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Copper binding components of blood plasma and organs, and their responses to influx of large doses of ^{65}Cu, in the mouse

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Abstract To establish for the first time how mice might differ from rats and humans in terms of copper transport, excretion, and copper binding proteins, plasma and organ cytosols from adult female C57CL6 mice were fractionated and analyzed by directly coupled size exclusion HPLC-ICP-MS, before and after i.p. injection of large doses of ^{65}Cu. Plasma from untreated mice had different proportions of Cu associated with transcuprein/macroglobulin, ceruloplasmin and albumin than in humans and rats, and two previously undetected copper peaks (Mr 700 k and 15 k) were observed. Cytosols had Cu peaks seen previously in rat liver (Mr > 1000 k, 45 k and 11 k) plus one of 110 kDa. ^{65}Cu (141 μg) administered over 14 h, initially loaded plasma albumin and mainly entered liver and kidney (especially 28 kDa and 11 kDa components). Components of other organs were less (but still significantly) enriched. ^{63}Cu/^{65}Cu ratios returned almost to normal by 14 days, indicating a robust system for excreting excess copper. We conclude that there are significant differences but also strong similarities in copper metabolism between mice, rats and humans; that the liver is able to buffer enormous changes in copper status; and that a large number of mammalian copper proteins remain to be identified.

Keywords Copper binding proteins · Mouse plasma · Cytosol · Heavy isotope · Excretion

Abbreviations
Mr Apparent molecular weight
k Thousands (as in apparent molecular weight)

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Introduction

Copper is an obligatory trace element in all living organisms and has been identified as important for the functions of a discrete number of redox enzymes involved in varied aspects of mammalian metabolism. These include mitochondrial cytochrome *c* oxidase—the terminal step of respiration; dopamine-beta-monooxygenase and alpha-amidating enzyme—required for the formation of specific neurotransmitters; lysyl oxidase—needed for the cross-linking maturation of

collagen and elastin in connective tissue; tyrosinase—which helps to form the pigment, melanin; and Cu/Zn superoxide dismutase and ceruloplasmin—which protect against reactive oxygen species (Harris 2000; Linder 1991, 2002). More recently, many copper binding proteins necessary for its uptake and transport have been identified, including the membrane transporters, CTR1 (Kuo et al. 2001; Lee et al. 2001) and DMT1 (Fleming et al. 1998; Gunshin et al. 1997); the copper "chaperones" HAH1, CCS and COX17 that shuttle Cu through the cytoplasm to specific sites (Amaravadi et al. 1997; Culotta et al. 1997; Huffman and O'Halloran 2001; Klomp et al. 1997; Linder 2002); and the copper "pumps" ATP7A and B that are defective in Menkes and Wilson diseases, respectively (Camakaris et al. 1999; Mercer 2001; Puig and Thiele 2002). Liver ATP7B is particularly important in helping mammals such as rats and humans efficiently eliminate excess copper (Linder and Roboz 1986; Turnlund et al. 1989, 1998) in the bile (Roelofsen et al. 2000; Schaefer et al. 1999) in a relatively non-re-absorbable form (Linder 1991). In the absence of effective ATP7B, Cu accumulates, particularly in the liver, causing cirrhosis and reduced organ function, leading to an early death in those with this genetic trait (Wilson disease). The identity and role of all the participants needed to transport excess copper to the bile are not yet understood; and other gene products must also be involved, including MURR1/COMMD1 (Burstein et al. 2005; Klomp et al. 2003), a multifunctional protein defective in the Bedlington terrier, a lack of which also results in liver copper accumulation and toxicity. In rats and humans, however, the ability to excrete excess copper via the bile is well developed and largely responsible for maintenance of whole body copper homeostasis (Linder 2001). Thus, there is only short-term storage of excess copper (in metallothionein), and excesses are readily excreted, as shown in rats with whole body counting after administration of radioactive ^{67}Cu (Linder and Roboz 1986) and in humans with the stable isotopic ^{65}Cu (Turnlund et al. 1989, 1998).

It has long been known that ceruloplasmin is the main copper-binding component in the blood plasma and most other body fluids of the mammal. This blue, multi-copper oxidase appears to have pleotropic functions and has been referred to as a prototypic "moonlighting" protein (Bielli and Calabrese 2002), capable of oxidizing a variety of substrates—from amines to Fe(II) (Harris 2000; Linder 1991, 2002); scavenging radicals (Linder 2001, 2002); and delivering copper to tissues (Campbell et al. 1981; Lee et al. 1993), probably via specific receptors (Kataoka and Tavassoli 1985; Linder 2002; Stevens et al. 1984). Its apparent role in Fe transport (attributed to its ferroxidase activity) has been of particular recent interest. Thus, in humans (Harris et al. 1995, 1998; Yoshida et al. 1995) or mice (Harris et al. 1999) lacking ceruloplasmin genetically or through severe Cu deficiency (Linder 1991, 2002; Osaki and Johnson 1969; Ragan et al. 1969) there is a gradual accumulation of excess iron in certain tissues, including the liver and retina, resulting in tissue damage. This has been explained on the basis that ceruloplasmin oxidizes Fe(II) as it leaves cellular storage sites, promoting the binding of the resulting Fe(III) to its blood plasma transporter, transferrin (Frieden 1970; Linder 1991). This concept is supported by experimental evidence that intravenous infusion of ferroxidase-active ceruloplasmin into organs of ceruloplasmin-deficient dogs (Osaki and Johnson 1969) or pigs (Ragan et al. 1969) results in the immediate release of iron into the blood, although it appears that only extremely low levels of active ceruloplasmin (<1% of normal) reduce iron transport and accumulation (Roeser et al. 1980).

