A novel electron paramagnetic resonance spin-probe technique demonstrates the relation between the production of hydroxyl radicals and ischemia–reperfusion injury

Masataka Eto a,b,*, Noriyoshi Kajihara a, Shigeki Morita a, Ryuji Tominaga a

a Department of Cardiovascular Surgery, Kyushu University, Fukuoka, Japan
b Department of Cardiovascular Surgery, University of Occupational and Environmental Health, Kitakyushu, Japan

Received 11 February 2010; received in revised form 27 July 2010; accepted 4 August 2010; Available online 17 September 2010

Abstract

Objective: Many previous studies have suggested an increase in hydroxyl radical (\(\cdot OH\)) production after myocardial ischemia–reperfusion; however, traditional techniques have not been able to conclusively prove this phenomenon. We investigated whether the production of \(\cdot OH\) was increased during myocardial reperfusion using a novel electron paramagnetic resonance (EPR) technique using an \(\cdot OH\)-specific spin probe. An \(\cdot OH\) scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), was used to examine the relationship between \(\cdot OH\) production and post-ischemic functional recovery or the degree of myocardial injury. Methods: We used an isolated rabbit-heart preparation perfused with support-rabbit blood, and the heart was reperfused after normothermic global ischemia. Heart samples were reacted with the EPR spin-trapping [6,7] for estimation of the amount of \(\cdot OH\) production in ischemia–reperfusion injury. We confirmed that \(\cdot OH\) production influenced cardiac function and myocardial ischemia–reperfusion injury.

Keywords: Free radicals; Antioxidants; Ischemia–reperfusion

1. Introduction

Many studies have shown that radical oxygen species (ROS) play an important role in myocardial reperfusion injury [1–3]. Hydroxyl radicals (\(\cdot OH\)) have the highest reactivity of all radical species and are thought to play a major role in causing cellular damage during ischemia–reperfusion. There have been difficulties, however, in measuring \(\cdot OH\) production because of its high reactivity [4].

There are various techniques such as high-performance liquid chromatography [5] or electron paramagnetic resonance spectroscopy (EPR) spin trapping [6,7] for estimation of the amount of \(\cdot OH\) production in ischemia–reperfusion injury. However, these methods can be unreliable for the measurement of metabolites, or use as an agent that acts as a radical scavenger.

Nitroxyl radicals react with \(\cdot OH\), which results in the loss of their EPR signal [8]. Using nitroxyl radicals as a probe, it is possible to measure the amount of \(\cdot OH\) produced from the clearance rate of the nitroxyl probe in an EPR spectrometer [9]. Several studies have used this technique, the EPR spin-probe technique, for the evaluation of \(\cdot OH\) [10,11]. However, myocardial ischemia–reperfusion has not been investigated using this novel technique.

In this study, we estimated the amount of \(\cdot OH\) production in myocardial ischemia–reperfusion by the EPR spin-probe technique. To the best of our knowledge, this is the first study to focus on the amount of \(\cdot OH\) production in myocardial ischemia–reperfusion using the EPR spin-probe technique. We investigated the relationship between \(\cdot OH\) production and post-ischemic recovery of cardiac function plus the degree of...
2. Materials and methods

2.1. Animals

Adult Japanese white rabbits weighing 3.0–3.5 kg were used in this study. All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, and were conducted in accordance with the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University.

2.2. Support rabbits and the cross-circulation system

The support rabbits were anesthetized by administration of 10 mg kg\(^{-1}\) sodium thiamylal to an ear vein, and were intubated with a tracheal tube connected to a mechanical ventilator (model SN-480-5, Shinano Industrial Co., Tokyo, Japan) with 100% oxygen. For further anesthesia, 1 mg kg\(^{-1}\) of vecuronium bromide and 70 \(\mu\)g kg\(^{-1}\) of fentanyl citrate were given intravenously. After heparinization (1500 U kg\(^{-1}\)) the common carotid artery and the external jugular vein were exposed. After heparinization (1500 U kg\(^{-1}\)), the heart was excised and quickly mounted onto the cross-circulation system. The blood draining from the system was returned to the jugular vein by another microtube pump. During the use of this system, anesthesia was maintained with a constant infusion of fentanyl citrate (200 \(\mu\)g h\(^{-1}\)) and vecuronium bromide (1.5 mg h\(^{-1}\)). In addition, 1000 U h\(^{-1}\) of sodium heparin was also infused. Arterial blood gases of the support rabbit were measured using a pH blood-gas analyzer (model IL-1304, Instrumentation Laboratory, Barcelona, Spain). The femoral artery pressure of the support rabbit was also continuously monitored.

