BioMetals **16:** 113–123, 2003.

Copper-binding proteins in human erythrocytes: Searching for potential biomarkers of copper over-exposure

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Published on line: September 2002

Key words: biomarkers, copper-binding proteins, copper exposure, erythrocytes

Abstract

The recognition that copper is essential but also potentially toxic to humans has prompted the search for biomarkers of copper excess. The experimental approach followed here involves the isolation and subsequent characterization of copper-binding molecules (CuBP) from human erythrocytes. Incubation (0–60 min) of freshly obtained erythrocytes in the presence of increasing concentrations of copper (10–50 μM; as ^{64}Cu-histidine) led to time- and concentration-dependent uptake of the radioisotope. A near-maximal incorporation was attained after 20 min, with 45–55% of the radioactivity being recovered in $20,000 \times g$ hemolysate supernatants (S-20). ^{64}CuBP from S-20 were separated by size exclusion and metal-affinity chromatography. Most radioactivity loaded into a Sephadex G-75 column was recovered in association with molecules of MMr greater than 60 KDa (largely accounted for by hemoglobin; Hb). Only negligible amounts of radioactive Cu were associated with metallothionein. With further purification, the higher MMr ^{64}Cu-binding fractions were resolved by Sephadex G-200 into two major peaks. The cpm/μg protein ratios of the first peak (high MMr) were proportional to the concentrations of copper presented to the erythrocytes. The second one contained mostly Hb molecules. Proteins from the first peak were concentrated in an affinity chromatography mini-column, suited to trap CuBP. The higher-affinity CuBP were eluted as a single peak which comprised around 60% of the load. An SDS-PAGE analysis of such peak reveals the presence of three bands, of which two are non-hemoglobin Cu-binding proteins. The latter, whose identity remains to be established, had MMr of approximately 30 and 40 KDa, respectively. Preliminary data indicate that the two bands bind ^{64}Cu within a range of concentrations, relevant to those expected to occur during copper over-exposure conditions.

Introduction

Copper is an essential trace element whose biochemical functions relate mostly to its ability to participate as cofactor of enzymes involved in electron-transfer processes (Linder & Hazegh-Azam 1996; Linder *et al.* 1998). Under physiological conditions, copper status is controlled by well-established homeostatic mechanisms (Peña *et al.* 1999; Harris 2000). However, under certain environmental or genetically defined conditions, such mechanisms can be overridden leading to an accumulation of copper in organs such as liver and brain (Luza & Speisky 1996; Bremner 1998). The recognition that such accumulation generally represents a common denominator preceding copper-induced cytotoxicity has prompted the search for biomarkers of copper excess. Presently there are no specific and reliable indicators of excessive copper accumulation except invasive and risky procedures to biopsy target organs for copper analysis (Milne 1998; Olivares *et al.* 1998). Thus, a biomarker would be non- or less invasive than a biopsy, and should function as a sensitive index of copper accumulations even in the absence of significant functional damage.

Copper in plasma is largely found as bound to ceruloplasmin (Cp) (Wirth & Linder 1985; Linder *et al.*

1998; Harris 2000). Since copper in plasma is in a dynamic equilibrium with hepatic copper, the liver is considered as the main copper storage and Cp secretory organ. Thus attempts have been made to use circulating Cp as a possible index of copper accumulation (Olivares *et al.* 1998; see also Milne 1998). However, whether immunoreactive or enzymatically measured, plasma Cp has been shown not to be a reliable indicator of copper status since its levels vary with factors such as age, gender and hormone use (Fisher *et al.* 1990; Milne & Johnson 1993; Milne 1994). Also, Cp levels are swiftly increased in response to factors other than copper excess, such as exercise, various inflammatory and infectious conditions (Harris 1997; Beshgetoor & Hambidge 1998). On the other hand, attempts to use copper-dependent enzymes as biomarkers of copper excess have been limited by the fact that their *de novo* synthesis is not induced by copper supplementation (Baker *et al.* 1999; Zhang *et al.* 2000), and by the recognition that their activities respond to copper only when a copper-deficiency condition pre-exists (Klevay *et al.* 1984; Milne 1998; Prohaska *et al.* 1997; Jones *et al.* 1997). A classical example of the latter is superoxide dismutase (SOD) in copper-deficient erythrocytes, in which supplementation with copper induces, both *in vitro* and *in vivo*, the rapid activation of a preexisting pool of SOD aproprotein molecules (Okahata *et al.* 1980; DiSilvestro 1989; Steinkuhler *et al.* 1991). Therefore, enzymes such as SOD in red blood cells and cytochrome-c oxidase in platelet (Johnson *et al.* 1993; Milne 1998) have been considered as indices to assess copper-deficiency.

An alternative biomarker-seeking approach could involve the identification and subsequent characterization of molecules capable of binding copper. Unlike most copper-dependent enzymes, such molecules may, at least hypothetically, respond to copper levels sensitively by binding copper beyond those levels associated with the expression of maximal activity of the copper-dependent enzymes. Human erythrocytes may represent one interesting avenue in the search for such molecules, as these cells are abundant, easy to access, and in immediate and continuous contact with copper in plasma. Thus, in the present study we have investigated *in vitro* the ability of human erythrocytes to take up copper, and have assessed the intracellular fate of the accumulated metal, with emphasis in copper-binding molecules relevant to the search for a biomarker.