In human and rat blood, ceruloplasmin accounts for approximately 70% of the total copper in the plasma (Barrow and Tanner 1988; Weiss and Linder 1985; Wirth and Linder 1985) rather than the 95% usually quoted. The remainder of the Cu is thought to be associated mainly with two other proteins that may make up most of the exchangeable copper pool in blood fluids. These are albumin, which in most mammals has a single, high affinity copper-binding site at its N-terminus involving a crucial histidine (Linder 1991, 2002); and a macroglobulin, designated alpha-1-inhibitor-3 in rodents and alpha-2-macroglobulin in humans (Liu et al. 2007). The macroglobulins have an even higher affinity for copper than albumin, and rapidly exchange copper with the latter, in vitro (Linder 1991, 2002; Trang Nguyen, Mizue Moriya, Ann Grana, Mimi Mak and Maria Linder unpublished results). Traces of other copper containing proteins are also present in human plasma/serum (Linder 1991), although their contributions to total plasma/serum copper is unclear. These include metallothionein, ferroxidase II, blood clotting factors V and VIII, extracellular SOD and

amine oxidase, as well as possibly histidine-rich glycoprotein and unknown small peptides (Linder 1991, 2002).

In the cell *cytoplasm*, several prominent copper binding proteins have also been separated by size exclusion chromatography (Freedman et al. 1989; Harris 2000; Norton and Heaton 1980; Sharma and McQueen 1981; Terao and Owen 1973). The two most commonly identified are Cu/Zn SOD (about 35,000 Da) and metallothionein (Mr about 15,000), although the previously mentioned (similar-sized) "chaperones" (HAH1, CCS, COX17) for shuttling copper to specific intracellular sites (Amaravadi et al. 1997; Culotta et al. 1997; Huffman and O'Halloran 2001; Klomp et al. 1997), and some other already mentioned soluble copper proteins/enzymes, are also present. A large (>100 kDa) unknown component has also commonly been observed.

Most of the information about copper in the various proteins described and the kinetics of its transport and excretion has been obtained from trace analysis of the copper in tissues and by following the distribution of radioactive copper in rats (Linder 1991, 2002; Terao and Owen 1973). In humans, there have also been some studies using the stable isotope, ^{65}Cu (Turnlund et al. 1989, 1998). Today, because of its size and ease of genetic manipulation, the mouse is the most widely used organism to model the transport and metabolism of specific nutrients or metabolites in humans; yet the Cu metabolism of mice is not well studied, particularly with regard to copper transport and blood plasma binding components. Indeed, earlier experimental work from our laboratory (Montaser et al. 1992) and that of Prohaska (1983) indicated that copper metabolism in the mouse might differ from that in humans and rats. Thus, the first objective of the studies reported here was to examine this issue. To accomplish this, size exclusion high performance liquid chromatography directly coupled to ICP-MS (inductively coupled plasma mass spectrometry) was used to compare the copper binding components in blood plasma from mice, humans and rats, as well as in cytosols of a variety of organs. This technique was also used to monitor the biochemical responses and long-term fluxes of copper, into and out of organs of the mice, after a large i.p. injection of ^{65}Cu. This stable isotope has been used in low doses in mice to study hepatic Cu turnover (Ting et al. 1990) but has not been used for temporal analyses to study Cu turnover in the blood or other organs (or their individual components). The results reported here are the first showing that the mouse differs considerably from the rat and human with regard to the proportions of copper associated with various plasma proteins; that the major mouse cytoplasmic binding proteins responsible for coping with larger doses of extraneous copper are similar to those previously reported in the rat; and that with high doses of Cu, it is possible to obtain significant, but not substantial, enrichments of copper proteins in organs peripheral to the liver.

Materials and methods

Materials

The stable isotope, ^{65}Cu, was obtained as CuO from Oak Ridge National Laboratory (Oak Ridge, TN). ^{64}Cu was from Mallinkrodt Institute for Radiology, at Washington University, St. Louis. Cibacron Blue beads (Affigel Blue) were from BioRad (Hercules, CA). Ultrapure nitric acid was from Fisher Scientific (trace element grade). Heparinized human blood plasma was obtained from healthy, adult volunteers at the university, according to a protocol approved by the California State University, Fullerton, IRB (Approval No. HSR # 05-004). Blood was taken at the Health Center and processed anonymously in the investigators' research laboratory. Plasma was separated by centrifugation and either used right away (fresh plasma) or stored frozen at −20 or −80°C, in aliquots, until use.

