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Chelatable iron and copper can be released from extracorporeally circulated blood during cardiopulmonary bypass

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During cardiopulmonary bypass surgery blood is extracorporeally oxygenated and circulated before returning to the systemic arterial circulation. Blood undergoing extracorporeal dilution and circulation is exposed to non-physiological surfaces, which cause the activation of several regulatory cascades. Cells are also subjected to damaging shear stresses. Under such conditions neutrophils can be 'activated' to release reactive oxygen intermediates such as O_2^- and H_2O_2, and other cells can release proteolytic enzymes and metalloproteins. Collectively, these events can result in the release of micromolar quantities of redox active iron and copper. Bleomycin-detectable iron and phenanthroline-detectable copper were found in two out of four mock bypass experiments. However, there was no correlation between the presence of chelatable iron and copper and the activation of neutrophils measured as elastase

Bypass surgery; Oxygen radical; Bleomycin-detectable iron, Phenanthroline-detectable copper; Reoxygenation injury; Antioxidants; Elastase, Caeruloplasmin; Haemoglobin

1. INTRODUCTION

There is now convincing evidence that organic and inorganic oxygen radicals are transiently formed when ischaemic heart tissue is reoxygenated (for review see [1,2]). In many cases oxygen radicals, and other reduction intermediates of oxygen can be prevented, intercepted or destroyed by a variety of chemical interventions added before or during the early part of reoxygenation (reviewed in [3]). The origins of many of these reactive forms of oxygen remain uncertain, but much evidence suggests they arise multifactorially as a result of biochemical changes triggered during the period of ischaemia. Studies in several experimental animal models of ischaemia–reoxygenation support a key role for chelatable forms of iron [4–6] and possibly copper [7] in expressing, or amplifying tissue damage caused by reactive forms of oxygen. In support of this concept animals placed in iron-overload show increased reoxygenation injury [8] and treatment with iron chelators appears to reduce such damage [8,9].

The purpose of the present study was to investigate whether chelatable forms of iron or copper can be released during the extracorporeal circulation of blood, for cardiopulmonary bypass surgery, prior to the reoxygenation of heart and lung tissue.

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2. MATERIALS AND METHODS

Calf thymus DNA, bleomycin sulphate, 1,10-phenanthroline, bovine serum albumin and apotransferrin were from Sigma Chemical Co., Poole, UK. All other chemicals were of the highest grades available from Fisons Scientific Equipment, Loughborough, UK. Liposomes were prepared from bovine brain phospholipids [10]. Sheep anti-human elastase and peroxidase-conjugated sheep anti-human α_1-antitrypsin were from The Binding Site, Birmingham, UK.

2.1. *Collection of blood*

A healthy male volunteer was placed supine and a tourniquet positioned around the arm. A bleb of lignocaine (1%) was introduced intradermally above the antecubital vein which was subsequently cannulated (Abbocath 14G, Abbot Ltd., Sligo, Ireland). Four-hundred ml of blood was allowed to drain into a haematological transfer pack (R2074, Baxter Healthcare, Thetford, UK) containing 4,000 U sodium heparin. The blood was added immediately to Hartmann's solution (Fresenius HC Group, Basingstoke, UK) to produce a final circuit-priming volume of 1 litre (concentration of heparin 4 U/ml).

The control for the effects of neutrophil activation occurring through the contact of blood with polyvinylchloride, a further 30 ml of blood was withdrawn into a syringe containing heparin in the same proportions to the transfer pack and diluted to the same degree with Hartmann's solution. This sample was kept in a static loop (see below), and also served as a control for blood not subjected to shear stresses.

2.2. *Bypass procedure*

In all experiments an extracorporeal circuit was used of the following composition: 0.75 metres of 3/8 inch i.d. polyvinylchloride tubing (Linx Medical Systems, Formby, UK), a bubble oxygenator incorporating a heat exchanger (William Harvey H1700, CR Bard Inc., MA, USA), an occlusive roller pump (Sarns Inc., Ann Arbor, MI, USA), a water heater–cooler (Sarns Inc., Ann Arbor, MI, USA) and a temperature probe connected to a continuous display recorder (Dideco, Italy).

