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SEQUENTIAL OXIDATIVE DAMAGE, AND CHANGES IN IRON-BINDING AND IRON-OXIDISING PLASMA ANTIOXIDANTS DURING CARDIOPULMONARY BYPASS SURGERY

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Cardiopulmonary bypass patients undergoing heart valve replacement surgery appear to be under oxidative stress, when compared with normal healthy controls, by showing increased levels of protein and lipid damage. During bypass surgery two further episodes of oxidative stress occur. The first is seen when patients are placed on extracorporeal blood circulation and oxygenation which results in a rise in lipid peroxides and thiobarbituric acid-reactive substances. The second phase of oxidative stress occurs during reperfusion of the myocardium following removal of the aortic cross clamp. Coincident with evidence of increased oxidative damage to lipids during these latter phases of oxidative stress were decreases in plasma iron-binding and iron-oxidising antioxidant activities.

KEY WORDS: Cardiopulmonary bypass, reoxygenation injury, antioxidants, free radicals, lipid peroxidation, transferrin, caeruloplasmin.

1 INTRODUCTION

During open-heart surgery blood is removed from the systemic venous circulation and oxygenated before being returned to the arterial circulation. When blood is pumped extracorporeally in contact with non-physiological surfaces it undergoes significant biochemical changes. These include "activation" of neutrophils to produce superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and the stimulation of several regulatory cascades including complement activation.[1] Shear stresses imposed upon red blood cells can cause the release of haemoglobin which, in the presence of hydrogen peroxide can release reactive forms of iron.[2] Reactive iron can decompose hydrogen peroxide and lipid peroxides to form aggressive oxygen-centred free radicals such as hydroxyl ($\cdot OH$), alkoxyl ($RO\cdot$) and peroxyl ($RO_2^{\cdot}$). Thus, extracorporeal blood circulation imposes severe oxidative stress on blood lipids,[3] proteins[4] and metalloproteins.[2]

Removal of the aortic cross clamp during bypass ends a period of ischaemia and subjects the myocardium to reperfusion or reoxygenation injury. This reoxygenation

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injury is amplified by prooxidant biochemical changes resulting from extracorporeal oxygenation and circulation of blood, and from the effects of haemodilution described above.

In the present study we examine coronary sinus blood for evidence of oxidative damage incurred during these two distinct phases of oxidative stress, and study for the first time changes in the iron-binding and iron-oxidising antioxidant activities of plasma occurring during these phases.

2 MATERIALS AND METHODS

2,4-dinitophenyl hydrazine, butylated hydroxytoluene (BHT), and the total protein assay kit (Lowry method) were obtained from the Sigma Chemical Co. Ltd., Poole, Dorset. All other chemicals were of the highest purity available from Fisons Instruments, Loughborough, UK. Bovine brain phospholipids were prepared as previously described[5] and used as a liposomal suspension containing 5 mg/ml in 0.15 M NaCl.

2.1 Collection of Blood Samples

Blood samples were collected from a cannula in the coronary sinus from thirty-two patients undergoing aortic valve replacement surgery (with or without artery grafting procedures). Blood was taken into lithium heparin tubes, stored at 4°C and transported to the laboratory at the end of surgery for immediate separation by centrifugation at 3000 rpm for 6 minutes. Batches of plasma, stored at −20°C, were analysed as soon as possible and no later than 4 weeks after collection. Sampling points during surgery were: (A) chest open (pre-bypass control sample), (B) bypass started, (C) cross clamp off (end of ischaemic period), (D) bypass ended, (E) off bypass before administration of protamine. The bypass circuit was primed with 1.5 litres of Hartman's solution, and the patient cooled to 25°C. Fifteen patients received cold crystalloid cardioplegia and seventeen cold blood cardioplegia. Institutional Ethical Committee approval was obtained for this study. Venous blood samples were also taken from 18 normal healthy volunteers (age range 21–36, mean 28).

2.2 Protein Carbonyl Measurements

Protein carbonyl functions were measured by the method of Oliver *et al.*[6] with the following changes. 200 μl of plasma were placed in a new clean glass tube with 1.0 ml 2,4-dinitrophenyl hydrazine 10 mM in 2 M HCl. 200 μl of plasma and 1.0 ml of 2 M HCl were included as controls. Tube contents were incubated at 37°C for 90 min in a shaking waterbath. Proteins were precipitated by adding 1.0 ml trichloroacetic acid 28% w/v, and centrifuging at 10,000 g for 5 min. The supernatant was discarded and the pellet remaining washed with ethanol: ethyl acetate 1:1 v/v on 3 occasions, centrifuging between each wash. The final pellet was suspended in 1.0 ml of guanidinium chloride 6 M in 2 M HCl, and left for 60 min at room temperature. Test and blank solutions were read at 360 nm, and carbonyls calculated using a molar absorption coefficient of $2.1 \times 10^3 M^{-1} cm^{-1}$.

