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DETECTION OF HYDROXYL FREE RADICALS IN THE REPERFUSED PRIMATE HEART

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Early reperfusion of an ischemic region can result in significant salvage of the area at risk. We show the presence of hydroxyl free radicals at the time of post ischemia reperfusion using electron paramagnetic resonance (EPR) spectroscopy in a macaque model. These free radicals may be formed as a result of reperfusion or may be an un-involved bystander. It is possible that they may be involved in reperfusion injury.

KEY WORDS: Ischemia; Reperfusion; Hydroxyl Free Radicals; Electron Paramagnetic Resonance.

INTRODUCTION

Preservation of the ischemic myocardium has become the "holy grail" of invasive cardiology and cardiac surgery. It has been well documented[1-4] that early reperfusion of an ischemic region of the myocardium can result in a significant salvage of the area at risk. Further attempts at preservation have led to the concept of "reperfusion injury" to account for the increase in tissue damage or myocardial dysfunction seen following revascularization of the ischemic tissue. Potential Etiologic agents for these events include prostaglandins, eicosanoids and oxygen free radicals.[5,6] Evidence supporting the role of oxygen free radicals (OFR) in this event have been

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indirect. Specifically, the beneficial effects of free radical scavengers or inhibitors of free radical generation on myocardial activity following ischemia and reperfusion have been cited to support the presence of OFR in the system.[7–9] Few investigators have produced direct evidence of OFR in the myocardial ischemia-reperfusion setting. Some have shown carbon centered radicals in the post reperfusion coronary effluent and inferred the presence of oxygen free radicals from this.[10–12] Animal models in which direct evidence of free radical production has been documented are limited to the rat and the rabbit and these too have been difficult to reproduce.

There is indirect evidence for the generation of oxygen free radicals in humans placed on cardio-pulmonary bypass machines.[13,14] The role of xanthine oxidase in the generation of free radicals in the human heart is controversial. Further, there is a lack of evidence for the presence of this enzyme in the human heart.[15–18] The leading theory for the formation of OFR in the ischemic myocardium requires the conversion of xanthine dehydrogenase to xanthine oxidase during ischemia with the formation of superoxide free radicals occurring at the time oxygen is reintroduced into the system. A chain reaction then ensues with the formation of hydrogen peroxide and results in the production of hydroxyl (·OH) free radicals causing lipid peroxidation and cell death. There are several other methods for the formation of oxygen free radicals such as through activated neutrophils, the electron transport chain and the arachidonic acid cascade.

MATERIALS AND METHODS

Surgical Protocol

Animals were cared for according to the guidelines of the American Physiological Society. Four rhesus monkeys (*Macaca mulatta*) of either sex were fasted for 24 hours before surgery. The animals were anesthetized using IM ketamine hydrochloride (Ketalar 10 mg/kg, Parke-Davis). Thereafter, anesthesia was maintained with repeated administrations using ketamine (5–10 mg/kg) and diazepam (valium, 1 mg) given intravenously as needed.

The animals were endotracheally intubated and ventilated using a Harvard NSH-34RH volume ventilator (Harvard Apparatus Company, South Natick, Massachusetts). Tidal volume used was 15 ml/kg at a rate of 10 breaths per minute. Supplemental oxygen was given and ventilation maintained to keep the oxygen saturation at not less than 95% and the pCO_2 at less than 45 mm Hg.

Hearts were excised through a median sternotomy following ligation of the superior and inferior venacavae. Catheters were introduced into the right ventricle through the pulmonary artery (PA) to collect blood from the coronary sinus drainage. The heart was perfused by way of a catheter in the aortic root with a modified Krebs-Henseleit room air oxygen tension solution warmed to 37°C at a pressure of 80–100 mm Hg at the aortic root. While the heart was being perfused, a snare was placed loosely around the left anterior descending artery (LAD) just distal to the origin of the circumflex artery and the accompanying coronary vein (CV) (Figure 1) was cannulated with a PE-50 catheter for the collection of specimens from the venous drainage of the area of ischemia.

