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DETECTION OF FREE RADICALS AND CHOLESTEROL HYDROPEROXIDES IN BLOOD TAKEN FROM THE CORONARY SINUS OF MAN DURING PERCUTANEOUS TRANSLUMINAL CORONARY ANGIOPLASTY

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Patients undergoing percutaneous transluminal coronary angioplasty (PTCA) were investigated for the production of free radicals and cholesterol hydroperoxides during reperfusion. Fifteen patients were studied. Ischaemia during balloon inflation was assessed by serial coronary sinus lactate analysis (mean maximal increase in anterior descending artery diltation was 130%), and by the demonstration of reperfusion hyperaemia (mean increase of coronary sinus oxygen saturation 74%).

Free radicals were detected by electron spin resonance (ESR) spin trapping using the spin trap PBN (N-t-Butyl-α-phenylnitrone). Radical adducts were detected in up to 50% of samples taken during reperfusion after anterior descending lesion angioplasty. No radicals were detected in control samples or during the ischaemic phase. Radical detection was positively correlated with the change in coronary sinus lactate ($p < 0.025$).

Coronary sinus cholesterol hydroperoxide analysis did not show a significant increase over control during reperfusion, due in part to unexpectedly high pre angioplasty levels.

This study provides clear evidence for the production of a burst of free radicals and evidence for lipid peroxidation in the minutes following myocardial reperfusion during angioplasty. A relationship between the severity of the ischaemic insult and the detection of radical adducts has also been found.

KEY WORDS: Angioplasty, electron spin resonance, spin trapping, hydroperoxides, ischaemia, reperfusion.

ABBREVIATIONS: PTCA – percutaneous transluminal coronary angioplasty; PBN – N-t-Butyl-α-phenylnitrone; HPLC – High pressure liquid chromatography; CS – coronary sinus; LAD – left anterior descending; ESR – electron spin resonance.

INTRODUCTION

It has been proposed that free radical species produced during myocardial ischaemia/reperfusion cause myocardial damage. To date only indirect evidence of their production is available from human studies.[1-3]

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Free radical species have been detected in animal myocardium either during ischaemia,[4] or during reperfusion,[5,6] by tissue freeze clamping and subsequent electron spin resonance (ESR) spectroscopy. Garlick *et al.*[7] unequivocally demonstrated free radical production in rat myocardium during reperfusion using ESR spin trapping. Rao *et al.*[8] detected free radical species in blood from the coronary sinus of the dog during left anterior descending ischaemia. Further evidence for the production of free radicals during myocardial reperfusion was provided by Bolli and McCay in dogs using perfusion with a spin trap.[9] They showed significant radical generation during the first ten minutes of reperfusion and a continued radical production for up to 3 hours after 15 minutes of ischaemia.

Lipid hydroperoxides, the primary molecular products of lipid peroxidation, are produced by the reaction of free radicals with cholesterol or polyunsaturated fatty acids (PUFAs) followed by an interaction of the primary carbon centered radical with oxygen. While they can be produced by enzymic lipid oxidation (for example by lipoxygenases), lipid hydroperoxides have not been found in the blood of caucasian volunteers using HPLC-chemiluminescence.[10–12]

Percutaneous transluminal coronary angioplasty (PTCA) is in many respects an ideal model of myocardial ischaemia/reperfusion. While the duration of ischaemia is short (< 3 min) physiological loading conditions and myocardial temperature are maintained, there is minimal associated tissue trauma and the duration of ischaemia can be precisely controlled.

PATIENTS AND METHODS

The study population comprised 15 patients (13 male: 2 female, mean age 56 years) undergoing PTCA for chronic stable angina pectoris (Table I). PTCA was performed according to a standard protocol. All patients received papavertium 15 mg and scopolamine 0.3 mg pre-medication, and heparin 10,000 iu immediately prior to the procedure. The electrocardiogram was monitored continuously using three limb leads, and a hard copy of the six limb leads recorded during each inflation. Three balloon inflations were normally performed, the first lasting between 30 and 90 seconds (mean 62 s), the remaining two lasting between 90–180 s (means 101 s and 117 s respectively). The duration of inflation and the interval between inflations depended upon patient tolerance of the procedure and electrocardiograph changes. Immediately before the angioplasty and 8F venous sheath was placed percutaneously in the left subclavian vein. A 7F Gensini catheter was then advanced to the coronary sinus and the position confirmed by contrast injection.

