to the end of the heart perfusion cannula [16]. DMPO solution was covered with aluminum foil to prevent light-induced degradation. The only vehicle in all the experiments was perfusion buffer. Hearts were removed from donor animals, and after cannulation 2 × 10 min of control perfusion periods were allowed for the heart to reach stable left ventricular function. During the first 10 min, a standard perfusion medium was used for all groups, while during the second 10-min period of preischemic perfusion, the composition of the perfusion fluid was changed, for treated groups, by switching to a second perfusion reservoir containing perfusion fluid to which SOD and/or CAT (5×10^4 IU/L, Sigma) had been added. The heart was then subjected to 30 min of normothermic global ischemia followed by reperfusion. During reperfusion DMPO was infused directly into the heart at an infusion rate of 1 mL/min of 50 mmol/L stock solution. This resulted in a final perfusate DMPO concentration of approximately 4.5–5.0 mmol/L (coronary flow was approximately 10 mL/min, 9.5 to 11.5 mL/min, during the Langendorff reperfusion period). In some additional experiments, DMPO was infused into the heart for 1 min before the induction of global ischemia to determine the presence or absence of free radicals in aerobically perfused hearts. To prevent spin adduct decay, the effluent was immediately frozen as it flowed from the heart, with an effluent sampling time of 30 sec. The ESR spectra were recorded at room temperature in a flat quartz cell with a JEOL (JEOL, Tokyo, Japan) JES-PE-1X spectrometer operating at X band (9.3 MHz) with a 100 KHz modulation frequency. The microwave power was maintained at 10 mW to avoid saturation. Unless otherwise indicated, the scans were traced with 0.2 mT of modulation amplitude with 2 min of scan time, and with 300 msec of response time. Hyperfine coupling constants were measured directly from the field scan using Mn^{2+} as a marker for calibration. Under *in vitro* conditions, the stoichiometric concentrations of H_2O_2 and DMPO were administered in the presence of xanthine oxidase (Fenton reagent), and the ESR signal intensity of the formed DMPO-$OH^{\cdot}$ radical correspond to a total oxygen free radical concentration of 2.1 ± 0.1 nmol/mL [15]. Free radical concentrations, in our study, were calculated according to the height of signal intensity.

HPLC studies

Detection of DMPO-$OH^{\cdot}$ adducts. Hearts (N = 8 in each group) were subjected to 20 min of aerobic perfusion followed by 30 min of global ischemia and reperfusion. Sampling time of the effluent was performed between 0 and 30, 60 and 90, 180 and 210, 270 and 300, 570 and 600 sec of reperfusion period, respectively, giving an effluent sampling time of 30 sec. DMPO was infused directly via a side arm of aortic cannula into the heart with an infusion rate of 1 mL/min of 50 mmol/L stock solution. This resulted in a final perfusate DMPO concentration of approximately 4.5 to 5.0 mmol/L because coronary flow was 9.5 to 11.5 mL/min during the Langendorff reperfusion period. The detection of DMPO-$OH^{\cdot}$ adduct was carried out as described by Pritsos *et al.* [17]. Immediately after the sampling, the effluent was filtered with a 0.22 μm pore size Nylon-66 sample filter (Rainin, Woburn, MA) and 20 μL was injected onto a Waters (Milford, MA) HPLC liquid chromatograph equipped with a model U6K injector, model 510 pump, model 460 electrochemical detector, and a model 740 Data Module. The detector potential was +0.6 V, employing a glassy

carbon working electrode against an Ag/AgCl reference electrode. An Altex Ultrasphere (3 μm ODS, 75 × 4.6 mm) column (Rainin) with a Brownlee RP-18 pre-column (Rainin) was used for the detection of the DMPO-OH· adduct at a flow rate of 1.0 mL/min. The mobile phase consisted of 0.03 mol/L of citric acid monohydrate, 0.05 mol/L of anhydrous sodium acetate, 0.05 mol/L of sodium hydroxide, and 8.5% of acetonitrile adjusted to pH 5.1 with glacial acetic acid and was filtered through a 0.22-μm pore-size Nylon-66 solvent filter (Rainin). In the drug-treated groups (5×10^4 IU/L of SOD or CAT), the perfusion of SOD or CAT was started 10 min before the induction of normothermic global ischemia and throughout reperfusion. The DMPO-OH· adduct peak was identified by injecting the DMPO adducts produced from a pure OH·-generating system [15].

