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Intestinal administration of copper and its transient release into venous rat blood serum concomitantly with metallothionein

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The molecular side of copper transport in biological systems is unknown. It was attempted to examine the copper and metallothionein (MT) release into the portal blood in rats *in vivo*. After direct administration of Cu(II) into the jejunum the copper and MT levels were distinctively higher in the portal venous serum compared with that of the *vena cava inferior*. MT in gel filtrated serum samples was analyzed immunologically employing ELISA and a monoclonal antibody to rat MT-I. Affinity chromatography on Protein A–Sepharose resulted in a higher immunoreactivity in the portal compartment as deduced from an elevated MT–antibody complex. It is assumed that MT serves as a genuine transport system for cuprous copper during the mucosal-to-serosal flux of this biologically important transition metal.

Keywords: copper transport, metallothionein, metallothionein-immunoreactivity, portal serum, rat jejunum

Introduction

The molecular mechanism of absorption, transport and storage of copper belong to the most important tasks in elucidating the biochemical role of this prominent transition metal. In this context the transfer of ingested copper from the intestinal mucosa into the portal blood is of great interest. The fate of orally administered copper was examined (Evans 1973, Ettinger 1984, Cousins 1985, Bremner 1987) and plasma copper was found to be coordinated as Cu(II) to albumin, histidine, threonine, His–Tyr, His–albumin (Sarkar & Kruck 1966, Neumann & Sass-Kortsack 1967) and Gly–His–Lys (Pickart *et al.* 1980). The best way for transporting Cu(I) in a non-reactive manner appears to be in the form of Cu(I)–thiolate complexes including Cu(I)–glutathione (Freedmann *et al.* 1989) and Cu(I)–metallothionein (MT) (Felix *et al.* 1989). MT was shown to be actively involved in both intestinal metal absorption (Starcher 1969, Evans *et al.* 1970, Cousins 1985, Elmes *et al.* 1987, Ohta *et al.* 1989), and in the mucosal-to-serosal flux of copper and zinc *in vitro* (Felix *et al.* 1990). The release of copper–thionein into the venous effluent was shown after perfusion of ligated intralumenal copper-exposed porcine small bowel. MT was characterized by chemical, physicochemical and immunological methods. An increased excretion of MT into the serosal fluid was measured immunologically using buffer-loaded sacs of porcine jejunum in the presence of Cu(II), Zn(II) and Cd(II).

Intralumenal administration of Cu(II) into the jejunum of anesthetized rats was carried out to examine the former mucosal-to-serosal copper transfer *in vivo*. Copper analysis and immunological detection of intact MT including ELISA and affinity chromatography on Protein A–Sepharose were performed in both the portal and the non-portal venous compartments.

Materials and methods

One dose of 5 ml phosphate buffered saline (P_i/NaCl) containing 5.5 mM glucose, 1 mM $MgSO_4$ 1 mM $CaCl_2$, 10 mM L-histidine and 0.5 mM $CuSO_4$ (158.7 μg Cu) were

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injected into the proximal section of the surgically exposed jejunum of male Sprague-Dawley rats (250 g). The rats were previously anesthetized by intramuscular injection of a mixture of Ketanest (2-(2-chlorphenyl)-2-methyl-amino-cyclohexanone-hydrochloride, Parke-Davis, Berlin, Germany) and Rompun (5,6-dihydro-2(2,6-xylidino)-4H-1,3-thiazine, Bayer, Leverkusen, Germany) at a dose of 100 and 16 mg, respectively, in the presence of 0.05 mg atropinsulfate kg^{-1} body weight. In general the anesthesis lasted for 90 min and, if necessary was prolonged using 25% of the initial dose. The control rats were treated with the same buffer omitting any copper. Blood samples of 200 μl were collected from both the *vena portae* and *vena cava inferior* over a period of 210 min at 30 min intervals. The coagulated blood was centrifuged and the sera were subjected to copper analysis and immunological assays.

Copper was quantified by flameless atomic absorption spectroscopy on a Perkin-Elmer 3030 spectrometer. Murine monoclonal antibodies to rat liver MT-I were prepared in the authors' laboratory (Nagel *et al.* 1990). The monoclonal antibody II-10a was employed. ELISA was carried out after gel filtration of serum samples according to Engvall (1980) and Felix *et al.* (1990). The microtiter plates were coated with 200 μl of sample solution at pH 9.6 for 4 h at 37 °C. A constant concentration of purified monoclonal antibody (2.6 μg/well) was added to all samples. Unspecific binding sites were blocked with milk powder (5% v/v) in P_i/NaCl. Alkaline-phophatase-conjugated goat anti-mouse IgG (Sigma, Munich, Germany) with a final dilution of 1:2000 was used as second antibody. The enzymic activity was measured on a Bio-Tek EL 309 microplate reader at 405 nm. IgG affinity chromatography was performed using Protein A–Sepharose CL-4B (Pharmacia). To 50 μl serum, 17 μg of the monoclonal antibody dissolved in 450 μl 3 M NaCl, pH 8.9, was added and the mixture incubated for 1 h at 25 °C. Samples were subjected to a column (1 × 1 cm) equilibrated with 3 M NaCl, pH 8.9. An amount of 12.5 ml of the same solution was needed to remove all non-binding serum components. Elution of the MT–antibody complex was carried out using 100 mM citric acid, pH 3.0.