Mice, rats and treatments

Adult, female C57-BL6 mice, and adult female Sprague Dawley and male Fisher rats were obtained from Simonson Laboratories (Gilroy, CA) and maintained on normal rodent chow (Harlan Teklad rodent chow, Madison, WI). Ceruloplasmin knockout mice were from our own colony, derived from heterozygous breeding pairs obtained from Z. Leah Harris at Johns Hopkins University. Protocols for treatments were approved by the university IACUC (No. 01-R1-05). Some of the C57BL6 mice were injected i.p. with five 25–30 μg doses of $^{65}CuCl_2$ in 0.9% NaCl (for a total of 141 μg) containing 50 mM nitrilotriacetate (NTA),

and they were euthanized in pairs at various times (30 min to 14 days) following the last injection. The NTA was added to assure solubility and bioavailability of the copper injected. Thus, the Cu-NTA complex readily transfers the copper to its plasma binding proteins, as repeatedly demonstrated by comparing additions of ^{67}Cu(II) or ^{67}Cu(II)-NTA to blood plasma (Lee et al. 1993; Weiss and Linder 1985), and comparing its tissue and protein distribution after i.v. administration to rats (Vargas et al. 1994; Weiss and Linder 1985). Euthanization was by bleeding from the vena cava under pentobarbital anesthesia after treatment with heparin, as previously described (Linder and Roboz 1986; Weiss and Linder 1985). Plasma was prepared and stored as described above. Liver, kidney, heart, lungs, spleen and brain were collected and homogenized with trace metal grade 0.15M NH_4Cl. Tissue cytoplasm was obtained after centrifugation for 1 h at 105,000*g* in a Beckman L8-70M ultracentrifuge, at 4°C. Portions of homogenate and cytoplasm were stored at −80°C until analyzed. Plasma was also obtained from normal adult, female Fisher and male Sprague Dawley rats, euthanized and bled as described for the mice.

Tissue, cytoplasm and plasma copper analysis

For total Cu isotope quantitation, either 100 or 250 µl portions of homogenate or cytoplasm, respectively, were dried to a constant weight at 60°C in acid-washed disposable tubes (Cat. No. 14-958-C; Fisher Scientific), then wet ashed on a hot plate with 250 µl of ultrapure concentrated HNO_3 until almost dry. Residues were dissolved in 4.5 ml of 2% ultrapure HNO_3 containing 10 ppb of internal standards (Ga, In, Tl and Y). Serum was diluted 100-fold with the same acid-internal standard solution. Individual Cu isotopes were quantified using a Perkin Elmer 6100 DRC ICP-MS from standard curves of Cu generated from dilutions of a multi-element standard with known isotopic ratios of ^{63}Cu:^{65}Cu (SCP Science, Champlain, NY). Analyses of bulk homogenates, serum and cytoplasm samples from control animals showed ^{63}Cu:^{65}Cu ratios consistent with that of the measured natural isotopic abundance of the standards, indicating minimal interference from $^{63}NaAr^+$ and other polyatomic adducts. Concentrations of ^{63}Cu and ^{65}Cu in the sample were expressed relative to protein content or dry weight. In the injected animals, the content of ^{65}Cu was partitioned into intrinsic ^{65}Cu (pre-existing) and extrinsic ^{65}Cu (experimentally administered). Intrinsic ^{65}Cu was calculated from the measured ^{63}Cu content of the sample using the ^{63}Cu:^{65}Cu isotope ratio in equivalent control samples. The extrinsic ^{65}Cu was determined by difference (total ^{65}Cu minus the calculated intrinsic ^{65}Cu content).

Electrophoresis and Western blotting

SDS-PAGE was carried out in 7% acrylamide resolving gels by standard procedures and transferred to PVDF membranes (BioRad) by semi-dry transfer (BioRad), in Tris Triton buffered saline, as previously described (Donley et al. 2002). Blocking was with 5% dry milk protein. Primary antibodies were raised in rabbits against human ceruloplasmin and albumin were from Dako (Carpinteria, CA). Secondary antibody was AP-conjugated goat-anti-rabbit IgG, from BioRad. Densitometry of immunoblots was performed with the BioRad Gel Doc XR System with Quantity One software.

Protein analysis

Protein content was determined using a Bradford dye binding assay (BioRad, Richmond, CA) and bovine serum albumin as a standard.

Directly coupled size exclusion HPLC-ICP-MS

Chromatographic separations and isotopic determination of samples were performed using a "biocompatible" Beckman 126 programmable solvent delivery module equipped with a Beckman 168 diode array UV/Vis detector that was directly coupled to ICP-MS (Mason and Borja 2002). PEEK$^@$ tubing was used to connect the components in the system. Aspiration was through a quartz Meinhard concentric nebulizer and a cyclonic spray chamber. Standard curves for apparent molecular weight (Mr) were obtained using gel filtration standards that included thyroglobulin (670 kDa), ferritin (480 kDa), bovine gamma-globulin (158 kDa), human ceruloplasmin (132 kDa), chicken ovalbumin (44 kDa), equine myoglobin (16.7 kDa), and vitamin B12 (1.34 kDa). Samples (100 µl) of 5-fold diluted plasma or tissue cytosol were filtered (0.45 µm) and fractionated

isocratically (20 mM Tris · HCl, pH 7.4) by size exclusion chromatography, using either a Biosep 2000 or 4000 SEC column (Phenomenex, Torrance, CA), or in some cases on 1 × 25 or 30 cm columns of Sephacryl S300 or Sephadex G150. The isotopic ratio of ^{63}Cu:^{65}Cu of the various peaks was determined at the centroid value of each peak. Analyses of samples from control animals showed protein peaks with ^{63}Cu:^{65}Cu ratios that were attenuated relative to the measured natural abundance of the Cu standards, indicating some inerference and augmentation of the signal at M/Z 65 from polyatomic adducts, possibly ^{48}Ca^{16}O^{1}H generated from the buffer.