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2.3 Lipid Peroxide Values

Plasma lipid peroxides were determined using the iodometric method of El-Saadani *et al.*[7] which spectrophotometrically measures the ability of lipid peroxides to convert iodide (I^-) to iodine (I_2), which reacts with excess I^- to form the tri-iodine anion (I_3^-) absorbing at 365 nm. Into new clean plastic tubes were added; 10 μ BHT, 100 μl plasma, and 1 ml of CHOD-iodine colour reagent (BDH-Merck). Controls were setup by adding a reagent blank for the "CHOD-colour reagent" (all reagents minus iodine) to plasma. Reaction tubes were left in the dark at room temperature (25°C) for 30 min, and then read in a spectrophotometer at 365 nm. Peroxide values were calculated using a molar absorption coefficient for the tri-iodine anion of $2.46 \times 10^4 M^{-1} cm^{-1}$.

2.4 Thiobarbituric Acid Reactivity as Malondialdehyde Equivalents

Plasma thiobarbituric acid (TBA)-reactivity was calculated as malondialdehyde (MDA) using a technique based on methodological observations by Asakawa and Matsushita,[8] and Gutteridge and Quinlan.[9] Into new clean glass tubes were placed: 20 μl plasma, 30 μl ferric chloride 17.9 mM, 10 μl BHT 2 mM, 0.5 ml of 1% w/v TBA in 50 mM NaOH, and 0.5 ml of 0.1 M potassium phthalate buffer pH 3.5. The tubes were mixed, capped and heated at 100°C for 60 min. When cool the pink chromogen was extracted into 1.5 ml of butan-1-ol and the fluorescence measured at 553 nm with excitation at 532 nm. An internal MDA standard was prepared from tetraethoxypropane (Aldrich Chemicals), and calculations were based on this value and the molar absorption coefficient of the MDA-TBA_2 adduct ($1.54 \times 10^5 M^{-1} cm^{-1}$).

2.5 Iron-Binding Antioxidant Protection

Normal plasma transferrin is only one-third loaded with iron and retains a considerable iron-binding capacity. The ability of transferrin to bind iron has been made the basis of an assay for plasma antioxidant activity.[10,11] Briefly, 0.2 ml of phospholipid liposomes (5 mg/ml), 0.2 ml of sodium phosphate buffer 0.1 M, pH 7.4 and 10 μl of plasma were mixed in new clean glass tubes. The reaction was started by adding 20 μl ascorbic acid 7.5 mM, and incubating at 37°C for 20 min. 0.5 ml of HCl 25% (v/v) and 0.5 ml of 1% TBA (w/v) in 50 mM NaOH were added and tubes heated at 100°C for 15 min. When cool, the chromogen was extracted into 1.5 ml of butan-1-ol and read spectrophotometrically against appropriate blanks and controls. Percentage inhibition was calculated relative to the control sample (not containing plasma) to which 100% damage occurred.

2.6 Iron-Oxidising Antioxidant Protection

The major iron-oxidising (ferroxidase) protein of plasma is caeruloplasmin, and this has been made the basis of an antioxidant assay.[10,11] 0.2 ml phospholipid liposomes (5 mg/ml), 0.2 ml sodium phosphate buffer 0.2 M, pH 6.5, 20 μl ferric chloride 1 mM, and 10 μl of plasma were mixed in new clean glass tubes. The reaction was started by adding 30 μl ascorbic acid 0.125 mM and incubating at 37°C for 20 min. TBA reactivity was developed and percentage inhibition calculated as in 2.5 above.

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2.7 Non-Specific Chain-Breaking/Radical Stimulating Effects of Plasma

When normal plasma is devoid of its iron-binding and iron-oxidising activities, it stimulates iron-ascorbate driven phospholipid peroxidation.[11] 0.2 ml phospholipid liposomes (5 mg/ml) 0.2 ml of sodium phosphate buffer 0.1 M pH 7.4, 20 μl ferric chloride 1.0 mM and 10 μl plasma were mixed in new clean glass tubes. The reaction was started by adding 20 μl of ascorbic acid 7.5 mM and incubating at 37°C for 20 min. Development of TBA reactivity and calculations of % inhibition were performed as above.

3 RESULTS

Total plasma proteins were significantly lower in patients presenting for bypass (69.9 ± 4.1 mg/ml) than in normal healthy controls (87.6 ± 0.6 mg/ml) (Table 1). Due to haemodilution with Hartman's solution, bypass protein values fell significantly (Table 1) and remained low throughout the bypass procedure. The haemodilution substantially affected all biochemical parameters and to correct for this, values were expressed, where possible, relative to protein content.