Blood samples were taken before coronary cannulation (control), during the final planned (usually the third) inflation, immediately after balloon deflation, and at intervals up to 10 minutes after reperfusion. 2 ml of blood for free radical analysis was added to 1.5 ml of freshly prepared 12 mM PBN, inverted and frozen on dry ice. Samples were defrosted, added to 1 ml of toluene and vortex mixed for 20 s, centrifuged for 10 min at 3000 rpm and the organic layer removed and stored under liquid nitrogen. Extraction was performed in all cases within 24 hours of collection of blood. These samples were analysed on a Bruker 200D ESR spectrometer. Figure 1 shows a typical three line ESR spectrum of a PBN radical adduct obtained during PTCA reperfusion.

Samples for the detection of lipid hydroperoxides were treated as follows: all procedures were carried out at 4°C. 2 ml samples of coronary sinus blood were mixed

TABLE I
Patient characteristics

Sex	M-13/F-2
Age	42–71 yr (mean 56 yr)
Duration of History ($N=$)	
< 6 months	6
< 2 years	8
> 2 years	1
Previous Myocardial Infarction	3
Drugs ($N=$)	
Aspirin	10
Beta blocker	8
Calcium Antagonist	7
Nitrate	4
Maxepa	4
Diuretic	3
Ferrous Sulphate (oral)	1
Lesions Dilated During PTCA	
Left Anterior Descending	13
Circumflex	2
Diag AD	1

Legend: PTCS = Percutaneous Transluminal Coronary Angioplasty, Diag AD = Diagonal Branch of the Left Anterior Descending artery, M = Male; F = Female

with freshly prepared butylated-hydroxytoluene (10 μM), desferroxamine (20 μM) and heparin (100 iu), and centrifuged within 2 hours (15 min at 3000 rpm) to obtain plasma. 0.5 ml of plasma was acidified to pH 3–3.5 (with 0.2 M citric acid) and lipid hydroperoxides extracted with 6 ml of hexane. After centrifugation (5 min at

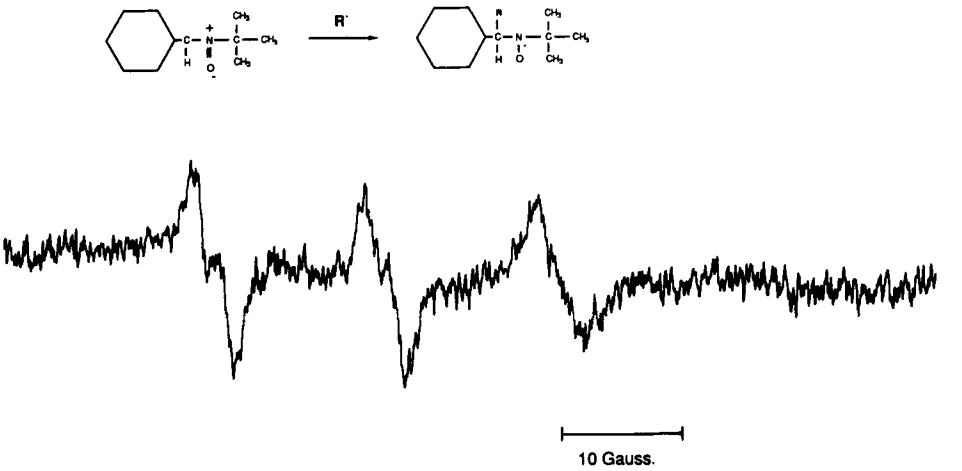

FIGURE 1 Figure showing ESR spectra of a PBN carbon centered radical adduct obtained from the blood of a patient undergoing PTCA-reperfusion. Insert shows a schematic representation of the PBN spin trapping reaction, where $R^{\cdot}$ is the reactive free radical. For a review of spin trapping in biological systems see.[17]

3000 rpm) the upper hexane phase was removed and dried under nitrogen. Any remaining hydroperoxides were then extracted by washing the sediment with a further 3 ml of hexane and repeating the above procedure. Using reverse phase HPLC the hydroperoxides were identified by chemiluminescence after reaction with isoluminal in the presence of microperoxidase. Using this method lipid hydroperoxides have not been detected in the plasma of normal volunteers (prepared and extracted as rapidly as possible) in this centre, the limit of detection for hydroperoxides is 0.05 μM in plasma.[12] Lipid peroxides were evaluated in only nine patients because of logistical problems in the early phase of the study. Lactate analysis was performed as follows: 2 ml of blood was added to Boehringer F1-EDTA preservative and placed on ice. Plasma separation was performed within 2 hours by centrifugation at 3000 rpm for 5 min. The plasma was stored at -20°C. Analysis was performed enzymatically using a Sigma diagnostic kit: pyruvate-lactate quantitative enzymatic method (340 nm). Oxygen saturation measurements were determined for each sample using a Radiometer OSM 2 analyser.