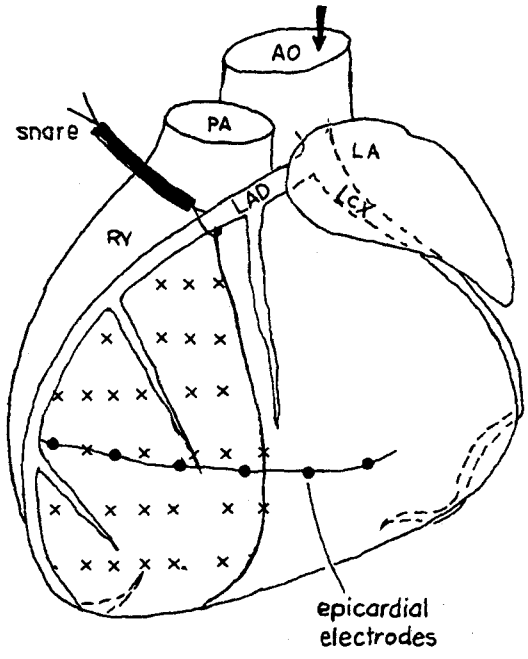

FIGURE 1 An anterolateral view of the heart for a schematic representation of the surgical procedure. The crossed area represents the anticipated area of infarct. The row of electrodes is laid over the infarct area as well as normal myocardium. Arrow shows the root of the aorta. AO = Aorta, LA = Left Atrium, LAD = Left Anterior Descending Coronary Artery, LCX = Left Circumflex Coronary Artery, LV = Left Ventricle, PA = Pulmonary Artery, RV = Right Ventricle.

Study Protocol

Baseline epicardial EKG readings were taken when the ST segment elevations due to electrode placement had fallen to a reasonable level of 5 mm or mv on the y-axis (0–30 minutes). Lidocaine (xylocaine 1 mg/kg, Astra) was prophylactically administered to reduce the possibility of ventricular fibrillation upon ligation. The LAD was then occluded following a 20 minute baseline period by tightening the snare. Evidence of a successful ligation includes (a) elevation of the ST segment, (b) cyanosis of the region at risk (perfusion bed) and (c) qualitative observation of an absence of blood flow in the LAD distal to the site of occlusion.

Two minutes before the release of the ligature, 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) (Sigma Chemicals, Saint Louis, MO, re-distilled) was infused at 100 mM with the perfusate. The snare was released following 20 minutes of ischemia. Samples were obtained from the PA and CV catheters at baseline and at 30 second intervals after reperfusion. These samples were frozen immediately in liquid nitrogen at −77°C and thawed individually just before reading their electron spin resonance (ESR) spectra. Samples were analyzed with ESR spectrometry set at 3,401G with a scan range of 100 Hz, a gain of 1.25×10^4, 10 Db power, f = 9.535 and a scan time of 16 minutes with a time constant of 2 seconds. Spectra were compared with reference values to identify the signals.

RESULTS

Epicardial Electrode ST Segment Changes

Following occlusion, the ST segments elevated significantly and remained so until reperfusion resulting in a significant drop in it followed by a gradual resolution towards the baseline readings. These changes were consistent with previous work done in our laboratory.[19,20]

Ventricular Arrhythmias

In some animals, ventricular arrhythmias – bigeminy and trigeminy in particular, were observed during the periods of early post occlusion and early post reperfusion. Lidocaine at 25 mg was administered to counter them. Additionally, bradycardia was observed in some macaques. Epinephrine (adrenaline) was applied topically on the heart to increase its sympathetic tone. Premature ventricular contractions or ectopic beats were seldom encountered and when seen did not warrant intervention (less than 6 premature beats per minute). Ventricular fibrillation and tachycardia were not observed upon reperfusion.

Electron Spin Resonance Data

Baseline signals from both the PA and CV effluents were consistent with background noise. At 30 seconds after reperfusion, the CV signal demonstrated the presence of ·OH free radicals in the effluent. This was not seen in the PA sample. The ·OH signal peaked in the CV fluid at one minute and rapidly declined there after. The PA effluent remained negative for the hydroxyl radical throughout the period of reperfusion. No other free radical signals were identified in any of the effluent samples. The DMPO-OH signal had the typical 1:2:2:1 pattern with $a_n = a_H = 14.9G$ (Figures 2 and 3).

DISCUSSION

Myocardial reperfusion injury has become clinically more important over the past few years. The benefits of early revascularization were initially shown in the cardiac surgery arena with bypass grafting of acute myocardial infarctions. Problems of reperfusion that were previously noted during the termination of cardiopulmonary bypass in routine open heart cases were more commonly seen in patients with acute myocardial infarcts. With the arrival of coronary angioplasty and effective thrombolytic agents, reperfusion problems including arrhythmias and ventricular dysfunction are being seen with increasing regularity.

The data reported here document the presence of hydroxyl free radicals at the time of post ischemia reperfusion. Whether the hydroxyl free radicals are the cause or the result of or an uninvolved bystander to reperfusion was not addressed in this study. Further studies need to be done to examine the role of an oxygen-free environment on the generation of OFR signals as well as the effect of free radical scavenger species on these signals and on myocardial performance.