Materials and methods

Reagents

Radioactive copper (^{64}Cu) was supplied as copper sulfate (5–6 mCi mg Cu) by the Chilean Atomic Energy Commission (Santiago, Chile). Non-radioactive $CuSO_4 7H_2O$ and L-histidine were purchased from Merck (Chile). Sephadex G-75 and G-200 gels and Sepharose HiTrap® columns (Code N° 17-0409-01) were obtained from Amersham Pharmacia Biotech. All other chemicals used were of analytical grade and purchased from Sigma, except for those used in electrophoresis procedures that were purchased from BioRad.

Incubation of erythrocyte suspensions

Blood freshly obtained from healthy human volunteers was collected in heparinized tubes and immediately centrifuged ($2,500 \times g$, 4 °C, 5 min). The plasma and buff coat were removed and packed red cells washed twice with NaCl (0.9%). Subsequently, red cells were gently suspended in pH 7.0 incubation buffer composed of 3-[N-morpholino] propanesulphonic acid (MOPS) 10 mM, D(+)-glucose 0.2% and NaCl 150 mM. Suspensions (8.4% v/v) were incubated (37 °C in air) under continuous and gentle shaking conditions. Copper was added as a 1:10 $CuSO_4$ plus L-histidine molar mixture. Hemolysis was monitored during and at the end of the incubation by measuring the changes in OD_{410} of samples taken from the extracellular medium. Hemolysis values were never greater than 2% in copper-exposed cells, and lower than 1% in control preparations. Samples of suspensions taken at various times were centrifuged (as above) and the supernatant used to determine cpm in the extracellular medium. The latter was estimated by subtracting the intracellular volume (8.4% of the suspension volume) from the total suspension volume, and it was expressed as cpm in the total initial extracellular volume. Pelleted cells were thoroughly washed with incubation buffer and centrifuged, repeating the procedure twice. Washed cells were added one volume of distilled water containing the protease inhibitor phenylmethylsulfonyl fluoride (50 μg ml final concentration), and lysed by vortexing. The hemolysates were centrifuged ($20,000 \times g$, 4 °C, 15 min) and cpm determined in aliquots of the supernatants (S-20). The latter allowed estimating the radioactivity associated with the total intracellular medium, which was calculated assuming that the cytosolic volume comprises

64% of the total intracellular volume (Bush *et al.* 1955). All data were corrected for quenching and for ^{64}Cu radioactivity decay (half-life 12.8 h.

Chromatographic isolation of copper-binding proteins

Unless indicated otherwise, all separation steps were carried out at 4 °C using columns pre-equilibrated and eluted with 10 mM Tris buffer, pH 7.0. S-20 samples were loaded into a Sephadex G-75 column (1.2 × 60 cm; flow rate of 15–20 ml h). One ml fractions were collected and cpm and OD (280, 410 and 540 nm) measured. Selected G-75 fractions were subjected to a second chromatographic separation in a Shepadex G-200 column (1.6 × 60 cm; flow rate of 7,5–10 ml h). Selected G-200 fractions were pooled and subjected to metal chelate affinity chromatography (MCAC) using a pre-packed HiTrap® column. Before sample loading, the column was charged with 100 mM $CuSO_4$ and equilibrated with a buffer consisting of 20 mM Na_2HPO_4, 1 M NaCl, pH 7.2. Elution (flow rate of 1.5 ml min) was initially carried out with a buffer containing 20 mM Na_2HPO_4, 1 M NaCl, 10 mM imidazole, pH 7.2, and immediately after with a buffer containing 20 mM Na_2HPO_4, 1 M NaCl, 500 mM imidazole, pH 7.2. Fractions were collected for protein determination.

Polyacrylamide electrophoresis

Samples were subjected to SDS-PAGE according to the method of Laemmli (1970). The molecular mass (MMr) of purified Cu-binding proteins was estimated using high-range MMr standards (2.3–212 KDa; Winkler Ltda., Chile).

Protein determination

Proteins were assayed by the Bradford method (Bio-Rad Kit assay).

Results

Uptake of radiolabelled copper by human erythrocytes: Time-course and concentration-dependence studies

To investigate the occurrence of intra-erythrocytic proteins with Cu-binding properties, the ability of red blood cells to take up *in vitro* radiolabelled copper from the extracellular medium was addressed. As shown in Figure 1A, the addition of increasing concentrations of copper (10, 25 and 50 μM; as ^{64}Cu-histidine) to the cell suspensions was followed by a time- and concentration-dependent decrease of radioactivity from the extracellular medium. Cpm dropped rapidly during the early phase of the incubation. After 20 min, less than 20% of the radioactivity remained in the extracellular medium. The cpm continued to drop thereafter, but at a substantially lower rate. By 60 min, around 85% of the radioactivity had disappeared. Figure 1B illustrates the intracellular uptake of ^{64}Cu, as expressed by the cpm associated with cytosolic S-20 fractions obtained from cell suspensions of the above-referred experiments. ^{64}Cu accumulated intracellularly in a time- and concentration-dependent manner. Copper swiftly entered the cells during the first 20 min, approaching a plateau thereafter. At 60 min, the radioactivity associated with the S-20 fractions was between 45 and 55% of that disappeared from the extracellular medium.