Analysis of hydroxylated benzoic acids. In a separate group of experiments, isolated rat hearts (N = 8 in each group) were subjected to 20 min of aerobic perfusion followed by 30 min of normothermic global ischemia and 30 min of reperfusion in the presence of 1 mmol/L of salicylic acid (Aldrich) [18]. Sampling time of the effluent was the same as for DMPO-OH· adducts. The presence of hydroxylated benzoic acids in the perfusate was determined as previously described [18, 19]. Briefly, the perfusate was filtered through a Rainin 0.22 μm pore size Nylon-66 sample filter, and a 25 μL volume of the sample was injected onto an Altex Ultrasphere (3 μm ODS, 75 × 4.6 mm) column (Rainin) protected by a Brownlee RP-18 pre-column (Rainin). The hydroxylated products of salicylic acid after interaction with hydroxyl radicals, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), were eluted with a buffer containing 0.03 mol/L sodium acetate and 0.03 mol/L of citric acid (pH = 3.6) at a flow rate of 1 mL/min [17, 18]. The detection potential was maintained at +0.6 V, employing a glassy carbon working electrode and an Ag/AgCl reference electrode. Retention times for the peaks of 2,5-DHBA and 2,3-DHBA were verified by injecting authentic standards (Aldrich) and by injecting the hydroxylated products of salicylic acid from a pure OH·-generating system [17].

Recorded indices

An epicardial electrocardiogram (ECG) was recorded by a Gould polygraph throughout the experimental period with the use of two silver electrodes attached directly to the heart. The ECGs were analyzed (N = 12 in each group) for the incidence and duration of ventricular fibrillation (VF) and ventricular tachycardia (VT), and also whether the fibrillation was nonsustained (spontaneously reverting to regular rhythm) or sustained (persisting throughout the reperfusion period). The diagnostic criteria for VT and VF were consistent with the recommendation of Lambeth Conventions [20].

Statistics

During reperfusion, a time-dependent, four-line signal (1:2:2:1) of the DMPO-hydroxyl radical adduct (DMPO-OH·) was recorded, $a_N = a_\beta^H = 1.48$ mT, using ESR spectroscopy. The oxygen radical concentration formed by the Fenton-reaction in the effluents of hearts was expressed in nmol/mL (N = 8 in each group). In the HPLC study, the signal intensity of the DMPO-OH· adduct was expressed in nA and calculated in nmol/mL (N = 8 in each group), and the perfusate concentrations of 2,3- and 2,5-DHBA were also calculated in nmol/mL. A one-way analysis of variance was carried out to test for any difference between the mean values of all the groups. If differences were established, the values of SOD- and CAT-treated groups were compared to that of the untreated control group using Dunnett's test [21]. Since the duration of VF, VT, and sinus rhythm (SR) was not Gaussian distributed, the Wilcoxon test (nonparametric distribution) was used. An analogous procedure was followed for distribution of binomial distributed variables, such as the incidence of ventricular fibrillation and ventricular tachycardia (N = 12 in each group): an overall chi-squared test for a 2 × n table was constructed followed by a sequence of 2 × 2 Chi-squared test in order to compare individual groups. If $P < 0.05$, the change was considered significant.

RESULTS

ESR studies

Figure 1 shows the time course of oxygen free radical generation in hearts reperfused after 30 min of normothermic global ischemia in control, SOD-, CAT-, and SOD plus CAT-treated hearts, respectively. In nonischemic hearts, the signals of the DMPO-OH· adduct were not observed (Fig. 1), represented by the spectra of "Before-ISA". Upon examination of the time course for the appearance of the DMPO-OH· signals, maximum signal intensity was observed at 180–210 sec of reperfusion followed by a gradual decline over the next few minutes in the control heart (Fig. 1, first panel). In the SOD-treated heart (Fig. 1, second panel), signals of the DMPO-OH· adduct were observed between 180 and 210 sec only, and its intensity was reduced in comparison with the time-matched control signals. Although a reduction of signal intensity was also observed in the CAT-treated heart (Fig. 1, third panel), this reduction was no greater than in the SOD-treated heart. When SOD was co-administered with CAT (Fig. 1, fourth panel), the formation of the DMPO-OH· adduct was not observable. After 0 sec, 60 sec, 180 sec, and 5 min of reperfusion, the calculated total oxygen free radical concentrations were 0 ± 0 (undetectable level), 0.852 ± 0.06, 1.64 ± 0.09, and 0.43 ± 0.06 nmol/mL in the effluents of ischemic/reperfused rat hearts (N = 8 in each group), respectively. In our studies, the calculated oxygen free radical concentrations were significantly lower than those of isolated rabbit hearts [22]. The difference between our data and the data of Zweier could be explained by the simple fact that under our experimental conditions, only the oxygen free radical concentration was measured from the myocardial effluent, while in their studies [22] the total free radical concentration, including carbon-centered