2.3. Heart isolation

The hearts were anesthetized and ventilated mechanically using the same method as the support rabbits. After performing a median sternotomy, the thymus and pericardium were carefully removed and the heart and aortic arch were exposed. After heparinization (1500 U kg\(^{-1}\)), the heart was excised and quickly mounted onto the cross-circulation system.

The hearts were perfused at a pressure of 70 mm Hg and blood temperature was maintained at 38 °C. A latex balloon was placed inside the left ventricle and the volume was adjusted to a left ventricular end-diastolic pressure (LVEDP) of 10 mm Hg. Thereafter, the balloon volume remained fixed throughout the experiment. The superior and inferior vena cava and the pulmonary veins were closed. The pulmonary artery was cannulated to drain the coronary venous blood. All hearts were paced arterially at 250 beats min\(^{-1}\). After an initial 30-min stabilization period, the aorta was clamped and the hearts were subjected to 30 min of normothermic (38 °C) global ischemia and then reperfused for 60 min. Left ventricular developed pressure (LVDP), LVEDP, and the first derivative of the left ventricular pressure (dP/dt) were measured from the intraventricular balloon connected to a pressure transducer. These pressures were monitored on an oscillograph (Polygraph system 365-12, NEC San-ei Inc., Tokyo, Japan) that was digitized online with an analog-to-digital converter (MacLab system, AD Instruments Ltd., Dunedin North, New Zealand) and recorded on a digital computer (PowerBook 1400c, Apple Computer Inc., Cupertino, CA, USA).

2.4. EPR experiments

EPR measurements were performed at room temperature using an X-band (9.45 GHz) EPR spectrometer (JES-RE-1X; Jeol Ltd., Tokyo, Japan). The EPR settings were as follows: a microwave power of 10 mW, a range of external magnetic field of 20 mT, and a scan rate of 10 mT min\(^{-1}\).

2.5. Quantification of *OH in myocardial tissue

Frozen left ventricular myocardial samples were homogenized in 50 mM sodium phosphate buffer (pH 7.4) containing protease inhibitors. The homogenate was immediately reacted with 0.1 mM 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxyl-TEMPO), and its EPR spectra were recorded for up to 10 min at intervals of 20–25 s. The rate of signal decay was measured to demonstrate the level of *OH generation.

2.6. Protocols

2.6.1. Protocol 1

Heart samples were taken before ischemia, just after reperfusion, and 30 min and 60 min after reperfusion to determine the time course of the amount of intra-myocardial *OH production. Six hearts were used for each time point. Total of 24 hearts were used. Each sample was homogenized and reacted with hydroxyl-TEMPO and the rate of signal decay was measured.

2.6.2. Protocol 2

The hearts were divided into two groups: one group received 3 mg kg\(^{-1}\) of the hydroxyl radical scavenger MCI-186 (MCI group, \(n = 12\)) and the other group received saline alone (control group, \(n = 12\)). In the MCI group, 3 mg kg\(^{-1}\) of MCI-186 was infused over a 10-min period immediately after reperfusion. In the control group, saline was infused instead of MCI-186 at the same time period. Myocardial samples were collected just after and 60 min after reperfusion, and the rate of signal decay was measured to evaluate the effect of MCI-186 (Fig. 1(A)).

2.6.3. Protocol 3

To clarify the effects of MCI-186 on post-ischemic left ventricular function, we measured LVDP, dP/dt, and LVEDP in the two groups (six hearts in each group) before ischemia, and 15, 30, 45, and 60 min after reperfusion (Fig. 1(B)). Coronary sinus effluent was also collected to elucidate the role of *OH in ischemia–reperfusion and to determine the effects on post-ischemic cardiac function.
measure creatine kinase isoenzyme-MB (CK-MB) levels to evaluate myocardial cellular damage before ischemia, just after reperfusion, and 15 and 45 min after reperfusion Fig. 1(B).

2.7. Statistical analysis

All data are presented as the mean ± standard deviation. One-way analysis of variance was used to evaluate the mean differences in the time course of intramyocardial *OH productions in conjunction with a post hoc test with the Scheffe correction. The generation of *OH radicals was compared between the control and MCI groups by an unpaired t-test. Comparison between the control and MCI groups was performed by repeated measures of analysis of variance. Specific time points were tested by an unpaired t-test when the overall trend was significant. p Values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Conventional hemodynamic variables

There were no differences in blood hemoglobin, hematocrit, platelet levels, pH, or any other parameters between the two groups throughout the experimental period.

3.2. Protocol 1

The rate of EPR signal decay, which provides a measure of the total amount of hydroxyl radical production in the heart, was significantly higher just after reperfusion than pre-ischemic values (2.00 ± 0.77 × 10⁻² min vs 0.11 ± 0.02 × 10⁻² min, p < 0.0001). This finding indicates that the highest hydroxyl radical production occurred in the very early phase of reperfusion. The rate of signal decay was still higher 60 min after reperfusion than pre-ischemic values (Fig. 2).