Chromatographic purification of cytosolic Cu-binding proteins

In order to characterize the cytosolic molecules associated with ^{64}Cu, S-20 fractions obtained from erythrocytes incubated in the presence of increasing copper concentrations were subjected to successive procedures of size exclusion gel filtration chromatography. The radioactivity elution profile of S-20 samples loaded into a Sephadex G-75 column is shown in Figure 2A. The ^{64}Cu-elution pattern suggests the existence of two major radioactivity-overlapping regions. A significant part of the loaded radioactivity eluted always close to or within the void volume of the column (exclusion limit $\sim$ 80 KDa). The other major radioactivity region corresponded to fractions where typically elute molecules whose MMr fall approximately within the 10–64 KDa range. As indicated in the figure, one of such molecules, presenting the greatest concentration in the S-20 loaded samples, corresponded to hemoglobin (64 KDa). From the radioactivity profiles, it is apparent that after loading the G-75 columns with equal amounts of S-20 proteins, the cpm that eluted near the void volume fractions maintained a direct relationship with the concentration of copper at which the erythrocytes had been exposed to. The radioactivity recovered within the 5–10 KDa region, in which a MT standard characteristically elute, was essentially

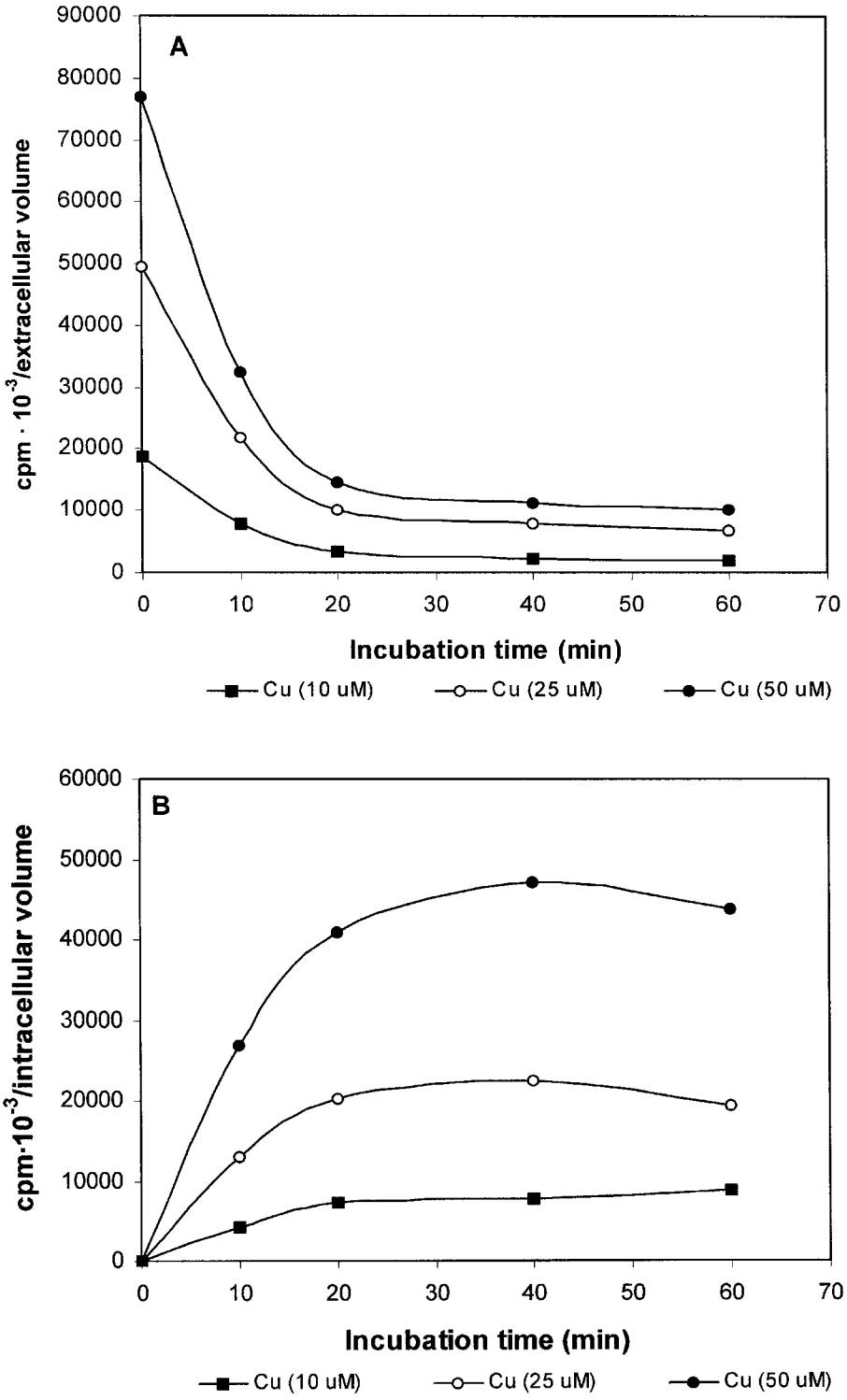

Fig. 1. Changes in radioactivity from the extracellular and intracellular medium of an erythrocyte suspension incubated in the presence of ^{64}Cu. Erythrocytes were incubated (0–60 min) in the presence of 10 (■), 25 (◯) and 50 μM (●) ^{64}Cu as $CuSO_4$. Cpm were determined in samples from extracellular (A) and intracellular (B) medium as described in methods. Data are expressed as cpm 10^{-3} per total extracellular or intracellular volume of incubation.