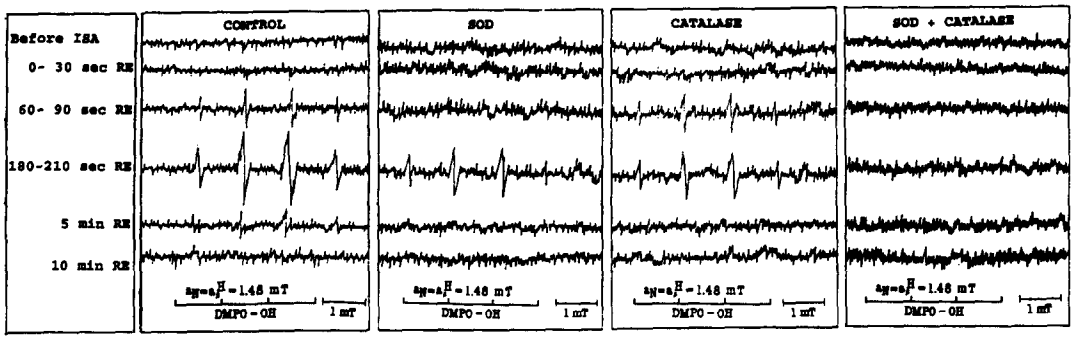

Fig. 1. Time course of oxygen free radical generation using ESR spectroscopy in control, SOD-, CAT-, and SOD plus CAT-treated hearts before ischemia (ISA) and after 30 min of normothermic global ischemia followed by reperfusion (RE). The first panel shows the spectra of DMPO-OH· in nonischemic hearts (before ISA) and after 30 min of global ISA followed by 30 sec, 60 sec, 180 sec, 5 min, and 10 min of RE; these represent the control drug-free values.

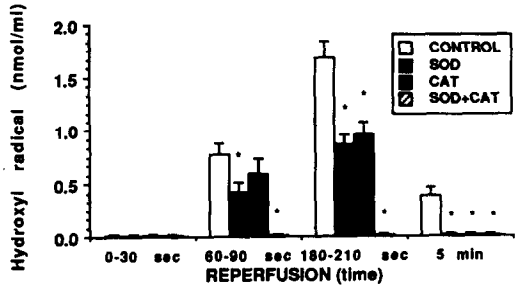

Fig. 2. DMPO-OH· concentration (N = 8 in each group) during reperfusion in control, SOD-, catalase (CAT)-, and SOD plus CAT-treated groups measured by ESR spectroscopy. Values are means ± SEM. Comparisons were made to the time-matched control values. *P < 0.05.

radicals, was measured from the ischemic/reperfused tissue. Thus, the results indicate (Fig. 2) that the treatment of hearts with SOD, CAT, and SOD plus CAT significantly reduced the formation of the DMPO-OH· adduct at 180–210 sec and 5 min of reperfusion, respectively. All hearts showed VF in the drug-free, SOD- or CAT-treated hearts (N = 8 in each group), while in the SOD plus CAT-treated hearts only three of the eight hearts were in VF.

HPLC studies

A chromatographic profile for the time course of the DMPO-OH· adduct in the untreated heart is shown in Fig. 3. Using the pure OH·-generating system as described in Materials and Methods, the peak retention time of the DMPO-OH· adduct was 5.9 min. Accordingly, the DMPO-OH· adduct peak (Fig. 3, A–F) and its intensity followed the appearance of the DMPO-OH· adduct in the effluent of reperfused hearts detected by ESR (Fig. 1) with a peak intensity at 180–210 sec of reperfusion. Figure 4 shows that the treatment of hearts with SOD or CAT significantly reduced the peak intensity of the DMPO-OH· adduct, respectively, but this reduction was not as great as found by Zweier [23] in his ESR study. These varying results using HPLC or the ESR technique could be explained by the simple fact of sensitivity differences in equipment used for the detection of DMPO-OH· adducts.