Statistics

Statistical analysis of the data was by one way ANOVA. Probability (P) values of <0.05 were considered significant.

Results

Comparisons of the copper binding components of mouse plasma in comparison with those of humans and rats

The distribution of copper among binding proteins of the blood plasma of the mouse has not been previously examined. To do this and compare the mouse with the more comprehensively studied rat and human systems, fresh and previously frozen samples from adult, female C57BL6 mice and from normal adult humans and rats were separated by HPLC size exclusion chromatography and analyzed by UV/Vis spectroscopy prior to coupled ICP-MS elemental analysis. This approach was taken as well to obtain more refined data for copper binding components in all three species than previously available in the literature. Initial fractionation of rat and human plasma using a Biosep 2000 column showed one major copper-containing peak, eluting at 11.3 min, having a narrow shoulder coinciding with the void volume (10.6 min) (Fig. 1a). Another (smaller) component eluted at about 14 min. The Mr of the major (11.3 min) and minor (14 min) components were 124 k and 72 k, respectively, which approximate to the molecular weights for ceruloplasmin (132 k) and albumin (69 kDa), respectively. Fractionation of mouse plasma (Fig. 1b) showed more copper associated with the presumed albumin peak (around 14 min) and proportionately less with ceruloplasmin, although the latter still predominated. In addition to this difference in Cu distribution, the overall copper content of mouse plasma was considerably lower than that of the human and rat. Concentrations of total copper (ng/ml) in the mouse samples were 400 ± 160 (Mean ± SD; $N = 4$), whereas those of humans and rats were in the range of 1000–1200 ng/ml (Lee et al. 1993).

To facilitate further separation of the larger proteins (including the macroglobulins) from ceruloplasmin, the plasma samples were fractionated on a Biosep 4000 column (Fig. 2). Typically, five copper peaks could be observed in chromatograms from fresh or frozen (not shown) samples of mouse plasma using this column (Fig. 2a). Beginning soon after the void volume, there was a small shoulder of copper

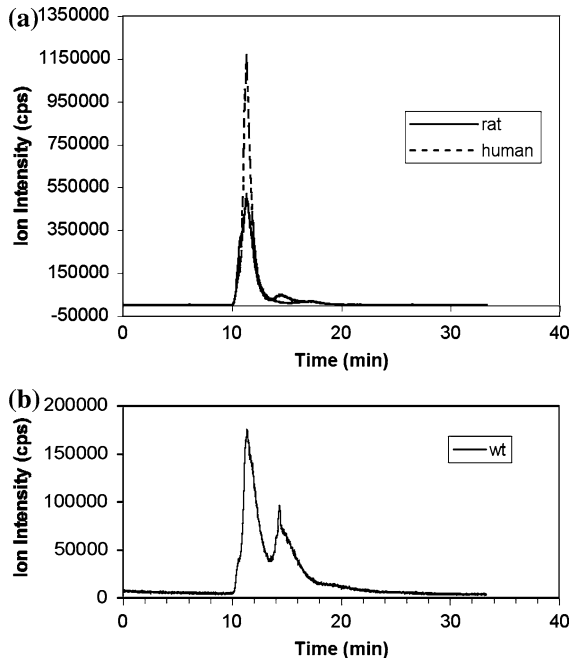

Fig. 1 Size exclusion HPLC separation of copper binding components of human, rat and mouse plasma/serum, on a medium-pore column. Fresh or pre-frozen samples of human (**a**), rat (**a**) and mouse (**b**) blood plasma were fractionated on a Biosep 2000 HPLC column, and the elution of copper-containing components was monitored by coupled ICP-MS. Elution time (min) on the x-axis is plotted against the ion intensity (cps) for ^{63}Cu. The main copper peak (11.3 min) and its shoulders at 10.6 (void vol) and 14 min had Mr values of 124, >200, and 72 k, respectively