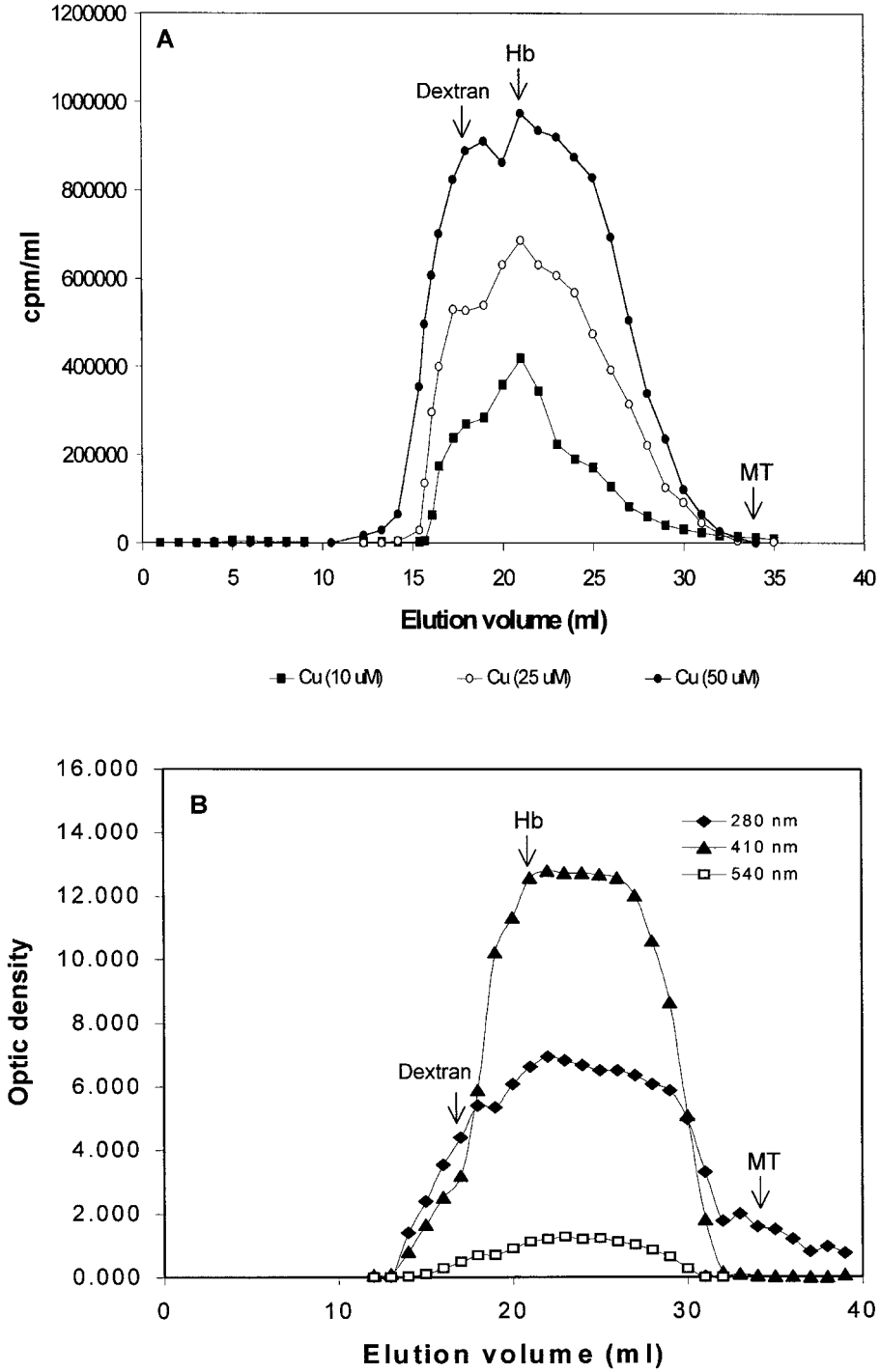

Fig. 2. Sephadex G-75 chromatography of S-20 cytosolic preparations: ^{64}Cu radioactivity and optic density elution profiles. S-20 supernatants, obtained from erythrocytes incubated (60 min) in the presence of ^{64}Cu, were loaded into a Sephadex G-75 column. Fractions (1 ml each) were collected and monitored for their cpm and optical density. Arrows refer to the volume of elution of standards of dextran, hemoglobin (Hb), and metallothionein (MT) applied to the column. Part A corresponds to the ^{64}Cu radioactivity profile of S-20 samples obtained after incubation of erythrocytes with 10 (■), 25 (○) or 50 μM (●) ^{64}Cu. Part B corresponds to the OD profile at 280 nm (♦), 410 nm (▲) and 540 nm (□) of S-20 samples obtained after incubation with 50 μM ^{64}Cu.

