Iron-binding antioxidant potential of plasma albumin

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1. The extracellular proteins caeruloplasmin and transferrin have important antioxidant properties by virtue of the fact that they inhibit iron-dependent free radical production, and ensuing damage to cells.

2. Albumin is a plasma protein which can loosely bind iron, but the redox activity of this iron has not been fully investigated.

3. The ability of albumin to bind iron and to prevent iron-dependent lipid peroxidation in vitro was investigated using liposomes and a rat brain homogenate system.

4. The iron-binding capacity of albumin was found to be substantial, and albumin inhibited lipid peroxidation in a concentration-dependent manner in both systems used.

5. This antioxidant property of albumin may be especially important in the plasma of babies born prematurely, in whom transferrin and caeruloplasmin concentrations are often very low, and in whom non-transferrin-bound iron has been detected in the plasma.

INTRODUCTION

Iron has two common valencies, iron(II) (ferrous) and iron(III) (ferric), the ferrous form being the most reactive in the generation of free radicals. Ferrous iron can participate in reactions leading to the production of the highly reactive hydroxyl radical (·OH) by the Fenton reaction, or alkoxyl and peroxyl radicals from the breakdown of lipid peroxides (reviewed by Gutteridge and Halliwell [1]). Two important plasma antioxidants are the iron-oxidizing protein caeruloplasmin and the iron-binding protein transferrin [1–5]. Transferrin tightly binds ferric iron so that it is unable to participate in radical-generating reactions [1–3, 5, 6]. In plasma from healthy adults, transferrin in only 20–30% saturated, therefore no iron ions should be available to catalyse free radical reactions [1, 3]. It has been demonstrated that albumin can bind iron although it is not regarded as a potent iron-chelating agent [7–12]. It therefore seems unlikely that albumin will bind iron in the presence of unsaturated transferrin. In abnormal conditions plasma may contain non-transferrin-bound iron (NTBI), and it is possible that this iron is associated with albumin.

The plasma of preterm babies has low concentrations of transferrin [13–17], which some studies show may be saturated with iron [14, 16, 17]. The bleomycin assay, a method developed by Gutteridge et al. [18] has been used to detect 'loosely bound' iron in the cord plasma of neonates [13, 14, 17]. A method for measuring NTBI has also been developed [19], and NTBI has been detected in the plasma of preterm babies over the first 14 days of life [20].

The ability of albumin to bind iron and prevent it from causing oxidative damage was investigated using liposomes and a rat brain homogenate system.

MATERIALS AND METHODS

Materials

Ferric nitrate nonahydrate, ascorbic acid, phosphatidyl choline, phosphatidyl ethanolamine, cholesterol, PBS, thiobarbituric acid, butylated hydroxytoluene, conalbumin and Bromocresol Green were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). BSA was obtained from Advanced Protein Products (Brierley Hill, West Midlands, U.K.) and disodium nitrilotriacetic acid was obtained from Aldrich (Gillingham, Dorset, U.K.). The kits for measuring caeruloplasmin and transferrin were obtained from The Binding Site (Birmingham, U.K.).

Sample collection and storage

Twenty-three venous blood samples were collected from 17 healthy adult volunteers aged between 20 and 45 years. Blood was taken into lithium heparin and immediately centrifuged at 800 g for 5 min. Plasma was removed and stored at −70°C until analysis.

Confirmation of the iron-binding activity of BSA

The iron-binding ability of albumin was confirmed by mixing BSA with a range of concentrations of...
iron, as ferric nitrate nonahydrate, at room temperature. Immediately after mixing, the BSA and iron underwent a two-step filtration process using 100 kDa and 20 kDa molecular weight cut-off Whatman ultracentrifuge filters [20]. Iron in the filtrate was measured using an HPLC method [20]. The experiment was repeated adding a low-affinity ligand disodium nitrilotriacetic acid (NTA) at 72 mmol/l to the BSA and ferric nitrate mixture. The final concentrations used were: BSA, 56.4 μmol/l (molecular weight, 66.5 kDa [21]) in PBS, pH 7.4; and ferric nitrate nonahydrate, up to 480 μmol/l.

**Measurement of lipid peroxidation**

The ability to prevent oxidation of liposomes and rat brain homogenate was used to evaluate the antioxidant potential of either BSA or plasma. Oxidation of the constituent lipid was assessed by measuring the production of thiobarbituric acid reactive substances (TBARS) after a 1 hour incubation at 37°C.