The correlation between frequency of radical detection and lactate production was evaluated using Spearman's rank correlation coefficient.

RESULTS

Seven patients developed chest pain, and eight developed ST changes consistent with myocardial ischaemia on a six-lead ECG. During LAD-angioplasty the lactate levels rose by a mean of 130% (SEM 16%) within seconds of balloon deflation and fell rapidly to normal within ten minutes. Oxygen saturation rose more slowly reaching a mean increase of 74% (SEM 18%) after 2 minutes of reperfusion. This fell to normal in most patients by 10 minutes, but remained elevated where contrast injections were performed at the time of the 10 minute sample (Figure 2). With circumflex and LAD diagonal PTCA, lactate levels rose by a mean of 32%.

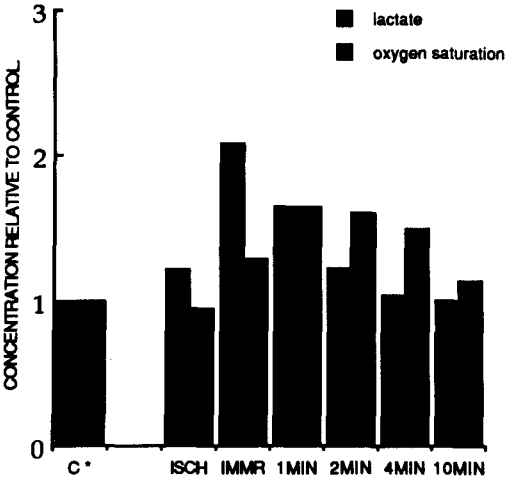

FIGURE 2 Relation coronary sinus lactate concentration and oxygen saturation during anterior descending ischaemia and reperfusion. CTL – control; ISC = ischaemia; 0–10 Time after balloon deflation (minutes). The control level for each patient has been assigned an arbitrary value of 1. Subsequent values for each patient are expressed as *x*/control, where *x* is the actual result.

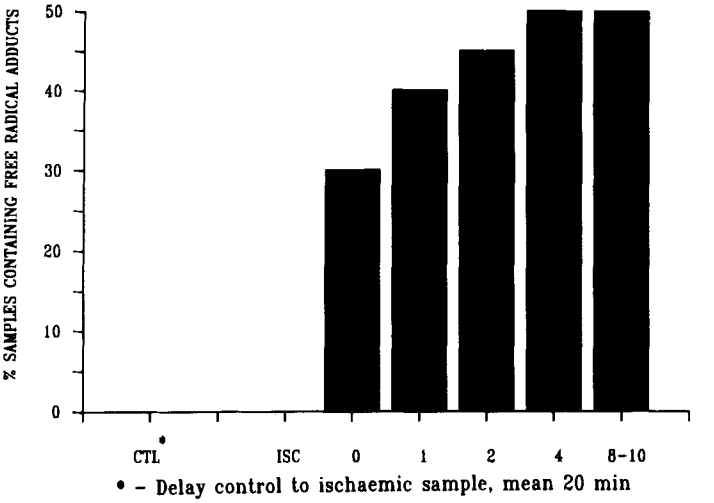

FIGURE 3 Frequency of free radical adduct detection in the coronary sinus blood during anterior descending PTCA. CTL = Control; ISC = ischaemia: 0–10 = time after balloon deflation (minutes).

ESR-Spin Trapping

In two patients artifactual signals, due to PBN contamination prevented interpretation of their results.

Of the remaining eleven patients undergoing LAD angioplasties, signals were detected during reperfusion in 10 (91%). More than one reperfusion sample was positive in eight patients. No signal was detected in any of the control samples (obtained prior to PTCA) or samples obtained during balloon inflation. Between 30 and 50% of reperfusion samples at each sampling period (0, 1, 2, 4, and 8–10 min) yielded a free radical signal (Figure 3). An example of the ESR spectra recorded from coronary sinus blood of one of the patients during PTCA is shown in Figure 4.

The hyperfine splittings for the radical adducts obtained were between 14.5 and 15.8 Gauss which is consistent with the expected splittings of a PBN trapped carbon centred radical in toluene.[13] In most spectra the beta splittings could not be accurately determined, where measurement of the secondary splitting was possible values of 1.5–1.6 Gauss were obtained.

As can be seen from Figure 4 signal intensity was small. Where radicals were detected in more than one reperfusion sample, these were not necessarily in successive samples. Thus it was not possible to determine total radical production curves for each patient. A relationship was, however, noted between the frequency of radical detection and the lactate production in these patients (Figure 5).