negligible and showed no correlation with the loaded ^{64}Cu. Figure 2B depicts the OD profile of fractions corresponding to the 50 μM ^{64}Cu S-20 samples whose radioactivity was depicted in Figure 2A. Standards of Hb elute with a maximal OD_{410} around fraction number 21. Since the S-20 profile shows also maximal OD_{410} at the 21 fraction, it is likely that a large part of the cpm associated with the non-void volume fractions be ^{64}Cu bound to hemoglobin. However, since Hb is known to non-specifically bind copper, we focused on further purifying those fractions containing Cu-binding proteins which, exhibiting MMr higher than Hb, presented also the highest cpm/OD_{280} ratio (generally, fractions 14–18). Thus, a pool of such fractions, obtained after incubating erythrocytes with ^{64}Cu (50 μM) was subsequently subjected to a Sephadex G-200 column. Radioactivity and protein elution profiles of the latter are depicted in Figures 3A & 3B. Both figures show two well-separated peaks, whether these are defined in terms of cpm or protein content. Based on the G-200 column calibration profile, the lower MMr peak corresponded to hemoglobin molecules. In turn, the higher MMr peak would comprise molecules eluted within the void volume of the column, which theoretically corresponded to proteins with a MMr near or greater than the gel exclusion limit (200 KDa). A rough estimate of the area under the lower MMr peak reveals that while such peak concentrates the larger amount of eluted proteins (near 5-fold higher), it accounts only for around 36% of the total eluted radioactivity. Thus, the erythrocyte cytosol contained high MMr proteins, which relative to Hb have a greater ^{64}Cu-binding efficiency (namely higher cpm/μg protein). However, not all the proteins associated with the higher MMr peak are necessarily Cu-binding molecules. Thus, in order to achieve a further purification of the Cu-binding proteins, the peak fractions were pooled and subjected to metal chelate affinity chromatography. This procedure allows the concentration of high affinity Cu-binding proteins and their separation from low-to-medium affinity Cu-binding proteins. The latter are either not trapped by the column or can be differentially eluted with low concentrations of imidazole. Figure 4 shows the protein profile of fractions eluted from a HiTrap® column loaded with the higher MMr Sephadex G-200 peak. A minor peak (fractions 1 to 7) was characteristically seen through the column loading, representing proteins that are not retained by the column, as they exhibit no Cu-binding properties. Low-to-medium Cu-binding affinity ligands were removed by elution with 10 mM imidazole (fractions 8–12). In turn, a clear and sharp peak was eluted when 500 mM imidazole was applied (fraction 14), representing around 60% of the protein load.

Electrophoresis of high affinity Cu-binding proteins

Proteins present in a pool of the higher MMr fractions eluted from a Sephadex G-200 column, and those obtained after passage through MCAC were subjected to SDS-PAGE (Figure 5). Lanes 2 and 3, corresponding to a sample of the G-200 peak, show an electrophoretic pattern that contains around a dozen bands. Many of these bands are also present in those fractions corresponding to proteins not retained by the HiTrap® column (lanes 4 and 5). However, when the 500 mM imidazole eluted peak was applied, only 3 bands were observed (lanes 7 and 8). Of these, the highest MMr band co-migrated with a standard of human Hb, had an estimated MMr close to 64 KDa, and featured absorption in the 410–420 nm region (not shown); thus, suggesting that such band would correspond to Hb. If such was the case, the presence of Hb molecules in the gel needs to be interpreted as an indication that the electrophoretic conditions employed by us were not stringent enough to denature all Hb molecules loaded into the gel. On the other hand, the concentration and running conditions of our gels precluded detection of-peptides with a MMr lower than 20 KDa (such as globin chains from Hb; MMr close to 16 kDa). Finally, the two remaining bands, whose MMr were estimated as 30 and 40 KDa, respectively, appear highly concentrated relative to those bands featured by both, the G-200 peak and those fractions not retained by the MCAC column.

Discussion

Studies conducted almost half a century ago by Bush *et al.* (1955 & 1956) were the first to demonstrate the ability of copper to enter the human erythrocyte. They observed that following the administration of a single oral dose of ^{64}Cu to normal individuals, radiolabelled copper levels increased in plasma and in erythrocytes in a parallel manner and roughly to the same extent. The present studies, conducted *in vitro*, corroborate the ability of human erythrocytes to take up ^{64}copper from the extracellular medium. In addition, they describe such uptake as a time- and concentration-dependent process, and demonstrate that the intracellular recovery of the radiolabelled is directly proportional to the concentration of