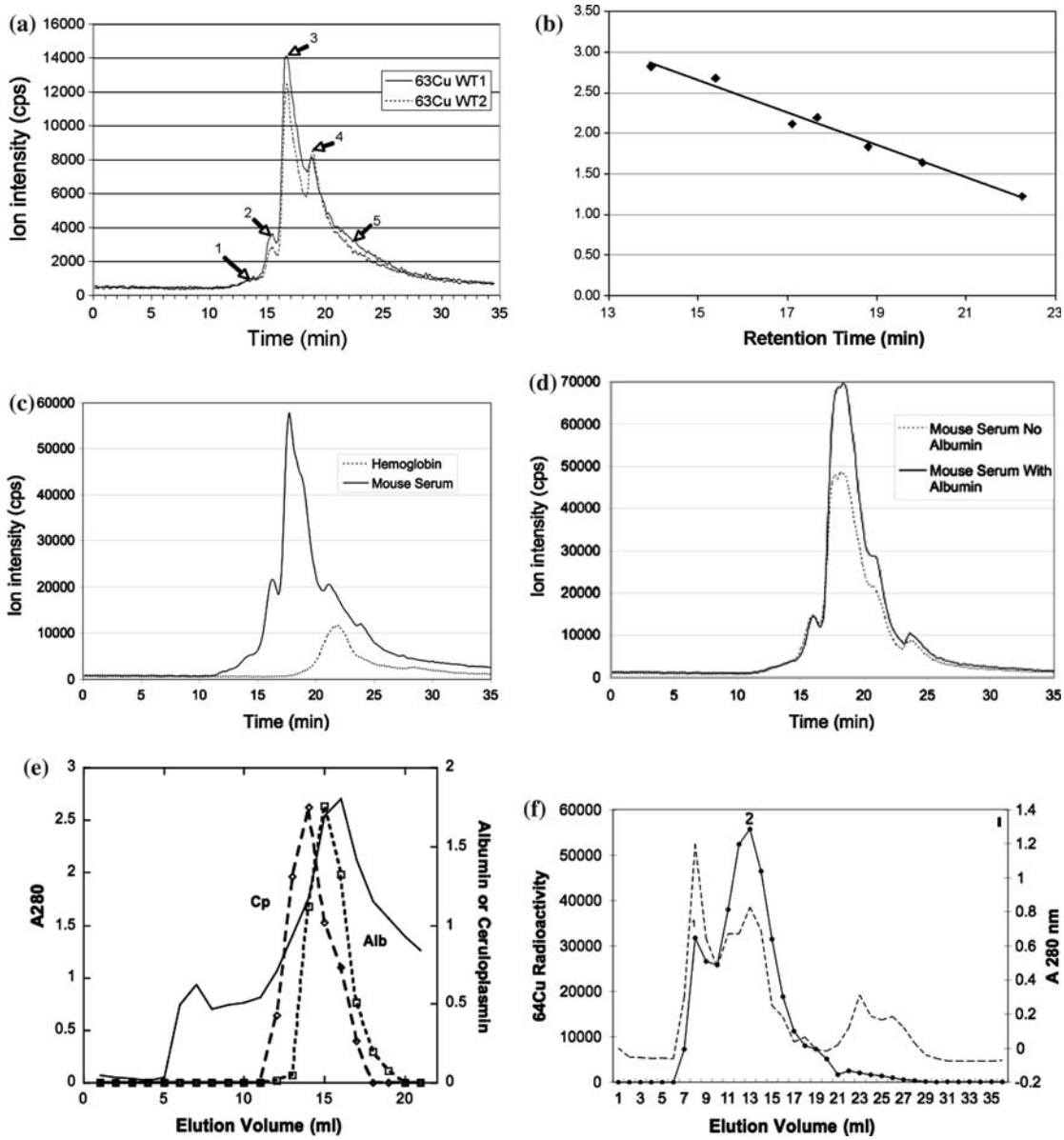

Fig. 2 Size exclusion HPLC separation of copper binding components of fresh mouse plasma, on a large-pore column. (**a**) Samples were fractionated on a Biosep 4000 HPLC column and the copper-containing components monitored by coupled ICP-MS. Data are presented as in Figure 1 for two representative samples. Similar results were obtained with multiple pre-frozen samples. Peaks/shoulders numbered 1–5 were calculated to have Mr values of about 700, 300, 190, 78, and 15 k, respectively. (**b**) Standard curve for the Biosep 4000 column, indicating elution of the following standards, from left to right: thyroglobulin (670 kDa), ferritin (480 kDa), bovine gamma-globulin (158 kDa), human ceruloplasmin (132 kDa), chicken ovalbumin (44 kDa), equine myoglobin (16.7 kDa), and vitamin B12 (1.34 kDa). (**c**) Fractionation of hemoglobin (^{57}Fe; dotted line), 64 kDa, compared with mouse serum copper (solid line). (**d**) Effect of removing albumin with Cibacron Blue beads: the same mouse plasma sample was treated (dotted line) or not treated (solid line) with an equal volume of PBS-equilibrated beads prior to HPLC-ICP-MS on the Biosep 4000 column, detecting ^{63}Cu. (**e**) Overlapping elution of ceruloplasmin (Cp) and albumin (Alb) in a 1 × 25 cm column of large pore Sephacryl S300, elution and quantitation of the proteins being by Western blotting and densitometry (see "Methods"). (**f**) Elution of radioactive ^{64}Cu (solid line) with transcuprein (peak 1) and albumin (peak 2) from a 1 × 30 cm column of Sephadex G150, following addition of ^{64}Cu(II)-NTA (1:3 molar ratio) to 0.5 ml mouse plasma. A_{280} is also indicated (dashed line)