Protein carbonyls tended to be higher in bypass patients (1.49 ± 0.12) than in normal healthy controls (1.30 ± 0.12 nmol/mg protein) but the difference was not statistically significant (Table 1).

Lipid peroxides and thiobarbituric acid-reactivity (TBAR) were measured in plasma as indices of lipid damage. Lipid peroxides were higher in the control bypass group (52 ± 7 nmol/g protein) compared with healthy controls (31 ± 2 nmol/g protein), and continued to rise during bypass reaching significant levels by the end of ischaemia when the aortic cross clamp was removed (Table 1). Consistent with increased levels of lipid peroxides were also rises in TBARS (Table 1), although increases in TBARS may not necessarily reflect lipid peroxidation.

Changes in specific and non-specific protein plasma antioxidant activities were measured in parallel with oxidative damage. Since these are expressed as percentage inhibitory activities, correction to plasma protein content is not necessary. The iron-binding and iron oxidising antioxidant activities of bypass plasma before haemodilution (81.64 ± 4.6% and 77.07 ± 3.6% respectively) were similar to those of normal healthy control subjects (83.63 ± 1.41% and 74.25 ± 3.25% respectively) (Table 2).

TABLE 1
Oxidative damage to plasma lipids and proteins during cardiopulmonary bypass surgery

Sampling point (number of patients)	Total plasma proteins mg/ml	Protein carbonyls nmol/mg protein	Lipid peroxides nmol/g protein	TBA-reactivity nmol/MDA/ g protein
Pre-bypass control (32)	69.9 ± 4.1+	1.49 ± 0.12	52 ± 7	44 ± 3+
Bypass on (28)	51.2 ± 4.2*	1.42 ± 0.11	69 ± 11	74 ± 23
Cross clamp off (32) (end of ischaemia)	51.2 ± 2.8*	1.36 ± 0.09	88 ± 11*	62 ± 5*
Bypass off (32)	52.5 ± 3.3*	1.39 ± 0.13	98 ± 10*	73 ± 6*
Prior to protamine (22)	54.0 ± 3.4*	1.37 ± 0.15	93 ± 11*	69 ± 6*

*indicates a significant change ($p < 0.05$) in value compared with the pre-bypass control.
+indicates a significant change in pre-bypass value compared with normal healthy controls.

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Haemodilution, however, significantly decreased the protein-specific antioxidant properties of plasma during bypass (Table 2).

When the plasma iron-binding antioxidant activities of patients on bypass were corrected back to their transferrin concentration (expressed as a percentage of the total proteins present), however, we see an overall loss of iron-binding antioxidant activity (Table 3). During bypass, transferrin increases its iron saturation,[12] and falls as a percentage of the total proteins present (Table 3).

When the iron-binding and iron-oxidising antioxidant properties of plasma are removed, plasma is observed to stimulate iron-driven phospholipid peroxidation.[11] This stimulation is, in part, attributable to protein thiol groups. Bypass patients as a group showed significantly lower stimulation of peroxidation (3.54 ± 2.3% Pre-bypass) when compared with normal healthy controls (12.75 ± 3.43%) consistent with their lower total plasma thiol groups (3.64 ± 0.43 nmol/mg protein) compared with normal healthy controls (6.55 ± 0.53 nmol/mg protein).

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4 DISCUSSION

Patients with heart disease undergoing routine valve replacement surgery, involving cardiopulmonary bypass, can experience cumulative oxidative stress at three different levels. Firstly, the cause of their heart disease may have originated from oxidative stress resulting from antioxidant depletion due to long term dietary habits,[13] and, or smoking together with a complex multi-factorial array of other-risk factors (reviewed in Gutteridge and Halliwell 1994).[14] The extracorporeal circulation and oxygenation of blood during bypass, exposes cells to non-physiological surfaces and shear stresses from suction pumps and cavitation. As a result, neutrophils are increased in the lungs[3] and are 'activated' to release superoxide and hydrogen peroxide, in the circulation. The most fragile red blood cells are prone to lyse and release haemoglobin. Hydrogen peroxide and haemoglobin can interact to generate pro-oxidant iron,[15] which in extreme cases can cause the iron overload of plasma transferrin.[12] Evidence for oxidative stress is frequently monitored by measuring damage to plasma lipids. Based on the measurement of thiobarbituric acid-reactive substances (TBARS), several groups have reported lipid peroxidation occurring either during bypass before cross clamping,[16] or after removal of the cross

TABLE 2
Plasma protein antioxidants during cardiopulmonary bypass measured as % inhibition or stimulation of phospholipid peroxidation