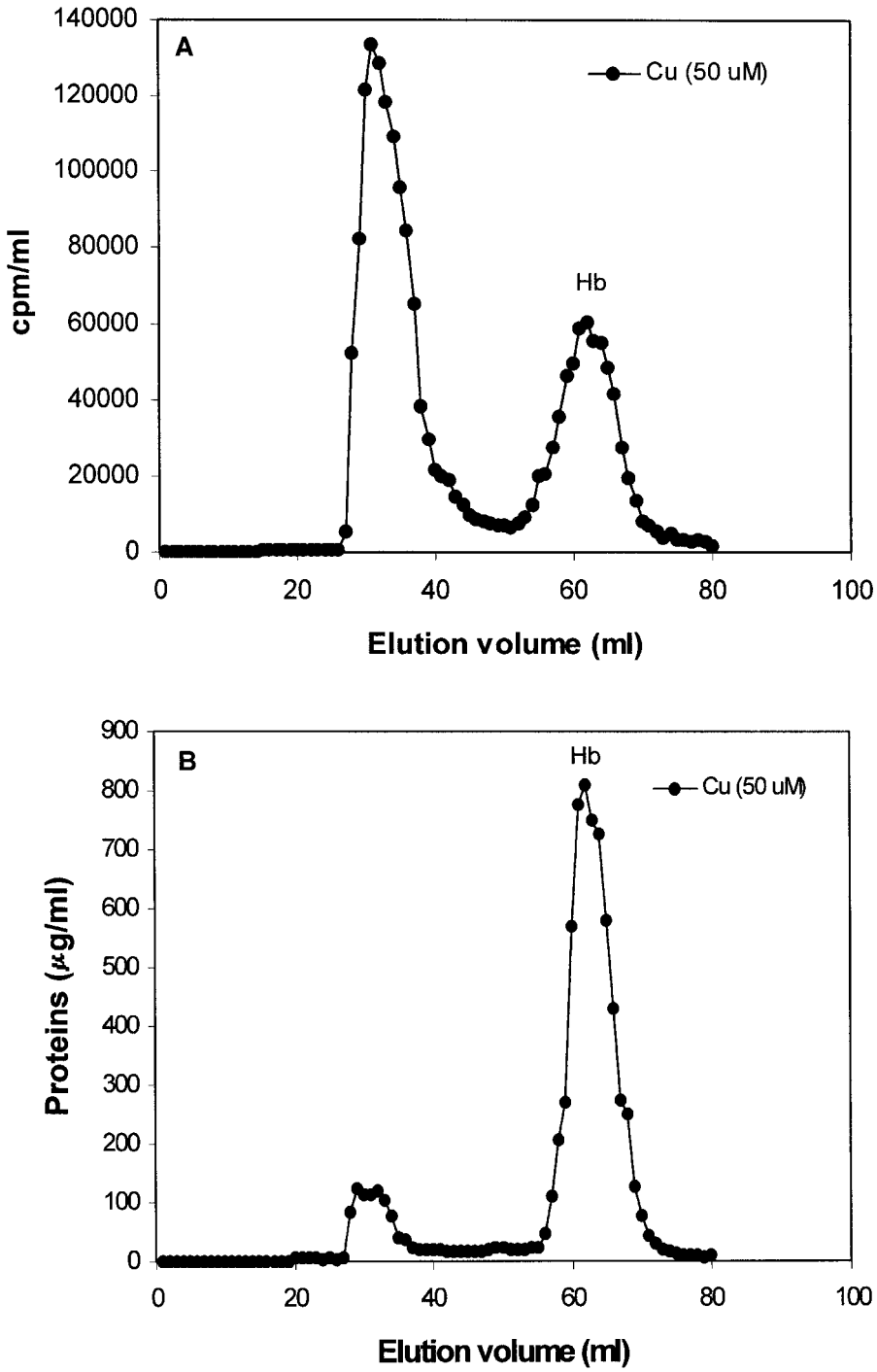

Fig. 3. Sephadex G-200 chromatography of higher MMr fractions obtained from a Sephadex G-75 separation: ^{64}Cu radioactivity and protein elution profiles. S-20 samples, obtained after incubation (60 min) of erythrocytes with 50 μM ^{64}Cu, were applied into a G-75 column. The higher MMr eluted fractions (generally N° 14 to 18) were subsequently applied into a Sephadex G-200 column. One ml fractions were collected and monitored for ^{64}Cu cpm (A) and for proteins (B).

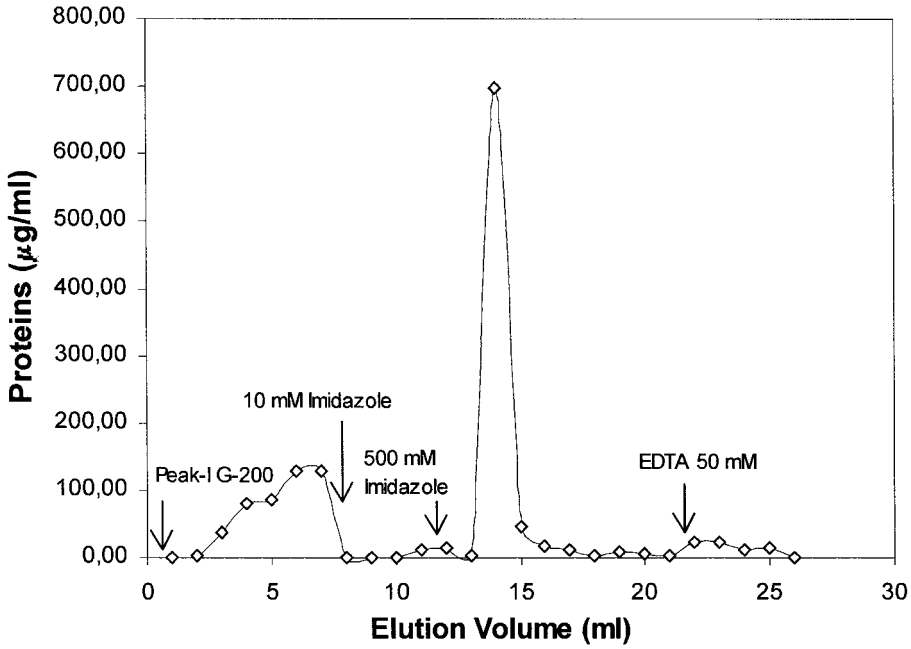

Fig. 4. Metal chelate affinity chromatography of higher MMr fractions obtained from a Sephadex G-200 separation: Protein elution profile. Fractions eluted from a Sephadex G-200 column as the higher MMr peak (generally N° 27–36) were pooled and applied into a HiTrap® column preloaded with copper. Following elution with 10 mM and 500 mM imidazole, fractions (1 ml each) were collected and monitored for proteins as described in methods.