The heart effluents were also subjected to HPLC analysis for the detection of OH· radicals because benzoic acid produces 2,3- and 2,5-DHBA in the presence of hydroxyl radicals. Using authentic standards and hydroxylated products of salicylic acid from a pure OH·generating system, the retention times for 2,5-DHBA and 2,3-DHBA were determined to be 7.7 and 8.5 min, respectively. Figure 5 shows the increase of 2,5-DHBA and 2,3-DHBA reaching a maximum peak at 180–210 sec of reperfusion. Treatment of the rat hearts with either SOD or CAT reduced the concentrations of 2,5- and 2,3-DHBA (Fig. 6) following a pattern similar to that seen in both ESR and HPLC studies. A higher reduction of reperfusion-induced 2,3- and 2,5-DHBA was found when SOD was coadministered with CAT. Because salicylic acid possesses an antiarrhythmic activity in isolated hearts [24] and because we were unable to detect free radicals in fibrillating hearts only [16], we, therefore, selected a subset of postischemic hearts in order to show ventricular fibrillation in the presence of salicylic acid in the control, SOD- and CAT-treated groups. Thus, Fig. 6 shows that SOD, CAT, and SOD plus CAT significantly reduced the production of 2,3- (Fig. 6A) and 2,5-DHBA (Fig. 6B) in fibrillating hearts. In nonfibrillating hearts, the detection of 2,3- and 2,5-DHBA was not possible (data not shown).

Arrhythmia studies

Hearts (N = 12 in each group) were subjected to 30 min of normothermic global ischemia followed by 30 min of reperfusion with 50,000 IU/L of SOD, 50,000 IU/L of CAT, and 50,000 IU/L of SOD plus 50,000 IU/L of CAT (combination group), respectively. Perfusion of drugs was started 10 min before the induction of normothermic global ischemia and maintained throughout the reperfusion period.

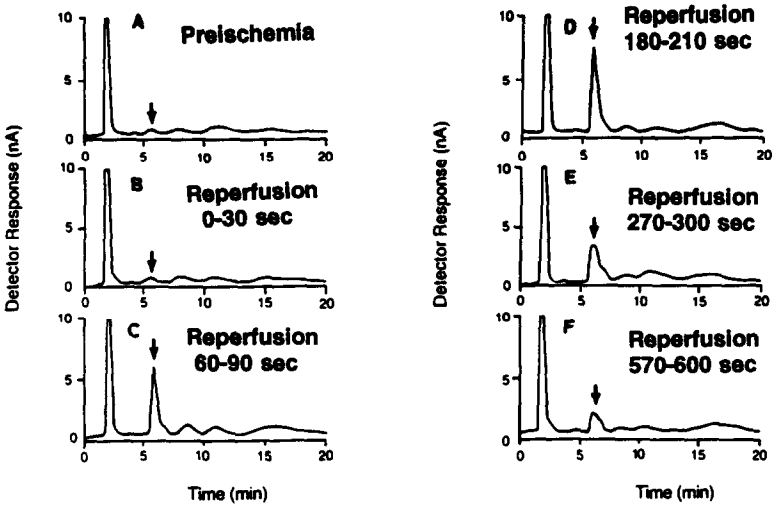

Fig. 3. HPLC detection of DMPO-OH· adduct production during reperfusion. Hearts were subjected to 30-min global ischemia followed by reperfusion. Effluents were collected: (A) before ischemia, (B) 0–30 sec, (C) 60–90 sec, (D) 180–210 sec, (E) 270–300 sec, and (F) 570–600 sec after reperfusion. The flow rate for the HPLC mobile phase was 1 mL/min, and the chromatographs were recorded at 1 cm/min. The electrochemical detector was set at a 10-nA sensitivity.

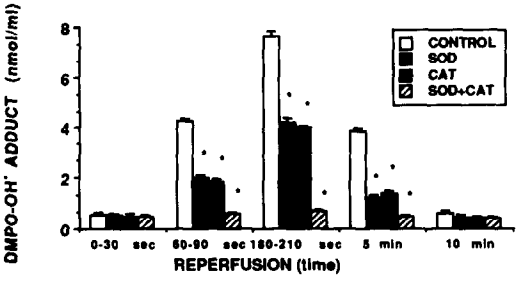

Fig. 4. DMPO-OH· concentration (N = 8 in each group) during reperfusion in control, SOD-, CAT-, and SOD plus CAT-treated hearts as measured by HPLC. Values are means ± SEM. Comparisons were made to the time-matched control values. *$P < 0.05$.