(peak 1) followed by a significant minor peak (peak 2) eluting at 15.5 min. The latter accounted for about 8% of the total copper and had an Mr of about 300 k (Fig. 2b). This is consistent with the macroglobulin, alpha-1-inhibitor-3 (Liu et al. 2007) which we have previously identified as the important copper transport protein in rat plasma, transcuprein, of Mr 270 k (Vargas et al. 1994; Weiss and Linder 1985). Peak 3 (Fig. 2a) eluted at 16.8 min with a Mr of approximately 190 k, contained about 55% of the plasma copper, and was tentatively identified as ceruloplasmin on the basis that it was markedly diminished in the plasma of knockout mice (data not shown). Peak 4 contained approximately 25% of the total plasma copper, and eluted at 18.8 min (Mr 78 k) just ahead of pure hemoglobin standard (64 kDa) (Fig. 2c), consistent with it being albumin. The final copper peak eluted as a broad shoulder centered around 22.5 min (approximately 15 kDa). The significance of mouse albumin for copper binding was further established by comparing the copper elution profile before and after treatment of mouse plasma with Cibacron Blue affinity gel (known to remove albumin specifically; Ma et al. 2005). A sizeable proportion of the total copper (about 20%) was removed in the region where albumin elutes (Fig. 2d). Copper was removed not only from the albumin "shoulder" but also from the region where ceruloplasmin elutes. This is explained by the fact that in this large pore gel, the two proteins overlapped with each other (Fig. 2e), as determined by Western blotting of mouse plasma fractionated on an open column of similar porosity. To further verify the binding of copper to transcuprein and albumin in the mouse, we added tracer ^{64}Cu(II) prior to fractionation. The elution profile in Fig. 2f is exactly the same as that found previously for the rat (Weiss and Linder 1985; Linder 1991, Liu et al. 2007). Two ^{64}Cu-binding peaks are evident: transcuprein (identified as alpha-1-inhibitor-3) eluting in the void volume on Sephadex G150; and albumin eluting later on.

Copper profiles of human and rat serum/plasma fractionated on the Biosep 4000 column showed certain differences from the mouse data. Analyses of human plasma obtained from five individuals showed one major and three minor copper peaks (Fig. 3a). Peak 1 eluted at 14.1 min (Mr about 680 k) and accounted for 5–10% of the total serum Cu. This Mr is close to that of the major human macroglobulin, alpha-2-macroglobulin (Sottrup-Jensen 1989), the human transcuprein (Liu et al. 2007), which is a tetramer of 180 kDa subunits—in contrast to the monomeric rodent macroglobulin, alpha-1-inhibitor-3, eluting at 15.5 min (Fig. 2a). Pure alpha-2-macroglobulin, purified from human plasma by size exclusion and Cibacron affinity chromatography (Lonberg-Holm et al. 1987) eluted with the same retention time. Peak 2 (Fig. 3a) contained approximately 65% of the total serum Cu and eluted at a time corresponding to ceruloplasmin (16.9 min). Peak 3 contained about 15% of the copper, and eluted at 18.7 min as a shoulder on peak 2, with a retention time comparable to albumin. An additional, minor copper-containing component, eluting at about

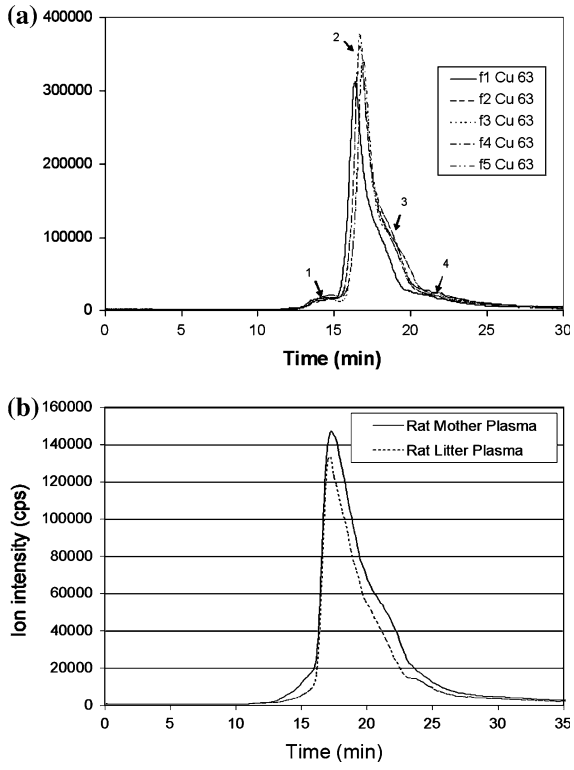

Fig. 3 Size exclusion HPLC separation of copper binding components of human and rat plasma, on a large-pore column. Results for fresh samples from 5 humans (**a**) and rats (**b**) (female Fisher lactating dam and her pups) are shown in the same way as for Figs. 1 and 2. Similar results were obtained with multiple pre-frozen samples. For the human plasma, peaks/shoulders 1–4 were calculated to have average Mr values of 676, 187, 82, and 15 k, respectively. Mr values for the rat peaks were about 700, 450, 178, with a broad shoulder ranging from about 80 to 15 kDa, and a small peak at 24 min (about 7 kDa)

22 min (about 15 kDa; also seen in mouse plasma) was observed as well.

Rat plasma had a very similar overall profile to that of the human (Fig. 3b), except that the copper eluting with macroglobulin/transcuprein (before the ceruloplasmin peak; peak 3) was approximately 450 rather than 680 kDa, consistent with rodent versus human macroglobulins (Sottrup-Jensen 1989). Collectively, the results confirm that ceruloplasmin is the most dominant copper binding protein in the blood plasma/serum of several mammals, but that it is only one of several proteins accounting for the total copper in this fluid, with other components carrying a substantial portion of the metal, and particularly so in the mouse.