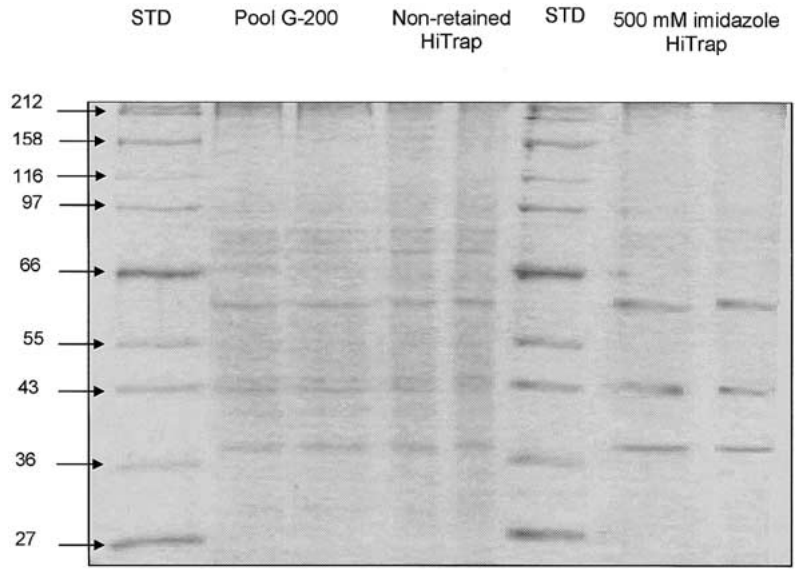

Fig. 5. SDS-electrophoretic pattern of high MMr Cu-binding proteins eluted from Sephadex G-200 and HiTrap® columns. Proteins (2.5 μg) from a pool of the Sephadex G-200 higher MMr fractions, and those present in fractions eluted from the HiTrap® column were subjected to 10% SDS-PAGE (100 V for 70 min, 22 °C). Gels were stained with Coomassie Brilliant Blue. Lanes 1 and 6 correspond, in decreasing order of MMr, to the following standards: myosin, MBP-β-galactosidase, β-galactosidase, phosphorylase b, BSA, glutamic dehydrogenase, MBP2, lactic dehydrogenase and triose-phosphate isomerase. Lanes 2 and 3 correspond to proteins contained in a pool of the higher MMr Sephadex G-200 fractions. Lanes 4 and 5 correspond to proteins not retained by the HiTrap® column, namely, eluted during its loading. Lanes 7 and 8 correspond to proteins retained by the HiTrap® column and eluted with Imidazole 500 mM.

copper to which the erythrocytes are initially exposed. Within the frame of our search for potential biomarkers, these observations might be construed as an indication that erythrocytes could represent a potentially suitable cell type to assess copper over-exposure conditions. Plasma and erythrocyte copper levels are quite similar in the normal healthy adult (Lahey *et al.* 1953; Shields *et al.* 1961; Hatano *et al.* 1982). However, for a number of conditions, whether physiological or not, these two parameters are, at least apparently, not necessarily bound or related. For instance, pregnant women (Shields *et al.* 1961) and growing children (Hatano *et al.* 1982) are known to exhibit normal copper levels in their erythrocytes despite presenting above-normal levels of copper in plasma. Copper in erythrocytes has been reported to be nearly double as high as that in plasma in the healthy newborn infant (Kleinbaum 1963). Another example is that of individuals affected by conditions such as Wilson's disease or by idiopathic hypocupremia which exhibit typically diminished plasma copper levels in association with normal levels of copper in their erythrocytes (Zipursky *et al.* 1958; Shields *et al.* 1961). This apparent lack of relationship between erythrocyte and plasma copper could be interpreted as an indication that ceruloplasmin, which accounts for the largest part of copper in circulating post-hepatic plasma (Wirth & Linder 1985; Linder 1998; Harris 2000) and for which receptors have been described on the surface of erythrocytes (Barnes & Frieden 1984; Saenko & Yaropolov 1990), would most likely play no significant role in transporting copper into these cells.