Sampling point (number of patients)	Iron-binding protection % Inhibition	Iron-oxidising protection % Inhibition	non-specific plasma 'antioxidant' effects % Stimulation
Pre-bypass control (32)	81.64 ± 4.6	77.07 ± 3.6	3.54 ± 2.3+
Bypass on (28)	59.60 ± 7.5*	58.66 ± 6.1*	3.47 ± 2.1
Cross clamp off (32) (end of ischaemia)	53.69 ± 7.1*	58.85 ± 5.0*	3.00 ± 2.4
Bypass off (32)	56.12 ± 7.7*	63.12 ± 5.6*	3.91 ± 1.9
Prior to protamine (22)	53.17 ± 9.75*	64.79 ± 6.2	2.69 ± 1.7

*indicates a significant change ($p < 0.05$) in valve compared with the pre-bypass control.
+indicates a significant change in pre-bypass valve compared with normal healthy controls.

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TABLE 3
Changes in transferrin during bypass surgery

Sampling point (number of patients)	Iron-binding antioxidant protection as % inhibition, corrected to pre-bypass transferrin (as a % of total proteins)	Transferrin g/l	Transferrin as a % of total proteins	Transferrin % saturation
Pre-bypass control (32)	81.6	2.02 ± 0.12+	3.00 ± 0.21	40.1 ± 3.9+
Bypass on (28)	72.4	1.21 ± 0.17*	2.47 ± 0.27	56.4 ± 6.1*
Cross clamp off (32) (end of ischaemia)	62.2	1.32 ± 0.13*	2.59 ± 0.21	52.3 ± 5.4
Bypass off (32)	63.5	1.38 ± 0.13*	2.65 ± 0.22	56.3 ± 5.9*
Prior to protamine (22)	57.4	1.48 ± 0.15*	2.78 ± 0.30	56.8 ± 7.9*
Normal healthy controls (12)	85.0 ± 1.28	2.91 ± 0.12	3.48 ± 0.23	20.4 ± 2.6

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clamp[4,17,18] as a result of reoxygenation injury. Our present data, based on iodometric measurement of lipid peroxides and supported by measurement of plasma TBARS shows that bypass causes two distinct phases of oxidative stress leading to increased molecular damage to lipids. In addition, the heart disease patients as a group, before undergoing bypass, have higher lipid peroxide concentrations suggestive of severe oxidative stress when compared with normal healthy controls.[19]

During cardiopulmonary bypass, formation of carbonyl residues on proteins were not significantly increased suggesting less damage or perhaps more efficient removal from the circulation. An important factor when considering *in vivo* oxidative damage to lipids and proteins, is the change in antioxidant activities of the patient. Several studies have shown that the lipid-soluble antioxidant α-tocopherol is depleted in plasma and tissue during human bypass[4,20,21] as well as that of ascorbic acid.[4] The authors, however, have argued that plasma molecules such as α-tocopherol and ascorbic acid are "secondary" antioxidants acting only once radicals are formed,[11] and that their important biological functions are protected by a hierarchy of "primary" antioxidants which exist to prevent radical formation (reviewed in Gutteridge and Halliwell 1988).[22] Plasma proteins such as transferrin and caeruloplasmin have the ability to bind and oxidise iron ions respectively, and prevent reactive and damaging free radicals forming in extracellular fluids by iron-driven reactions. Dramatic changes in iron chemistry have recently been shown to occur during bypass,[12] and since iron plays a pivotal role in oxygen radical formation (reviewed in Halliwell and Gutteridge 1990)[23] we have studied changes in primary plasma antioxidants protecting against iron-mediated damage. Our data show a significant loss of iron-binding and iron-oxidising antioxidant activity when expressed as a percentage of the total proteins present. When non-specific plasma antioxidant activities are measured (by fully saturating transferrin with iron and inhibiting the ferroxidase activity of caeruloplasmin), plasma is seen to stimulate iron-promoted lipid peroxidation.[11] This stimulation is, in part, due to iron-reducing molecules, including protein thiols, which are extremely low in cardiopulmonary bypass patients before surgery.[19] Thiols are known to decrease during thrombolysis[24] and tissue reoxygenation.[4] Most of the iron-reducing molecules present in plasma, such as protein thiols, are potent scavengers or organic and inorganic oxygen radicals, although our assay shows them as iron-reductants stimulating lipid peroxidation.

Our present study shows that patients with heart disease undergoing routine valve replacement surgery, are as a group oxidatively stressed. These patients then appear to receive two further oxidative insults during cardiopulmonary bypass. A protocol to manage iron release[25,26] and introduce appropriate antioxidant interventions before, during and after bypass surgery may be strongly indicated in cardiopulmonary bypass patients.

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