Copper binding components of organ cytoplasms

To detect and compare (with published data for the rat) the major copper binding components of mouse tissue cytoplasms, samples of 100,000*g* supernatants from kidney, liver, heart, spleen, lung and brain homogenates of the same mice used for plasma analysis were fractionated on a Biosep 4000 HPLC column and analyzed by ICP-MS. Comparable copper profiles were observed for all of the tissues (Fig. 4). A significant copper binding component (peak 1) eluted close to the void volume (12 min; >1000 kDa) and, with the exception of the brain, a second peak was observed at approximately 18 min (Mr about 110 k). In all but heart and brain, the dominant copper peak (peak 3) eluted at about 20 min (Mr about 45 k). In the heart (Fig. 4b), there was a separate peak at about 23 min (Mr about 11 k) (peak 4), which coincided with shoulders in that region of the elution profiles for other tissues (Fig. 4a–c). The brain, which had the lowest amounts of cytosolic copper, had a broad peak encompassing the region of peaks 3–4 in the other tissues (Fig. 4c). The major copper peak at 20 min (45 kDa) coincided precisely with the largest peak for Zn in the kidney (Fig. 4d) and liver (data not shown) and was tentatively identified as Cu/Zn superoxide dismutase (about 35 kDa). Zn also eluted with peak 2 for copper in the kidney, and in the void volume. There was no evidence for a discrete peak for metallothionein at 7 kDa (~24 min), although an 11 kDa peak containing Cu with traces of Zn was observed in heart cytosol (Fig. 4e), which is consistent with the known retention characteristics of metallothionein in size exclusion chromatography (Kagi and Nordberg 1979). Lack of reliable antibodies for mouse Cu/Zn superoxide dismutase and metallothionein prevented confirmation of the identities of these components.

Effects of injections of excess copper, as ^{65}Cu

To determine how large doses of copper would be accommodated by and eliminated from these various binding proteins and whole organs, mice were injected with a total of 141 μg ^{65}Cu(II)-NTA, given in five injections (of 25–30 μg) over 14 h. The total dosages injected were estimated to be 3–4 times the copper already present in a 30 g mouse and were administered to ensure sufficient enrichment of ^{65}Cu in organs and cytosolic components beyond the liver for analytical precision. The excess copper produced no obvious changes in the behavior or activity of the mice, indicating a good toleration.

Pairs of mice were euthanized at various times after the last ^{65}Cu injection to analyze the distribution of ^{65}Cu. Figure 5 shows the calculated values for the distribution of injected (*extrinsic*) ^{65}Cu in the various mouse organs, as well as for preexisting, *intrinsic* ^{63}Cu and ^{65}Cu, 2 h (Fig. 5a) and 24 h (Fig. 5b) after the last ^{65}Cu injection. Values were calculated from the measured ^{65}Cu and ^{63}Cu concentrations and the natural ^{63}Cu/^{65}Cu ratio (see "Methods"). Analysis of the partitioning of the extrinsic ^{65}Cu in the various tissues 2 h after the last injection showed that the liver had by far the highest concentration, followed by the kidney, with minimal accumulation in heart and brain. Extrinsic ^{65}Cu in the plasma was also greatly elevated, being 3× the intrinsic content 2 h post injection, as would be expected, since the copper was distributed to the tissues through the blood. Indeed, analyses of plasma samples 30 min after the last ^{65}Cu injection showed levels of extrinsic ^{65}Cu in plasma 5× higher than 90 min later (at 2 h; data not shown). This implied rapid loss of the copper from the circulation. Levels of extrinsic ^{65}Cu in plasma fell to about twice normal levels by 24 h (Fig. 5b), while minimal losses were observed from the liver. The total concentrations of copper in heart, spleen and brain increased significantly between 2 and 24 h post injection. These increases were caused by an influx of intrinsic copper, suggesting that the injected ^{65}Cu had