In both patients who had single vessel angioplasty to non LAD vessels free radicals were not detected either during control or reperfusion.

Lipid Hydroperoxides

Table II shows the levels of cholesterol hydroperoxides found in the nine patients during angioplasty. Hydroperoxide levels greater than 0.05 μM were found in one patient during ischaemia and on balloon deflation, but no hydroperoxides could be

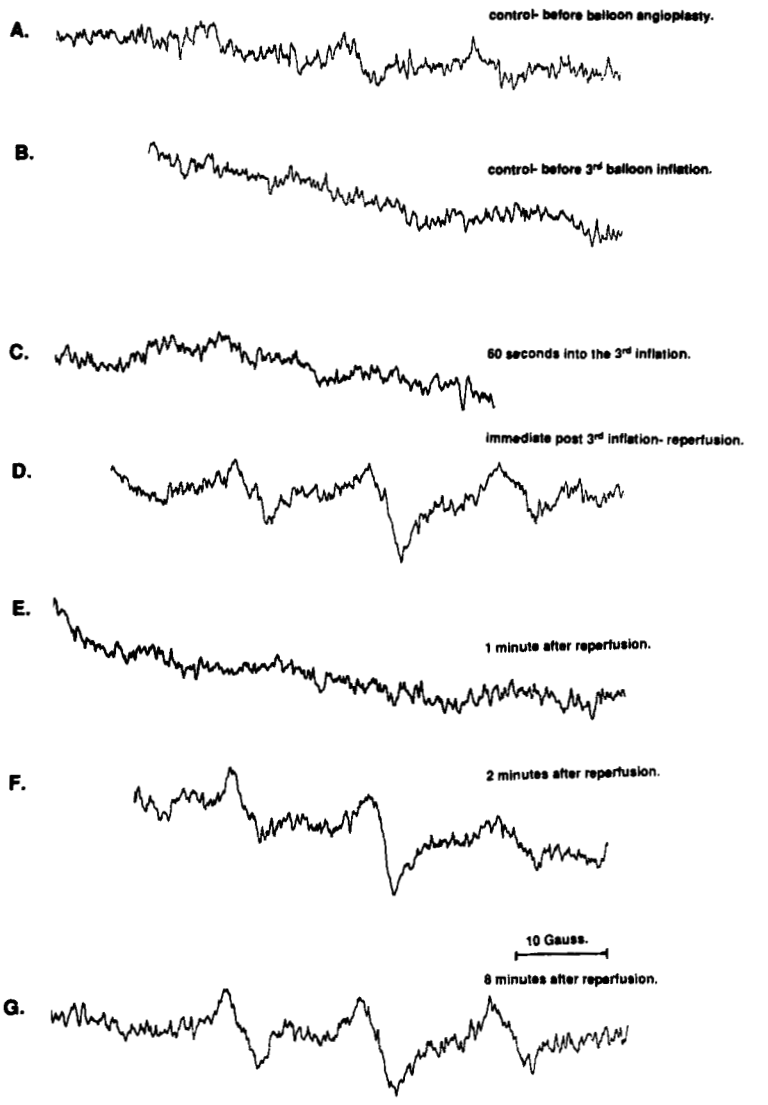

FIGURE 4 Electron spin resonance spectra of spin adducts extracted into toluene from coronary sinus blood of a patient during PTCA. machine 'noise' only is seen in (A) control, (B) immediately prior to 3rd inflation, (C) during the 3rd inflation, and (D) immediate reperfusion. Three line signals (approx 15G) are seen in (E) 1 min, (F) 2 min and (G) 8 min reperfusion samples. Spectrometer conditions were: 10 dB microwave power, 9.52 GHz microwave frequency, 100 KHz modulation frequency, 2 gauss modulation amplitude. Measurements were made on a Bruker ER 200 D ESR spectrometer fitted with a general purpose cavity and operating at a temperature of 213°K.

detected in the other eight patients at these times. During reperfusion hydroperoxides were detected in all but three patients.

It should be noted that control levels of cholesterol hydroperoxides, taken from the coronary sinus before balloon inflation, were abnormally high ($> 0.10\,\mu$M) in three of the patients studied.