The static loop control consisted of 0.5 m 3/8 inch i.d. polyvinylchlo-

ride tubing connected end-to-end with a Connecta-TH 3-way tap connector (Viggo-Spectramed, Helsingburg, Sweden). After the circuits and the static loop (control) were primed, the pump was started and a flow of 3 l/min maintained for 2 h at 37°C, while filtered air was passed through the oxygenator at a rate of 2 l/min. The static loop was placed in a waterbath maintained at 37°C for 2 h Blood samples were taken from the circuit and static loop at zero time and after 60 and 120 min.

2.3. *Measurement of chelatable iron and copper*

Bleomycin-chelatable iron was determined as previously described [11] and quantitated by measuring the amount of malondialdehyde (MDA) released from DNA by bleomycin-iron and ascorbate. Iron in the biological sample that can bind to bleomycin and damage DNA is considered to be loosely-bound and redox active Phenanthroline-chelatable copper in biological samples was measured using a similar molecular recognition assay [12] specific for chelatable redox active copper

2.4. *Antioxidant assays measuring iron-binding and iron-oxidising protection*

2.4.1. Iron-binding

By adding high concentrations of ascorbate to inhibit the ferroxidase activity of caeruloplasmin, the iron-binding antioxidant activity of transferrin can be specifically measured in plasma [13]. Two detector molecules were used to assess radical damage and resulting antioxidant protection: (a) Phospholipid peroxidation using, 0.2 ml liposomes 5 mg/ml, 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.4, 10 μl of sample and 20 μl of ascorbate 7.5 mM in new clean plastic tubes, incubated at 37°C for 20 min; (b) DNA damage using, 0.2 ml of DNA 1 mg/ml, 20 μl of bleomycin sulphate 1.5 units/ml, 20 μl of sample, 0.1 ml of $MgCl_2$ 50 mM, 0.1 ml of 1.0 M Tris buffer, pH 7.4, and 50 μl of ascorbate 7.5 mM. The samples were incubated in new clean polypropylene tubes for 30 min at 37°C. Damage to both detector molecules was assessed by measuring the release of malondialdehyde (MDA) and its reaction with thiobarbituric acid (TBA) to form a pink chromogen absorbing at A_{532nm}.

2.4.2. Iron-oxidising

By saturating the plasma transferrin with ferric ions, and lowering the ascorbate concentration, the iron-oxidising (ferroxidase) antioxidant activity of plasma can be specifically measured [13], using mixed phospholipids as a detector molecule 0.2 ml of phospholipid liposomes 5 mg/ml, 0.1 ml of 0.2 M sodium phosphate buffer, pH 6.5, 10 μl of plasma and 20 μl of ferric chloride 1 mM are mixed in new clean plastic tubes and the reaction started by the addition of 30 μl of ascorbate 0.125 mM. Tube contents were incubated at 37°C for 20 min, and damage to phospholipids assessed by the development of TBA reactive material as described above. The results of typical experiments performed in duplicate are shown. The precision of the assays has been described in detail elsewhere (see references).

2.5. *Ferroxidase assays*

Total plasma ferroxidase assays were measured kinetically as the oxidation of a ferrous salt and its loading onto apotransferrin to form the pink diferric complex [14]. Ferroxidase activities not due to caeruloplasmin (ferroxidase I) were measured by performing the assays in the presence of azide.