**Detection of TBARS**

TBARS were measured using a method based on that developed by Stocks and Dormandy [22, 23]. A 0.8 ml volume of the constituent lipid was mixed with 0.4 ml of 11.5 mmol/l HCl, then centrifuged at 1000 g for 5 min. A 0.8 ml portion of the resulting supernatant was added to 0.4 ml of 1% thiobarbituric acid, prepared in 50 mmol/l sodium hydroxide, and heated for 30 min at 80°C. The TBARS were extracted into an equal volume of butan-1-ol and detected by measuring the absorbance at 532 nm (CamSpec, M330). The presence of other substances that may absorb light at 532 nm is corrected for by subtracting the absorbance at 600 nm [22, 23].

**Liposome preparation and assay conditions**

Multilamellar vesicles were prepared in PBS, pH 7.4, by a method based on that outlined by New [24]. This involves the following stages: 12 mg of phosphatidyl choline, 5 mg of phosphatidyl ethanolamine and 4 mg of cholesterol were dissolved in 1 ml of organic solvent (2:1 chloroform:methanol); the lipids were dried down in a large round-bottomed flask, using a rotary evaporator, to obtain a thin, even film of lipid; they were then dispersed in 5 ml of PBS, pH 7.4, to form multilamellar vesicles. The liposomes were sonicated in a bath-type sonicator (sonomatic®, Jencoms) in order to produce small unilamellar vesicles.

Incubation of liposomes alone did not generate TBARS, indicating that the liposomes do not undergo significant auto-oxidation (results not shown). The production of TBARS was stimulated by incubating the liposomes with 100 μmol/l ascorbic acid. In all results, ascorbic acid-induced lipid oxidation is referred to as ‘maximum oxidation’.

Each investigation was carried out on three separate occasions using a freshly prepared liposome suspension or a freshly thawed aliquot of brain homogenate. Within a single experiment all incubations were carried out in triplicate.

**Modulators of lipid peroxidation in a liposome preparation**

The effects of BSA, ferric nitrate nonahydrate and conalbumin (apotransferrin) on lipidosome oxidation were assessed. All incubations were carried out at 37°C for 1 h before the measurement of TBARS.

Liposomes (780 μl), prepared as described, were incubated with 10 μl of BSA to give a final concentration of up to 22.6 μmol/l BSA in PBS, pH 7.4 [BSA (10 μl) 56.4 μmol/l] that had been pre-mixed at room temperature with ferric nitrate nonahydrate (30–480 μmol/l) was also incubated with 780 μl of liposomes, giving a final concentration of 0.71 μmol/l BSA. The experiment was repeated using BSA prepared in PBS at pH 4.0; this was incubated with ferric nitrate nonahydrate at room temperature for 15 min and the pH was adjusted to 7.4 before incubation with liposomes. The antioxidant activity of conalbumin was explored by incubating 780 μl of liposomes with 10 μl of conalbumin at final concentrations of 0.016–0.25 μmol/l (molecular weight, 80 kDa [25]). A final concentration of 100 μmol/l ascorbic acid was also included in all the incubation mixtures. The total volume of each incubation mixture was 800 μl. Maximum and minimum controls contained liposomes with 100 μmol/l ascorbic acid only or 0.75 μmol/l butylated hydroxytolulene (BHT) respectively.

Inhibition of ascorbic acid-induced lipid oxideation was calculated as follows:

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\text{Percentage inhibition} = \left\{1 - \frac{A_{532\ sample} - A_{532\ minimum\ control}}{A_{532\ maximum\ control} - A_{532\ minimum\ control}}\right\} \times 100
\]

**BSA as an inhibitor of lipid peroxidation in a brain homogenate assay**

The antioxidant activity of BSA was assessed by its ability to inhibit the peroxidation of rat brain homogenate using a modification of an assay described by Silvers et al. [26]. BSA (40 μl) was incubated at a range of final concentrations up to 88.5 μmol/l with 2 ml of ice-cold brain homogenate [1:20, (v/v) in PBS, pH 7.4] for 1 hour at 37°C. Controls containing brain homogenate alone or homogenate with 0.75 μmol/l of BHT were also incubated as the maximum and minimum controls respectively. TBARS were measured as described, and the extent to which brain homogenate auto-oxidation was
inhibited was calculated. The assay was also performed substituting conalbumin for BSA, at concentrations up to 0.858 µmol/l. Inhibition of rat brain homogenate oxidation was calculated.

Human plasma as an inhibitor of lipid peroxidation in a liposome preparation

Liposomes (780 µl; 4.1 mg/ml lipid in PBS) were incubated with 10 µl of plasma and 10 µl of 8 mmol/l ascorbic acid for 1 hour at 37°C. Controls for maximum oxidation (liposomes and ascorbic acid only) and minimum oxidation (liposomes and 0.75 µmol/l BHT only) were also incubated. TBARS were measured and the amount of inhibition of ascorbic acid-induced liposome oxidation was calculated.