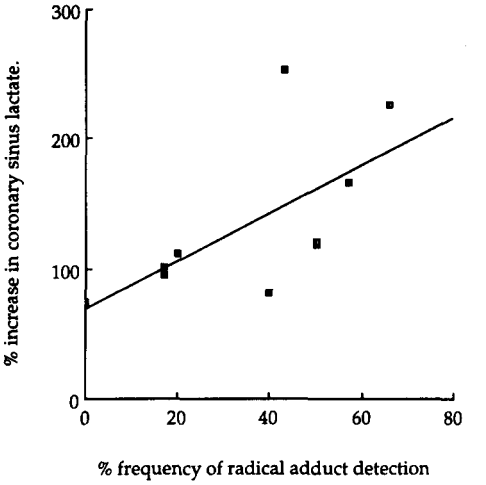

FIGURE 5 Figure showing relationship between frequency of free radical detection in reperfusion samples (percentages) and maximal percentage increase in coronary sinus lactate levels $-\rho = 0.699$, $p < 0.025$.

TABLE II
Coronary sinus cholesterol hydroperoxide concentrations in relation to mycardial ischaemia

Pat	CTL	ISC	0	1	2	4	8–10
1	–		–		–	0.22	
2	–	–	–	–	–	0.13	0.20
3	0.16				0.28	0.41	
4	0.10	–	–		–	0.11	–
5	0.58	–	–	–	0.13		
6	0.92	0.12	0.13	0.10	0.21	0.21	0.41
7	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–
9	0.07	–	–		–		–

Legend: CTL = Control, ISC – Ischaemia, 1–10 = Time after balloon deflation (minutes). Pat = patient (1–9), "–" = hydroperoxide level $< 0.05\,\mu M$.

DISCUSSION

This study shows that free radical species are directly detectable in the human coronary sinus, after only 2–3 minutes of coronary occlusion. The frequency of detection bears a direct relationship with the severity of the ischaemic insult (lactate production), and occurs only during reperfusion.

Cholesterol hydroperoxides are also found, but the relationship with the reperfusion is less clear in view of the relatively high control levels found in three patients.

The cellular source and the metabolic origins of these free radicals must now be determined. In particular whether they originate from damaged endothelium or from reoxygenated myocardium.

Three studies of lipid peroxidation during angioplasty have been published to date. One used the relatively non specific Yagi method[1] and documented a small increase in coronary sinus malonylaldehyde levels during the ischaemic washout phase (< 15 s)

after reperfusion. The second documented a small increase in the levels of malonyladehyde in the coronary sinus during reperfusion (peak levels detected at 5 minutes reperfusion).[3] The final paper[14] failed to show any significant change in conjugated dienes or malonylaldehyde during reperfusion in 10 patients undergoing LAD PTCA. The authors of this study pointed out that the total ischaemic time was short (162 s) and that only half of the patients showed an increase in coronary sinus lactate.

In this communication we directly demonstrate the formation of free radical species and subsequent production of cholesterol hydroperoxides. The spin trapping technique is highly specific as only free radical species are detected by this method. The technique is also highly sensitive, nanomolar quantities of radical adduct being detectable.[15]

The half-life of free radical species in body fluids is very short (< 1 s). Their detection in the coronary sinus in these patients demands some explanation. The radicals detected in the coronary sinus of these patients may have been produced indirectly from lipid hydroperoxides. Thus, primary radicals react with lipids giving rise to hydroperoxides, these molecules can reach the coronary sinus (being less reactive and having a greater diffusing capacity). If small amounts of metal ions, or low molecular weight metal ion complexes are available, these hydroperoxides may then give rise to a free radical flux at a considerable distance from the 'parent' radical reactions (the site of reperfusion injury).

These results obtained are entirely consistent with the findings of Bolli and McCay using a canine model[9] – no signals were observed in controls, only small signals obtained during ischaemia and large signals found during the first 10 minutes of reperfusion. Results from control dogs showed that reperfusion was the necessary condition for significant radical detection.

The less consistent results obtained from cholesterol hydroperoxide analysis is, perhaps, not surprising since any lipid membrane damage can cause peroxidation. It may well be that instrumentation of the arterial tree is responsible for the initial peak of cholesterol hydroperoxides, a possibility we are currently investigating. There have been reports of extensive cholesterol embolisation after catheterisation in patients with severe atherosclerosis.[16] Thus, using a technique as sensitive as HPLC-chemiluminescence one might anticipate the detection of circulating cholesterol hydroperoxides during cardiac catheterisation.

Nevertheless, there appears to be a late rise which would be consistent with reperfusion injury.

This study demonstrates that it is possible to measure free radical production in human subjects using ESR spin trapping. We further demonstrate a highly accurate method for measuring lipid peroxidation in man. This model is now being used to determine the time course of radical generation during reperfusion, and may well be the ideal model for future studies of reperfusion injury. Our preliminary results suggest that radical species appear only during reperfusion and that a correlation exists between the severity of the ischaemic insult and myocardial radical production.

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