3.3. Protocol 2

The rate of decay in the signal just after reperfusion in the MCI group was significantly lower compared with that in the control group (2.00 ± 0.77 × 10⁻² min vs 0.86 ± 0.14 × 10⁻² min, p = 0.004, Fig. 3). This suggests that MCI-186 inhibited *OH production in the myocardium just after reperfusion. As long as 60 min after reperfusion, *OH production was still observed and had not returned to the levels observed during the pre-ischemic period.

3.4. Protocol 3

LVDP and dP/dt were significantly higher in the MCI group than those in the control group 60 min after reperfusion (95 ± 9 mm Hg and 1843 ± 200 mm Hg s⁻¹ vs 60 ± 6 mm Hg

![Graph](https://example.com/graph1.png)

**Fig. 2.** Rate of signal decay for each reperfusion period. The rate of signal decay just after reperfusion was significantly greater compared with that in the other periods. The rate of signal decay remained higher at 60 min after reperfusion than that in the pre-ischemic period.

![Graph](https://example.com/graph2.png)

**Fig. 3.** Rate of signal decay in the 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI) and control groups just after and 60 min after reperfusion. The rate of signal decay in the MCI group was significantly lower compared with that in the control group at both reperfusion periods.
and 1182 ± 127 mm Hg s⁻¹, Figs. 4 and 5). LVEDP was lower in the MCI group than that in the control group 60 min after reperfusion (30 ± 13 mm Hg vs 52 ± 7 mm Hg, Fig. 6). These results indicated that left ventricular function was better preserved in the MCI group. The concentration of CK-MB was lower in the MCI group than that in the control group at both 15 min (12 ± 3 vs 21 ± 6 ng ml⁻¹) and 45 min (28 ± 7 vs 44 ± 9 ng ml⁻¹) after reperfusion (Fig. 7).

4. Discussion

In the present study, we clearly demonstrated that *OH production was significantly increased in the early phase of reperfusion. The major findings in the present study were as follows: (1) The production of *OH was increased during myocardial reperfusion as observed by a novel EPR technique with an *OH-specific spin probe. The rate of *OH signal decay was significantly increased just after reperfusion compared with that of pre-ischemia. (2) Administration of MCI-186 reduced the rate of *OH signal decay. (3) Cardiac function (left ventricular diastolic pressure and dP/dt) was significantly improved using MCI-186. (4) Hearts treated with MCI-186 showed a better recovery of function and reduced CK-MB leakage.
defined as the signal decay rate (Fig. 8 (C)). To interpret the peak height of the EPR spectra of hydroxyl-TEMPO were signals reduce at time (Fig. 8 (B)). Semilogarithmic plots of lines, and the peak signal intensity was stable with time (Fig. 8(A)). In the presence of a condition that generates EPR signals of hydroxyl-TEMPO were observed as three sharp OH was increased during myocardial reperfusion using the novel EPR spin-probe technique with an OH-specific spin probe. We used hydroxyl-TEMPO, a nitroxyl radical that is stable at room temperature, water soluble, and capable of entering cells. These properties of hydroxyl-TEMPO enable the detection of OH formed not only in the blood but also in the vascular, endothelial and myocardial tissue, as demonstrated previously by Ide et al.[10]. Hydroxyl-TEMPO is known to react with OH resulting in the loss of its EPR signal. EPR signals of hydroxyl-TEMPO were observed as three sharp lines, and the peak signal intensity was stable with time (Fig. 8(A)). In the presence of a condition that generates OH, EPR signals reduce at time (Fig. 8(B)). Semilogarithmic plots of the peak height of the EPR spectra of hydroxyl-TEMPO were defined as the signal decay rate (Fig. 8(C)). To interpret the rate of EPR signal decay accurately and to confirm its efficiency in the quantification of ROS, Ide performed in vitro validation studies on the metabolism of hydroxyl-TEMPO in the presence of the exogenous ROS-generating system. The EPR signal did not change under the superoxide-generating system or the H2O2-generating system. A dose—response study was also done by Ide. These properties have made it possible to detect OH formed in heart tissue [11—13].