The incidence of reperfusion-induced arrhythmias is shown in Table 1. Despite the "early" administration (drugs given 10 min before ischemia) of SOD or CAT, the incidence of reperfusion-induced VF and VT was not reduced, but when SOD was administered in combination with CAT, a significant reduction in the incidence of reperfusion-induced VF (both sustained and total) was observed. Furthermore, a reduction in the incidence of reperfusion-induced VF was reflected in a striking increase in the mean time that hearts were in SR during reperfusion (Table 1). Upon reperfusion, in isolated rat hearts, a maximum vulnerability was found after 30 min of normothermic global ischemia [25]; therefore, we adapted this ischemic duration for our study.

DISCUSSION

The deleterious effects of reperfusion of the previously ischemic myocardium have been linked to the production of oxygen-derived free radicals [2, 3, 8]. The free radical hypothesis derives from the results of numerous studies indicating beneficial effects of various free radical scavengers and spin traps on post-ischemic myocardial functions and cellular metabolism [1, 26–31]. Although contradictory reports are also available [32–34], there is no doubt that oxygen free radicals are indeed generated during the reperfusion of ischemic tissue [3, 35, 36], and that these free radicals probably initiate a sequence of pathological reactions indicating the maldistribution of ions resulting in cell membrane dysfunction, and ultimately, myocardial functional derangement.

In the last 4–5 years we have witnessed significant development of the free radical hypothesis of myocardial reperfusion injury. Advanced instrumentation has enabled scientists to directly demonstrate the presence of these oxygen free radicals, particularly hydroxyl radicals. Until very recently the ESR technique has been the only method of choice for this purpose. This technique conclusively demonstrated the presence of OH· in the post-ischemic reperfused heart [6–8, 25, 37]. Although the ESR technique has been proven to be a useful tool for the proper identification of OH· in biological tissue, its application has been extremely limited. As mentioned earlier, there are certain disadvantages of ESR, the primary one being availability. The ESR spectrophotometer is an extremely expensive instrument, and is not readily available to many investigators. In addition, both use of the instrument and interpretation of the data need adequate experience and skill, and without it there are often erroneous results. Finally, the sensitivity of ESR for oxygen free radical detection is not high enough and, thus, relatively smaller signals of superoxide

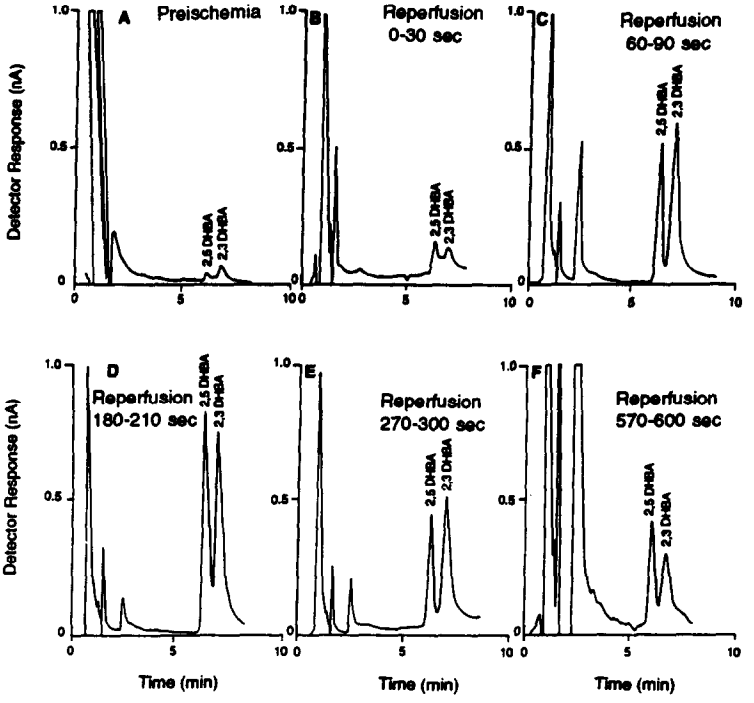

Fig. 5. Detection of 2,3- and 2,5-DHBA by HPLC during reperfusion. Hearts were subjected to 30-min global ischemia followed by reperfusion. Effluents were collected: (A) before ischemia, (B) 0–30 sec, (C) 60–90 sec, (D) 180–210 sec, (E) 270–300 sec, and (F) 570–600 sec after reperfusion. The flow rate for HPLC mobile phase was 1 mL/min and the chromatographs were recorded at 1 cm/min. The electochemical detector was set at a 1-nA sensitivity.