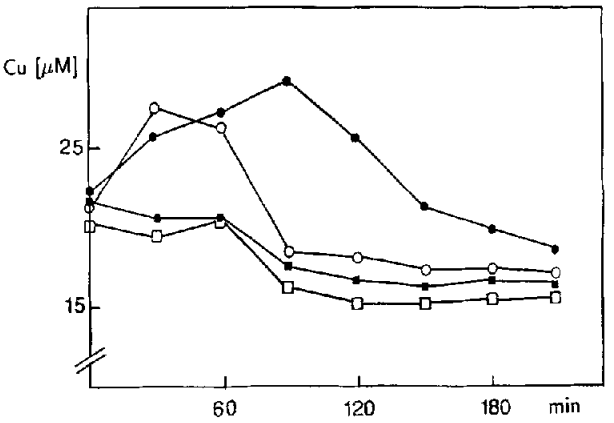

Figure 1. Rat serum copper concentrations in the vena portae (●, ■) and vena cava inferior (○, □): (●, ○) values after intestinal copper application and (■, □) control (copper omitted). Blood samples (200 μl) were removed every 30 min using heparinized catheters. Coagulation was completed within 6 h at 4 °C. The supernatants of the centrifuged samples were analyzed for copper. Data are expressed as mean values from four independent measurements. The standard deviation was less than 6%.

Results and discussion

The possible copper mediated excretion of MT from the rat intestinal mucosa into the portal vein was examined *in vivo*. After small bowel intralumenal Cu(II) exposure different copper and MT concentrations in the portal blood were compared with those of the vena cava inferior, which represents the non-portal venous compartment. Injection of a buffered Cu(II) solution into the proximal jejunum of anesthetized rats led to a significant rise of the serum copper concentration in the vena cava inferior for 60 min (Figure 1). This was followed by a rapid and progressive decline to concentrations even lower than normally observed at the beginning of the experiment. The copper absorption profile was in accordance with earlier studies where ^{64}Cu was orally applied to rats and the maximum plasma concentration was reached after 30 min (Marceau *et al.* 1970). By way of contrast, elevated copper levels were noticed for more than 90 min in the portal serum. In the absence of additional copper no detectable rise of copper was seen and the serum concentration profiles proceeded in a virtually parallel fashion although the copper values of the portal sera were slightly higher. It is assumed that at least part of the elevated portal copper in the previously metal-treated animals is associated with MT.

Ceruloplasmin was monitored by following the *N,N*-dimethyl-phenylenediamine oxidase activity (Curzon & Vallet 1960) and was found to be essentially identical in the sera of both the vena portae and vena cava inferior throughout the course of the investigations. This indicates that the observed elevated copper concentrations are not attributed to ceruloplasmin.

Substantial support of elevated Cu–MT levels was expected from immunological measurements. For comparative reasons, 90 min after the copper injection blood samples were exclusively collected from either the vena portae and vena cava inferior. The processed sera were chromatographed on Superose 12 and were immunologically analyzed for MT by ELISA. An immunoreaction was noticed which was

clearly elevated in the eluate of the portal serum (Figure 2).

The elution patterns exhibited three immunoreactive fractions corresponding to $M_r > 250$, 60–70 and 10–12 kDa, respectively. The latter fraction is attributed to monomeric MT which is generally eluted in this M_r range. The phenomenon of higher M_r MT species, although at a substantially diminished concentration, was consistent with earlier observations where a serosal release of MT was noticed using perfused ligated segments of porcine small intestine (Felix *et al.* 1990). The occurrence of oligo- and polymeric MT species can be explained by the well-known inter- and intramolecular polymerization via disulfide bridges (Hartmann & Weser 1977). Due to partial oxidation of the copper–thiolates this behavior is often concomitant with an uncontrolled release and rebinding of metal ions from the original coordination site. It should be emphasized that, unlike in the *in vitro* studies (Felix *et al.* 1990), the monomeric MT is the most prevalent immunoreactive species.

In the present experiment good agreement of the elution patterns of the copper containing fractions with that of the respective immunological pattern was seen. Most of the copper was found in the 10–12 kDa region in both the portal and the non-portal gel filtrated sera. The elution pattern also exhibits copper and immunologically detectable low M_r components (<10–12 kDa). Whether or not this is due to MT fragments or phenomena related to hydrophobic interactions of the protein to the Superose 12 column is unknown.