Other measurements

The concentrations of caeruloplasmin and transferrin in the adult plasma were determined using immunoturbidimetric kits (The Binding Site; product codes NK045 and NK070 respectively) according to the manufacturer's recommendations. The plasma albumin concentration was measured using Bromocresol Green, which binds quantitatively with human albumin forming a blue–green complex with an absorbance maximum at 628 nm (Sigma Chemical Co.; product code 631). Each of these measurements were made on a Cobas Bio centrifugal analyser (Roche Products, Welwyn, Garden City, U.K.).

Statistics

The relationships between protein concentrations and inhibition of oxidation were investigated using analysis of variance. Linear regression was carried out in order to analyse the relationship between plasma albumin and inhibition of liposome oxidation.

RESULTS

The reproducibility of measurements of lipid peroxidation within a single experiment was partly determined by the extent of lipid peroxidation; where this was slight, variation tended to be higher. The coefficient of variation within experiments, where each incubation was carried out in triplicate, ranged from 0 to 34.6%, with a median of 2.7%. Graphs are presented showing mean values and SDs of three separate experiments.

Iron-binding activity of BSA

The iron-binding activity of BSA is illustrated in Fig. 1. In the absence of NTA (Fig. 1A) no iron was detectable in the filtrate from BSA previously mixed with ferric nitrate (80 µmol/l). This was true for all concentrations of ferric nitrate investigated, up to 480 µmol/l. It was thereby calculated that BSA could bind at least 8.5 moles of iron per mole. When 72 mmol/l NTA was added to the 56.4 µmol/l BSA/80 µmol/l ferric nitrate mixture a peak appeared at a retention time of 3.29 min (Fig. 1B), equivalent to 38.8 µmol/l iron. Some of the sites at which albumin binds iron evidently have a higher affinity for iron than has NTA.

Fig. 1. Chromatograms from HPLC analysis representing iron eluted at a retention time of 3.292 min. (A) 3.75 mg/ml BSA + 80 µmol/l Fe(NO₃)₃·9H₂O filtered without the chelator NTA, and (B) 3.75 mg/ml BSA + 80 µmol/l Fe(NO₃)₃·9H₂O filtered with NTA.

(A) (B)
As seen in Fig. 4, there was an increase in the inhibition of liposome oxidation with increasing conalbumin concentrations. A 75% inhibition of liposome oxidation was achieved at a conalbumin concentration of 0.25 pmol/l.

BSA as an inhibitor of lipid peroxidation in a brain homogenate assay

Figure 5 shows that BSA inhibited the oxidation of rat brain homogenate in a concentration-dependent manner. At a BSA concentration of 88.4 μmol/l, 96% inhibition of rat brain homogenate was achieved.

Figure 6 shows that conalbumin inhibits rat brain homogenate oxidation in a concentration-dependent manner. Conalbumin (0.74 μmol/l) inhibited the oxidation of rat brain homogenate completely.

DISCUSSION

These results demonstrate that BSA can bind iron in a manner which makes it unavailable for reactions leading to the generation of free radicals. BSA...
performed as a powerful antioxidant in both a liposome and a rat brain homogenate system. It was effective even at a molar ratio of iron to albumin of 8.5.

The binding coefficient of albumin for iron is not known, although it is considered to bind iron only rather loosely, unlike transferrin or ferritin. Evidently, some of the binding sites on albumin have a higher affinity for iron than has the low-affinity ligand NTA, which succeeded in removing only about 50% of albumin-bound iron.

Liposome oxidation was induced by the addition of ascorbic acid. The induction of liposome oxidation in this manner was believed to be due to ascorbic acid-dependent reduction of endogenous iron. Ferrous iron is more reactive than ferric iron at some endogenous iron in the liposome system, or iron already albumin bound, was not all bound in a redox inactive state.

BSA also inhibited oxidation in a rat brain homogenate assay. Oxidation of brain homogenate is largely prevented by apotransferrin [28], the antioxidant activity of which depends on its ability to bind iron [5, 6]. Oxidation of brain homogenate is also prevented by desferrioxamine [28], an electron-donating antioxidant as well as iron-chelating agent [29]. The ability of BSA to inhibit oxidation of the brain homogenate provides further support for the argument that albumin can bind iron in a form in which it cannot readily catalyse lipid peroxidation. BSA 88.4 µmol/l inhibited the oxidation of rat brain homogenate by 96% compared with 89% inhibition by only 0.49 µmol/l conalbumin. This indicates that conalbumin is about 180 times more effective as an antioxidant in this system. The mean concentrations of albumin and transferrin measured in the plasma from 23 healthy adults were 40.7 ± 5.87 mg/ml (612 µmol/l) and 1.98 ± 0.650 mg/ml (25 µmol/l) respectively. In human plasma, albumin may only be seven times less effective as an antioxidant compared with transferrin. Transferrin is considered by some authors to be one of the most important plasma antioxidants [30].