2.6. *Circulating neutrophil elastase*

In plasma, free neutrophil elastase is immediately bound to α_1-antitrypsin. The circulating levels of this complex were measured by an ELISA technique. 96-well plates (Greiner, Germany) were coated with 100 μl of sheep anti-human elastase (15 μg/ml in a 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6, for 24 h at room temperature, and then washed with 0.1 M sodium phosphate in 0.15 M sodium chloride containing 0.1% v/v Tween 20 (PBST), and blocked with 1% w/v bovine serum albumin. Standards and samples were diluted 1.100 to 1:500 with PBST, added to the wells and incubated for 2 h at room temperature. The wells were washed with PBST and further incubated for 1 h with peroxidase-conjugated sheep anti-human α_1-antitrypsin (1:1,000). After further washes with PBST the substrate (0.5 mg/ml of *o*-phenylenediamine dihydrochloride in 0.05% w/v hydrogen peroxide, 0.1 M citric acid and 0.2 M disodium hydrogen phosphate) was added. The reaction was stopped after 20 min by adding 4 M sulphuric acid. The plates were read at an absorbance of 492 nm

3. RESULTS

Plasma was separated from diluted blood, undergoing extracorporeal oxygenation and circulation for up to 120 min, and examined for the presence of chelatable iron and copper ions. In two out of four mock bypass experiments (A and B) low micromolar concentrations of bleomycin-chelatable iron and phenanthroline-chelatable copper were detectable (Tables I and II). A static loop control sample which was not oxygenated or circulated did not contain chelatable iron or copper (Tables I and II). The plasma samples from experiments A and B, which showed the presence of chelatable iron and copper, lost antioxidant activities in assays designed to detect iron-binding and iron-oxidising protection, and usually stimulated damage to the DNA and phospholipid detector molecules (Tables I and II).

For measurement of ferroxidase activities suitable plasma was only available from experiment A (showing chelatable copper) and experiment D (not showing chelatable copper). Both samples showed a tendency for ferroxidase activities to fall slightly during bypass (A fell from 0.187 to 0.165, and D from 0.200 to 0.186 IU/ml) although insufficient data were available to assess the significance.

Plasma levels of elastase–α_1-antitrypsin complex increased significantly ($P = 0.05$) in samples A, C and D during the 120 min extracorporeal circulation of blood when compared with the static loop controls (Fig. 1), confirming neutrophil activation was occurring. However, in sample 'B' elastase levels did not appear to increase (Table II).

4. DISCUSSION

During cardiopulmonary bypass surgery, blood is removed from the systemic venous circulation and pumped through an extracorporeal oxygenator before returning to the systemic arterial circulation. Blood undergoing extracorporeal oxygenation is known to encounter abnormal stimuli, such as massive exposure to non-physiological surfaces and shear stresses [15]. Exposure of blood to any foreign surface activates clotting, fibrinolytic, complement and kallikrein/kinin cascades, and the shear stresses generated by blood pumps, suction systems and cavitation at the end of arterial cannulae lead to red blood cell lysis and the release of haemoglobin. A combination of such events can facilitate the release of proteolytic enzymes and the formation of reactive forms of oxygen, and some or all of

Table I

Release of bleomycin-detectable iron from blood during bypass pumping

	Plasma bleomycin-detectable iron (μmol/l)	Plasma iron-binding-dependent antioxidant activity (oxo-iron species) %Inhib (I); %Stim (S)	Plasma iron-binding-dependent antioxidant activity (lipid radicals) %Inhib (I); %Stim (S)
Control blood in static loop			
0 min	0	16% I	46% I
60 min	0	17% I	43% I
120 min	0	12% I	32% I
Bypass circulated blood			
Sample A 0	0	10% I	27% I
60	0.39	10% S	8% I
120	1.37	33% S	56% S
Sample B 0	0	21% I	30% I
60	0	9% I	6% S
120	0.21	4% S	33% S
Sample C 0	0	17% I	73% I
60	0	10% I	69% I
120	0	6% I	34% I
Sample D 0	0	10% I	73% I
60	0	11% I	59% I
120	0	8% I	57% I

these may contribute to several of the recognised adverse effects of bypass surgery, such as those occurring during the proposed widespread intravascular inflammatory response [16]. During bypass it is known that complement is activated [17], as was confirmed in our study (data not shown). In addition, extracorporeal ox-