In order to support the observed differences in MT content the sera were subjected to affinity chromatography. The IgG monoclonal antibody to rat liver MT-I was added to the respective serum samples and after incubation for 1 h separated by affinity chromatography on Protein A–Sepharose. MT was detected indirectly by copper analysis of the eluted MT–IgG fraction (Figure 3). The total amount of copper associated with the MT–antibody complex in the portal serum sample was clearly increased compared with that of the vena cava inferior.

Unfortunately, no detectable differences between either venous compartment were noticed in the immunoblot. Weak immunopositive bands were seen throughout. This phenomenon might be explained in considering MT levels under different physiological conditions. In blood plasma MT is known to be 1–8 $\mu g\,ml^{-1}$ in adult rats (Bremner *et al.* 1987) and is usually not detectable employing this method. However, due to the certainly existing

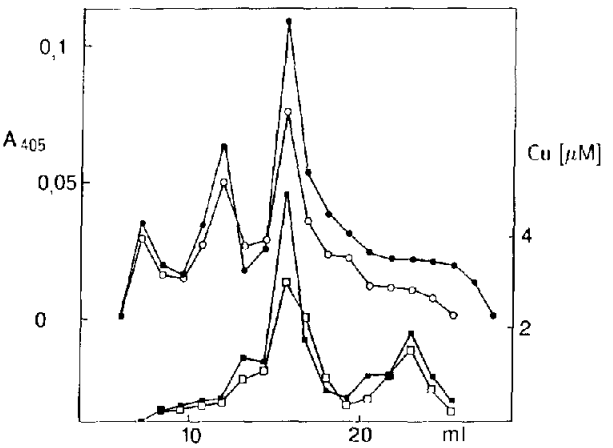

Figure 2. ELISA for MT (●, ○) and copper (■, □) of gel filtrated rat serum after 90 min intestinal copper application: (●, ■) vena portae and (○, □) vena cava inferior. Samples (100 μl) were fractionated on Superose 12 (1 × 30 cm). The column was equilibrated with P_i/NaCl in the presence of 0.1% 2-mercaptoethanol (v/v) and operated with 1.0 MPa at 20 °C. The fraction volume was 1.2 ml. The measurements were carried out in triplicate. The standard deviation was better than 8%.

stress situation during the course of the experiment, the level may rise up to 20-fold above normal (Sato *et al.* 1984). The observed indistinguishable immunopositive bands in the above immunoblot were close to the detection limit of this technique.

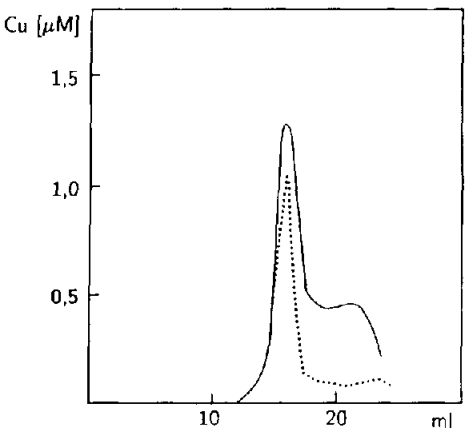

Figure 3. Copper elution profiles of affinity chromatographed rat serum: (——) vena portae and (· · ·) vena cava inferior. To 50 μl of serum originating from blood after 90 min of intestinal copper application, 450 μl of 3 M NaCl, pH 8.9 was added and the mixture subjected to a Protein A–Sepharose CL-4B column (1 × 1 cm) equilibrated with 3 M NaCl, pH 8.9. The non-binding serum components were removed with 12.5 ml of the same buffer. The MT–antibody complex was eluted with 100 mM citric acid, pH 3.0, at 20 °C. The fraction volume was 1 ml.

Thus, possible occurring differences are hardly seen.

Nevertheless, there is evidence from both the present results and a recent study *in vitro* (Felix *et al.* 1990) that part of the ingested copper enters portal circulation in the form of copper–thionein. MT can be considered a genuine extracellular transport system for this biochemically essential transition metal. It functions as a safe vehicle for cuprous copper which is coordinated in stable oligonuclear Cu(I)–thiolate binding centers to minimize the many undesired reactions of unspecifically bound copper. Whether or not the claimed transcuprein a copper-binding plasma protein (Weiss & Linder 1985), is related to oligomeric MT species awaits to be elucidated.

Intestinal application of traces of copper given as ^{64}Cu or ^{67}Cu were exclusively recovered in the portal albumin fraction, indicating that MT was not affected (Gordon *et al.* 1987). In the present study the employed copper concentration was sufficiently high enough to substantially affect Cu–MT release. In other words, elevated copper levels are efficiently controlled by MT. Attention should also be placed on the phenomenon that excessive copper administration resulted in the excretion of Cu–MT into the intestinal lumen from mucosal cells. Cu–thionein was also detected in the cellular supernatants of leucocytes (Hartmann *et al.* 1989) and yeast (Felix *et al.* 1989) which had been pretreated with copper.

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