4.1. Novel EPR spin-probe technique

In this study, we investigated whether the production of OH was increased during myocardial reperfusion using the novel EPR spin-probe technique with an OH-specific spin probe. We used hydroxyl-TEMPO, a nitroxyl radical that is stable at room temperature, water soluble, and capable of entering cells. These properties of hydroxyl-TEMPO enable the detection of OH formed not only in the blood but also in the vascular, endothelial and myocardial tissue, as demonstrated previously by Ide et al.[10]. Hydroxyl-TEMPO is known to react with OH resulting in the loss of its EPR signal. EPR signals of hydroxyl-TEMPO were observed as three sharp lines, and the peak signal intensity was stable with time (Fig. 8(A)). In the presence of a condition that generates OH, EPR signals reduce at time (Fig. 8(B)). Semilogarithmic plots of the peak height of the EPR spectra of hydroxyl-TEMPO were defined as the signal decay rate (Fig. 8(C)). To interpret the rate of EPR signal decay accurately and to confirm its efficiency in the quantification of ROS, Ide performed in vitro validation studies on the metabolism of hydroxyl-TEMPO in the presence of the exogenous ROS-generating system. The EPR signal did not change under the superoxide-generating system or the H2O2-generating system. A dose—response study was also done by Ide. These properties have made it possible to detect OH formed in heart tissue [11—13].

4.2. Classical EPR spin-trapping technique

Many studies have evaluated the amount of free radicals using the EPR spin-trapping technique [6,7,14]. Spin traps are chemical probes that react with free radicals and form a stable spin adduct. Blood samples that react with spin-trap agents have been quantified by EPR. However, these methodologies have the following limitations. The spin-trap agent most commonly used is 5,5-dimethyl-1-pyrroline N-oxide (DMPO). DMPO is unstable and its radical adduct has a short half-life. The DMPO—superoxide adduct, DMPO-OOH, breaks down into the DMPO-OH adduct, which is an OH-derived radical adduct. Some DMPO-OH signals could be the result of direct trapping of superoxides rather than hydroxyl radicals. Another spin-trap agent commonly used is N-tert-butyl-α-phenylnitrone (PBN). This compound is toxic in vivo and this toxicity might cause injury to the myocardium, thereby influencing myocardial function.

4.3. Superoxide dismutase and free-radical scavengers

Among the radical scavengers, superoxide dismutase (SOD) has been the most extensively investigated [15,16]. The reported effects of SOD on ischemia—reperfusion, however, have not been consistent [1]. The suggested reasons for variation in the results using SOD are: (1) SOD is too large a molecule to enter into the interstitial and/or the intracellular spaces, (2) the serum half-life of SOD is too short to react with superoxides, and (3) the superoxides that are scavenged by SOD are low in reactivity and less toxic. On the other hand, OH is thought to be one of the most toxic radicals because of its high reactivity.

4.4. Blood-perfused rabbit-heart model

With regard to the experimental model, we used the blood-perfused cross-circulation model developed in our laboratory. Many studies have used the Langendorff system with Krebs—Henseleit buffer for demonstrating the effect of the drugs. However, for evaluating cardiac function, there are significant differences between Krebs—Henseleit buffer and blood as mentioned previously [17]. Our blood-perfused model is well suited to investigate the process of ischemia—reperfusion, which involves numerous cellular components such as leukocytes and platelets, and our technique is established [18,19].

4.5. OH production during ischemia—reperfusion injury

Our finding of a greater recovery of cardiac function shown in the group treated with MCI-186 is in agreement with previous studies using OH scavengers [3,17,20]. MCI-186 is a developed antioxidant that has a low molecular weight, is highly diffusible, has a long half-life, and scavenges hydroxyl radicals [20—23]. However, previous studies did not examine the level of radicals in the myocardium, or whether free-radical scavengers truly scavenge free radicals. Thus, the relation between free-radical scavengers and cardiac function in those studies is unknown. We showed an OH scavenging effect of MCI-186 and its beneficial effect on cardiac function with measurements of OH production in the myocardium using our EPR spin-probe technique. In the current study, we showed that OH production was highest just after reperfusion. There have been conflicting studies regarding the timing of OH production in ischemia—reperfusion injury. Some studies have reported that peak OH production occurs in the late phase of the reperfusion period (1—3 h after reperfusion) [24], whereas others have suggested that peak production occurs just after reperfusion [25]. By measuring OH production with the spin-probe technique, we determined that OH production was increased just after reperfusion. Moreover, 60 min after reperfusion, we confirmed that OH production in the myocardium remained greater than that of the pre-ischemic period. Further studies are required to clarify the factors...
contributing to the production of \*OH during each phase of reperfusion.

4.6. Limitation of the study

Using the EPR spin-probe technique, we investigated the production of the hydroxyl radical after myocardial ischemia—reperfusion injury. However, this method is not standard at this time. Further study is needed to investigate the real time \*OH production in the myocardium after ischemia—reperfusion injury.

5. Conclusion

The present study provides a novel methodology using an EPR spin probe for quantifying \*OH production during ischemia—reperfusion injury. Using this method, we demonstrated that \*OH production was highest just after reperfusion and remained elevated 60 min after reperfusion. The level of \*OH production was related to the recovery of cardiac function and myocardial injury, indicating that \*OH plays a major role in ischemia—reperfusion injury.

References