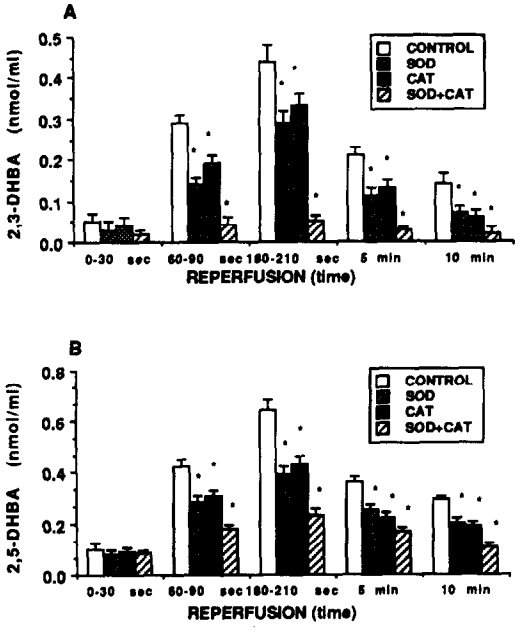

Fig. 6. (A) 2,3- and (B) 2,5-Dihydroxybenzoic acid concentrations in the effluents of reperfused hearts subjected to 30-min normothermic global ischemia followed by reperfusion in the presence of 1 mmol/L of salicylic acid in the control, SOD, CAT-, and SOD plus CAT-treated groups. The sampling time was: 0–30, 60–90, 180–210, 270–300, and 570–600 sec, respectively, during reperfusion. Values are means ± SEM. Comparisons were made to the time-matched control values. *$P < 0.05$.

anion or $OH^{\cdot}$ are often overpowered by other larger free radical signals.

Recently, many laboratories have attempted to develop an alternate method to detect oxygen free radicals. A number of reports are now available demonstrating the presence of $OH^{\cdot}$ by HPLC. The pioneers in this field are probably Floyd *et al.* [38] and Grootveld and Halliwell [19], who developed the methodology for the $OH^{\cdot}$ detection by HPLC. Many laboratories [11–13], including our own [2, 10], have successfully adapted these methods and demonstrated the presence of $OH^{\cdot}$ in post-ischemic myocardium. Two methods are currently available, both of which use the electrochemical detection technique. However, the biochemical approaches are quite different; one uses a stable chemical trap, salicylic acid, whereas the other uses a spin trap, DMPO.

The success of the salicylic acid method depends on the formation of two stable compounds, 2,3- and 2,5-dihydroxybenzoic acid, upon the interaction of $OH^{\cdot}$ and salicylate [19, 38]. These hydroxylated products of benzoic acid are very stable and can produce electrical signals when analyzed by HPLC using an electrochemical detector. Using this technique, many investigators have demonstrated the presence of $OH^{\cdot}$ in ischemic/reperfused heart as mentioned earlier.

However, it has been pointed out recently that the cytochrome P450 system, which may be present in the heart, can also form 2,5-dihydroxybenzoic acid [39]. Nevertheless, the 2,3-dihydroxybenzoic

Table 1. Incidence and duration of reperfusion-induced ventricular fibrillation and ventricular tachycardia after 30 min of normothermic global ischemia followed by 30 min of reperfusion in the untreated and drug-treated groups

Groups	Incidence of VF (%)			Incidence of VT (%)	Duration of VF (sec)		Duration of VT (sec)	Duration of SR (sec)
	Sustained	Nonsustained	Total		Sustained	Nonsustained		
Untreated	83	8	92	100	1428 ± 32 n = 10	485 n = 1	187 ± 18 n = 12	43 ± 40 n = 12
SOD (50,000 IU/L)	75	8	83	83	1392 ± 26 n = 9	392 n = 1	200 ± 29 n = 10	216 ± 55 n = 12
CAT (50,000 IU/L)	67	17	83	92	1463 ± 18 n = 8	380 n = 2	159 ± 21 n = 11	248 ± 79 n = 12
SOD (50,000 IU/L) plus CAT (50,000 IU/L)	25*	8	33*	50*	1500 ± 12 n = 3	477 n = 1	98 ± 14* n = 6	1215 ± 178* n = 12

N = 12 in each group; n indicates how many of the 12 hearts were in VF, VT, and sinus rhythm (SR) during reperfusion. When n ≥ 3, values are given as means ± SEM.