The ability of adult plasma to inhibit liposome oxidation showed a strong positive correlation with plasma albumin concentration. The albumin in the plasma may be capable of binding iron in the liposome system, thereby preventing it from taking part in redox reactions. Surprisingly, neither plasma transferrin nor plasma caeruloplasmin concentrations were correlated with inhibition of liposome oxidation. Under the particular conditions of this assay system, plasma albumin may be a more effective antioxidant than plasma transferrin or caeruloplasmin. Gutteridge [31] showed very clearly that the relative effectiveness of plasma transferrin and caeruloplasmin in preventing iron/ascorbate-stimulated oxidation of phospholipid was dependent on the concentrations of iron and ascorbic acid in the assay mixture.

**Fig. 6.** Inhibition of rat brain homogenate oxidation in response to increasing conalbumin concentrations. Data are means ± SDs of three independent experiments.

**Fig. 7.** Association between the inhibition of liposome oxidation and plasma albumin concentration. Percentage inhibition = 55.11 ± 5.1068 albumin; r = 0.629, P = 0.001.
Little work has previously been carried out on the effect of albumin on iron-mediated lipid oxidation. Albumin is known to bind copper tightly and prevent copper-mediated damage; it can also prevent hypochlorous acid (HOCl)-mediated damage and can bind non-esterified fatty acids and bilirubin [32]. Van der Heul et al. [11] demonstrated that plasma albumin was capable of binding iron, even in the presence of unsaturated transferrin, when plasma iron saturation was above 30%. Recent work by Hulea et al. [7] has shown that in a low-density lipoprotein mixture undergoing oxidation catalysed by 10 μmol/l (NH₄)₂Fe₂(SO₄)₃, 100 μmol/l (6.6 mg/ml) BSA could reduce the oxidation by 46%. Sellak et al. [12] showed that BSA was protective against iron-induced lysozyme inactivation, and concluded that this was because it was competing with lysozyme for iron binding. Gutteridge [31] has also shown albumin to have a modest inhibitory effect on the iron-stimulated oxidation of phospholipid, the presence of bilirubin, which, they concluded, was protective against iron-binding and iron-oxidising proteins. Gutteridge et al. [10] demonstrated that the ability of albumin to inhibit lipid peroxidation was greatly enhanced in the presence of bilirubin, which, they concluded, was important for the secure binding of iron to albumin. In vivo, bilirubin is tightly bound to albumin and is distributed throughout the circulation and extravascular space [33]. In vitro, bilirubin is a powerful antioxidant: it can scavenge peroxo radicals [34], form complexes with transition metals [35] and protect albumin-bound fatty acids against peroxidation [36]. Gopinathan et al. [37] have demonstrated a correlation between plasma antioxidant activity and bilirubin concentration in the plasma of term babies at 5 days postnatal age [37]. In view of bilirubin's important antioxidant functions, it is possible, as Hulea et al. [7] suggest, that the antioxidant activity of the albumin–bilirubin complex is more important than that of albumin alone [7]. Preterm babies often have high concentrations of plasma bilirubin because of low concentrations of glucuronyl transferase, which catalyses its breakdown [32].

The iron-binding property of albumin could be especially important in the preterm neonate, as potentially catalytic iron, measured as bleomycin-detectable iron and NTBI, has been detected in plasma and cord plasma from preterm neonates [13, 14, 17, 20]. Lackman et al. [38] showed that the plasma of babies who developed intracerebral haemorrhage had significantly higher iron saturation than the plasma of control babies; the average plasma saturation of these sicker babies was 54%. Van der Heul et al. [11] showed that at higher plasma iron saturation more iron is distributed over albumin. These findings, together with other studies which have shown that the plasma of some preterm babies has iron-saturated transferrin [14, 17], suggest that albumin could have an important antioxidant role in the plasma of preterm babies. Measurements of ‘free’ iron, which only takes transferrin into account, may underestimate the role of iron binding by other proteins and thereby overestimate the amount of redox active iron in the plasma.

This study has shown that BSA has considerable iron-binding capacity, which conferred significant antioxidant activity in lipid peroxidation systems in vitro. When the percentage iron saturation of transferrin is high, or in the presence of NTBI, the iron-binding potential of plasma albumin may make a significant contribution to plasma iron-binding activity and in this way contribute to plasma antioxidant activity. This may be particularly important in babies born prematurely.

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