Table II

Release of phenanthroline-detectable copper from blood during bypass pumping

	Plasma phenanthroline-detectable copper (μmol/l)	Plasma iron-oxidising dependent antioxidant activity (lipid radicals) %Inhib (I); %Stim (S)	Plasma elastase (ng/ml)
Control blood in static loop			
0 min	0	18% I	155
60 min	0	14% I	160
120 min	0	6% I	180
Bypass circulated blood			
Sample A 0	0	10% I	155
60	0.20	1% I	360
120	0.20	13% S	600
Sample B 0	0	2% I	125
60	0.10	3% S	125
120	0.20	6% S	130
Sample C 0	0	12% I	140
60	0	11% I	390
120	0	26% I	740
Sample D 0	0	39% I	135
60	0	21% I	285
120	0	31% I	510

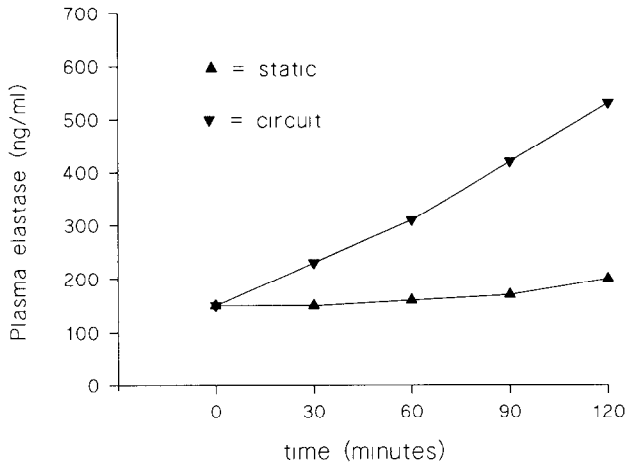

Fig. 1. Plasma elastase values (ng/ml) are shown at time points from '0' to 120 min in a typical bypass where blood was circulated extracorporeally (▼) or left in a static loop (▲).

ygenation and circulation in most cases leads to a significant activation of neutrophils leading to increased levels of plasma elastase.

Human plasma from normal healthy individuals does not contain chelatable forms of iron or copper [11,12]. Iron is tightly sequestered to the iron-transport protein transferrin, occupying only one third of its iron-binding potential, whereas copper exists predominantly as part of the structure of the plasma protein caeruloplasmin. The iron-binding capacity of transferrin gives it a considerable antioxidant potential to inhibit oxygen radical formation driven by iron salts [13]. This antioxidant activity is lost when the transferrin becomes fully iron saturated, as occurs in conditions of iron-overload. The origin of the chelatable iron present in two of our mock bypass experiments is not entirely clear, but may involve haemoglobin released from red blood cells by shear stresses. When hydrogen peroxide is released from activated neutrophils, in sufficient quantities it has the potential to facilitate the release of chelatable iron from haemoglobin [18].

Caeruloplasmin, the major copper-containing protein of human plasma, can catalyse the oxidation of ferrous ions to the ferric state (ferroxidase I activity) [14]. This activity, however, can be rapidly lost when caeruloplasmin is degraded. Proteolytic degradation of caeruloplasmin begins as soon as blood is withdrawn from the body, and accelerates when plasma is stored or mishandled [19], causing release of chelatable copper fragments which cause lipoprotein oxidation in vitro [19]. The resulting oxidised lipid–protein–copper complex has an azide non-inhibitable ferroxidase(II) activity [20]. Normal plasma contains around 17.0 μmol/l of copper and almost all of this is associated with caeruloplasmin. Red blood cells, on the other hand, contain some 10.0 μmol/kg of copper which is mostly associated with the enzyme superoxide dismutase. The origin of the chelatable copper detected in two of our bypass experiments is, at present, unclear.

Extracorporeal oxygenation and circulation of blood in Harmann's solution is here shown to lead to the release of chelatable iron and copper. Both iron and copper ions can amplify oxidative damage when tissue is reoxygenated. Attempts to limit the involvement of such metal complexes in oxidative damage, by intervening with antioxidants and metal chelators, is at present being considered.

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