Transfer of copper across the intestinal basolateral membrane is swiftly followed by its appearance in portal blood plasma as copper bound to albumin and to histidine molecules (Basuoka & Saltman 1994). Histidine, which forms a ternary complex with Cu(II)-albumin in plasma, displace Cu from proteins to release copper as a Cu(II)-histidine complex (Sarkar & Kruck 1966; Sarkar 1980). Our observation that the erythrocyte *in vitro* readily takes up copper when presented as Cu-histidine suggests that such complex may function *in vivo* as a significant carrier to deliver metal into the red cells. Since liver cells readily access copper from Cu-histidine (Sarkar 1980; Waldrop & Ettinger 1990), the concentration of the complex is likely to be higher in the pre-hepatic (portal) blood and, therefore, the transfer of copper into the erythrocyte to be greater during the intestinal absorption of the metal. The latter may, conceivably, explain a close correlation between plasma and erythrocyte copper as reported by Bush *et al.* (1955 & 1956) after the administration *per os* of a radiolabelled form of copper to humans. Approximately half of the total copper present in circulation can be accounted for by its occurrence in erythrocytes. Under normal exposure conditions, copper occurs within these cells both as a minor diffusible and as a major non-dialyzable fraction (Neumann & Silverberg 1967). While the chemical nature of the labile or diffusible pool of copper has not been yet established, most of the firmly-bound pool of copper is believed to be accounted for by copper structurally-bound to superoxide dismutase molecules (Markowitz *et al.* 1959; Gartner & Weser 1983). Therefore, the existence of a relationship between plasma and erythrocyte copper would depend, not only on whether the copper-absorbed from intestine is transported as a form capable of delivering the metal to the erythrocytes, but also, on the nature of the copper binding ligands in red blood cells. These Cu binding ligands may represent a labile pool of copper that can diffuse from the erythrocyte to the plasma and thereby distribute to various tissues. The latter contention is, in fact, supported by the observation that the *in vitro* incubation of human red blood cells pre-loaded *in vivo* with ^{64}copper is followed by a time-dependent diffusion of the radiolabel into plasma (Bush *et al.* 1956; Neumann & Silverberg 1967). Consequently, the existence of such diffusible pool of copper in erythrocyte may limit the use of total erythrocyte Cu as an adequate indicator of the plasma level of copper.

Under the experimental conditions used in the present studies about half of the radioactivity, on average, that had disappeared from the extracellular medium was recovered in association with the cytosolic S-20 fractions. The remaining radioactivity could be accounted for by ^{64}Cu adsorption onto the erythrocyte surfaces and by its binding to the pelleted membranes. Based on recovery calculations, we have estimated that the intracellular radiolabelled Cu reached a concentration up to 4-fold higher than that presented to the red blood cells at zero time. Since such accumulation took place in the absence of any significant cell lysis (not shown), this result suggests the potential existence of Cu-binding molecules in the erythrocyte that may retain large amounts of copper transiently within the cytosol. Given the enormously high concentration of hemoglobin in erythrocytes, and its ability to bind copper (Bemski *et al.* 1969), large amounts of the radiolabelled Cu are likely to occur in association with Hb. In fact, as observed during the Sephadex G-75 chromatographic separation of

S-20 samples, most of the ^{64}Cu was found in association with fractions where Hb molecules co-eluted. Hb would act as a 'sink' not only to excess copper from the plasma, but also metals such as cadmium and silver ions (Eaton & Cherian 1991; Scheuhammer & Cherian 1991), thus, precluding its use as a potential biomarker to assess the excessive exposure of copper entering the erythrocytes. On the other hand, the radioactivity pattern of the Sephadex G-75 separation revealed that no significant amounts of radiolabelled copper are associated with metallothionein. In liver cells, MT molecules can sequester copper under abnormal conditions (Hunziker & Sternlieb 1991; Mulder *et al.* 1992; Jimenez *et al.* 2002). Therefore, there is no direct relationship between MT and copper accumulation in erythrocytes.

Because of the high amount of hemoglobin present in the S-20 samples, it was not possible to resolve SOD from Hb in Sephadex G-75 chromatography. We did not pursue further the separation of SOD molecules in this study because measurement of its activity is useful only to assess copper deficiency. However, in the absence of deficiency, an over-exposure to copper results in no further increases in SOD activity (Baker *et al.* 1999; Rock *et al.* 2000). Since our objective was to identify Cu-binding molecules as biomarkers of over-exposure, we had focused on molecules, other than Hb, MT or SOD. As shown in the radioactivity profile of the Sephadex G-200 chromatography, the pool of high Cu binding fractions was resolved from Hb molecules as a peak of considerably high MMr proteins. Remarkably, these non-Hb molecules were associated with levels of radioactivity that corresponded with the concentrations of ^{64}Cu retained and taken up by the erythrocytes. Moreover, on a *per* μg of protein basis, these high-MMr molecules exhibited a substantially greater binding to ^{64}Cu than Hb, suggesting the existence within the erythrocyte of molecules capable of binding copper with a higher affinity. The observation that such binding was related to the extracellular copper concentrations prompted their further purification. Their separation by MCAC, which depends on metal ion-complex formation with the proteins' histidine residues, led to the isolation of those proteins with the highest affinity for copper in erythrocytes. An SDS-electrophoretic analysis of ^{64}Cu-binding proteins obtained in the Sephadex G-200 separation reveals that such proteins comprised about a dozen protein bands, of which only three were separated by the MCAC procedure. One of the three bands, the higher MMr, is likely to be Hb; the two other bands, whose identity also remains to be established, were ascribed MMr of approximately 30 and 40 KDa, respectively. Preliminary data (not shown) indicate that, upon their separation from the higher MMr band (by means of a Sephacryl S-300 column), the two bands are able to bind ^{64}Cu from Cu-histidine, and that such binding occurs within a range of copper concentrations relevant to those expected to occur during copper over-exposure conditions.

Acknowledgements

This work was supported by grants from DID #I008-99/2 (University of Chile) and Fondecyt #1010705.

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