It is important to note that the DMPO-OH signal observed by electron spin

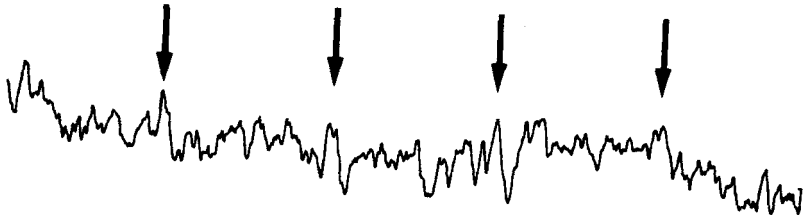

FIGURE 2 Typical background EPR signals with the 1:2:2:1 pattern (arrows) generated due to DMPO-OH from a non-ischemic specimen. Db = 10, Gain = $1.25 \times 10^4 \times 10$, f = 9.536, Scan Range = 100 Hz.

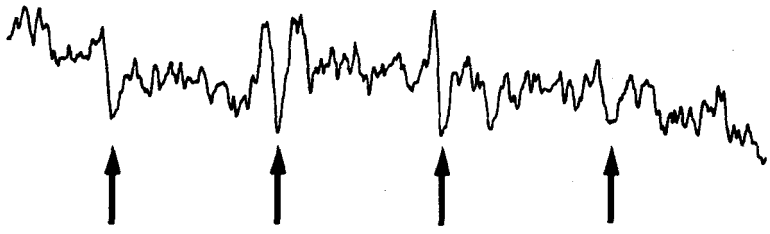

FIGURE 3 EPR.DMPO signals with the 1:2:2:1 pattern (arrows) generated from a ischemic-reperfused specimen. Db = 10, Gain = $1.25 \times 10^4 \times 10$, f = 9.536, Scan Range = 100 Hz, Scan Range = 100 Hz.

resonance may have arisen from the breakdown of DMPO-OOH that can be formed by the reaction of $\cdot O_2^-$ with DMPO or by the reaction of $\cdot$OH with DMPO as shown below:

$$\text{DMPO} + \cdot O_2^- \longrightarrow \text{DMPO-OOH} \longrightarrow \text{DMPO-OH}$$

$$\text{DMPO} + \cdot\text{OH} \longrightarrow \text{DMPO-OH}$$

Therefore, one could argue that this parallel is a potential limitation of this detection system. However, the $\cdot$OH radicals are formed from $\cdot O_2^-$ radicals (see below) and hence both pathways for the signal have a common ancestor.

There have been attempts to modify the effects of reperfusion injury using free radical scavengers. However, the source of these free radicals is unclear. Studies to date have failed to demonstrate the presence of xanthine oxidase in myocardial tissue in primates and other animals. In the absence of xanthine oxidase, other mechanisms for the generation of free radicals may be present. Recent studies have reported on the effects of ischemia, shock, endotoxin and other agents on the endothelium. If the endothelial cells are stimulated, they are able to initiate the activation of the platelet activating factor (PAF) as well as the complement cascade, specifically C_3, C_4 and C_5.

Either PAF or the complement can stimulate leukocytes to undergo an "oxygen burst" resulting in the production of superoxide radicals (O_2^-). Dismutaion of $\cdot O_2^-$ may then occur resulting in the production of hydrogen peroxide (H_2O_2). Hydroxyl radicals ($\cdot$OH) can be formed from a reaction involving $\cdot O_2^-$ and H_2O_2 through the superoxide driven Fenton reaction.

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + \cdot\text{OH} + OH^- \qquad \text{Fenton Reaction}$$

The ferrous iron (Fe^{++}) used in the above reaction can be generated by a reduction reaction involving ferric iron (Fe^{+++}) and the superoxide radical.

$$\cdot O_2^- + Fe^{+++} \longrightarrow Fe^{++} + O_2$$

This pathway does not require xanthine oxidase to be present in the myocardium while all agents involved are known to be present there. Leukocytes are readily available in the form of tissue macrophages and need not have time to migrate into the area.

Support exists for OFR as the key to myocardial reperfusion injury but definitive data to prove their role in reperfusion problems is lacking. Indirect evidence does not suffice when the agents being used may have other known and unknown physiologic effects. Additionally, the ESR work that has been done in this area is frequently suspect. As such, more disciplined study must continue before we conclude that free radicals are the key to reperfusion injury of the myocardium and other tissues.

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