* Significantly different from the untreated control value, $P < 0.05$. Note: because the distributions of the duration of VF, VT, and SR were not Gaussian, the Wilcoxon test (nonparametric distribution) was used.

acid signal is due to the presence of OH· only. This method is easily adaptable and does not require a great deal of skill; furthermore, it is inexpensive and can be set up within a short period of time. Most importantly, it is a highly sensitive method, capable of detecting OH· in the picomole range as compared to the nanomole detection capability by ESR.

Another HPLC technique which has also been employed recently for the detection of OH· uses DMPO to form DMPO-OH· spin adduct, similar to ESR. This spin adduct is then analyzed by HPLC using an electrochemical detector. Similar to the salicylate method, this technique is also inexpensive, simple, easily adaptable, and extremely sensitive (pmol OH· detection limit). However, because the DMPO-OH· adduct has a shorter half-life, the processing of the sample must be performed quickly, and requires special handling as described in Materials and Methods.

In this study we compared the results obtained by using the above three techniques under identical conditions. In all cases, a burst of OH· generation was observed during the early period of reperfusion, with a peak concentration at the third minute. Previous reports [7, 40] have documented a similar time course of free radical generation with a maximum peak between 2 and 4 min after the onset of reperfusion. The important issue, however, is that all three methods have yielded similar results. The OH· signals were also reduced by either SOD or CAT.

An interesting finding that emerged from our experiments is that OH· production occurred only in the fibrillating hearts, and not in the non-fibrillating hearts. Thus, if fibrillating and non-fibrillating hearts had not been differentiated, the results regarding the close relationship between free radical production and arrhythmias would have been completely misleading. It seems to be absolutely essential to dissociate the non-fibrillating hearts from the fibrillating hearts for interpretation of the results. The relative high variability of the results obtained by other researchers [6, 23, 41] concerning the genesis and role of the free radicals and their influence when challenged by different scavengers during reperfusion may be due, in fact, to the failure to make a differentiation between fibrillating and non-fibrillating heart populations. In view of the unique electrophysiology of the rat heart it is very important to concede that the results obtained from our study should not be directly extrapolated to other animal species, or humans, without confirmatory data.

In our study, the results from both ESR and HPLC methods indicate that although either SOD or CAT could reduce the OH· formation, when given prior to ischemia and during the reperfusion, neither had any effect on the reperfusion-induced VF and VT. On the other hand, when SOD was coadministered with CAT, a significant reduction was observed in the incidence of reperfusion-induced VF and VT. It is also known that certain spin traps such as DMPO may possess antiarrhythmic properties [42]. However, in ESR and HPLC studies, when DMPO was used for the detection of DMPO-OH· radical adduct in high concentrations, this spin trap

by itself could not reduce the reperfusion-induced VF and VT. It is probably due to the fact that a relatively higher concentration of DMPO (mM) is necessary for spin-trapping purposes, and it is likely that at this high concentration the antiarrhythmic property of DMPO is lost [16].

Animal studies have shown consistently that high-grade ventricular arrhythmias are associated with an abrupt release of coronary artery occlusion. In the clinical setting, the introduction of thrombolysis, together with the ever increasing use of surgical manipulations of the myocardium, have also stimulated interest in the consequences of reperfusion, and the possibility that the restoration of flow may not always be beneficial and may at times even become harmful. Baum *et al.* [43] and Elharrar and Zipes [44] suggested that the predominant mechanism of death after hospital discharge in patients not receiving reperfusion therapy was sudden cardiac death, and clinical autopsy data from the victims of VF had shown that acute coronary thrombosis or acute myocardial infarction occurred in only a few cases, thus suggesting that the cause of VF was probably reperfusion-induced arrhythmias. Despite a flurry of investigations, the mechanism of reperfusion-induced arrhythmias remains uncertain. However, evidence continues to accumulate suggesting a role of oxygen free radicals in the genesis of reperfusion-induced arrhythmias [42, 45, 46]. The results of our study indicate that free radical generation was observed only in the fibrillating hearts and the combination of SOD with CAT can reduce the incidence of VF and VT.

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