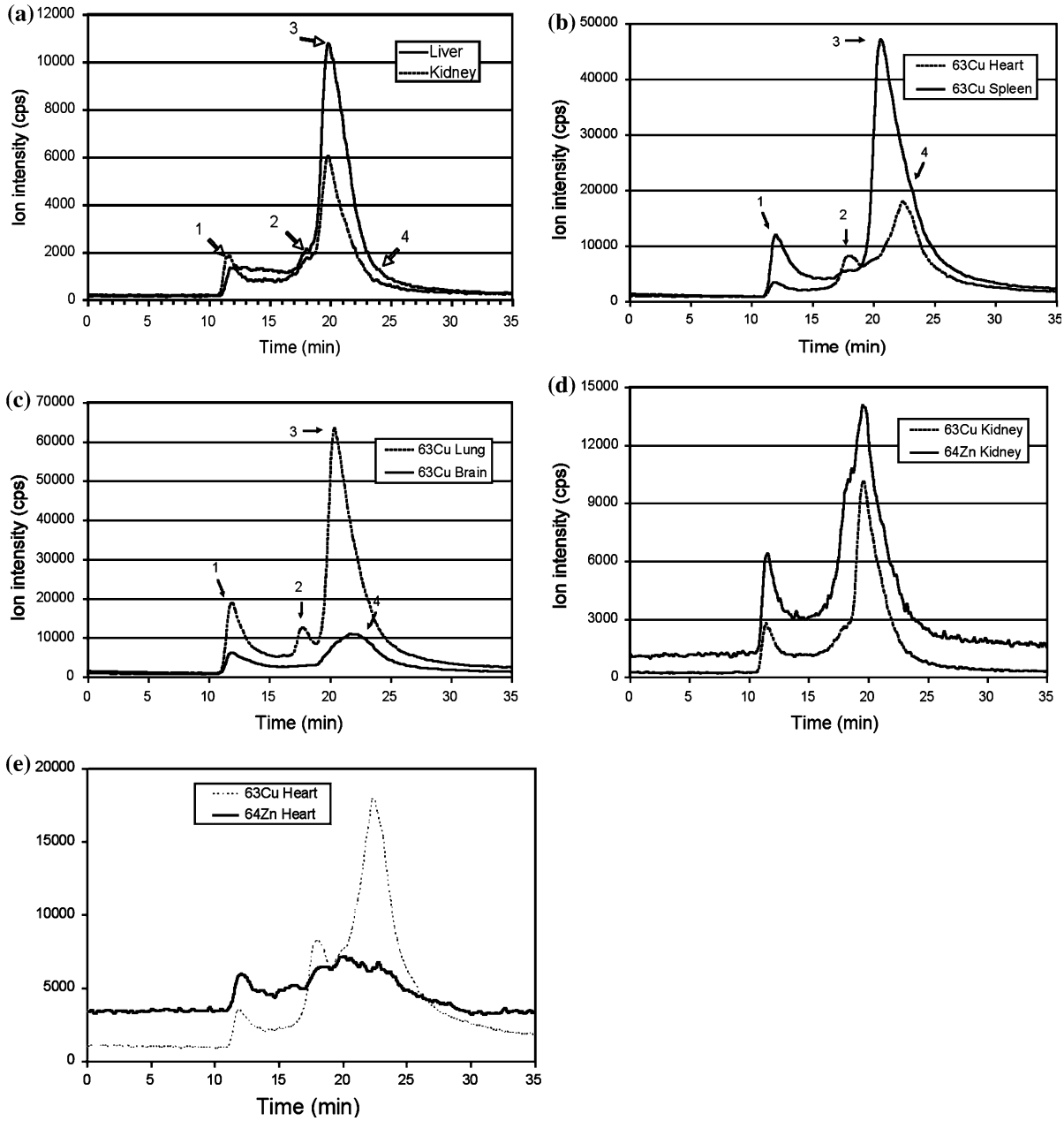

Fig. 4 Size exclusion HPLC separation of copper (and zinc) binding components in cytoplasm of tissues of normal adult female mice. Samples of cytoplasm obtained by centrifuging fresh homogenates for 1 h at 105,000*g* (see "Methods") were applied to the same large-pore size exclusion HPLC column as for plasma (Figs. 2, 3) and plotted in the same way. Representative ^{63}Cu elution profiles for (**a**) liver and kidney, (**b**) heart and spleen, (**c**) lung and brain have been superimposed. The Mr values for peaks/shoulders 1–4 were calculated to be >1000, 110, 45, and 11 k, respectively. In **d** and **e**, the zinc elution profiles for the same samples of kidney (**d**) and heart (**e**) cytosol have been superimposed on those for copper

induced displacement and transfer of preexisting metal from other sites in the animal.

Figure 6 shows the temporal changes in extrinsic ^{65}Cu in the tissues and plasma from 30 min after the last injection until 24 h (a) or 2 weeks (b) later, expressed as a percentage of the administered dose. (Values for plasma and brain in the figure have been multiplied for visual clarity.) Again, the

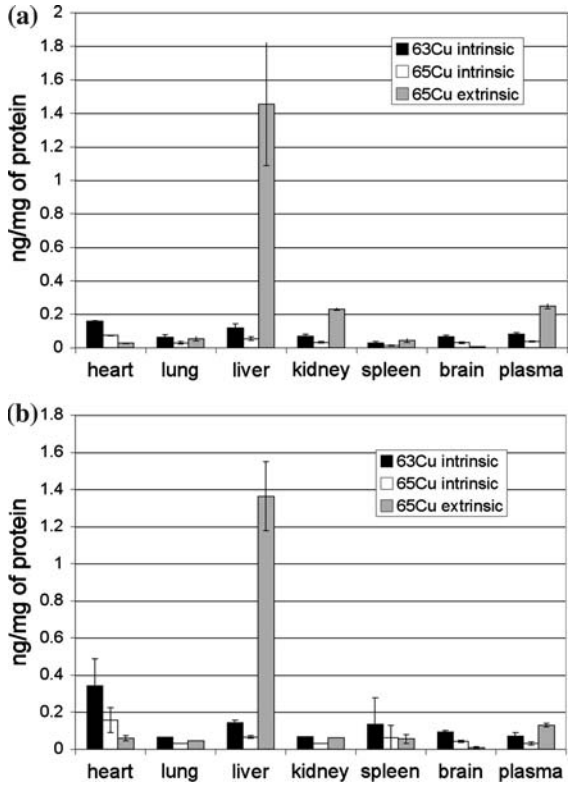

Fig. 5 Copper contents of mouse tissues 2 and 24 h after injections of large doses of ^{65}Cu. Concentrations of intrinsic ^{63}Cu (black bars), intrinsic ^{65}Cu (clear/white bars), and extrinsic (excess) ^{65}Cu (grey bars), in nmol/mg tissue protein, were determined 2 h (**a**) and 24 h (**b**) after the last i.p. injection of a 25–30 μg doses of ^{65}Cu. Error bars are average deviations of values for pairs of mice

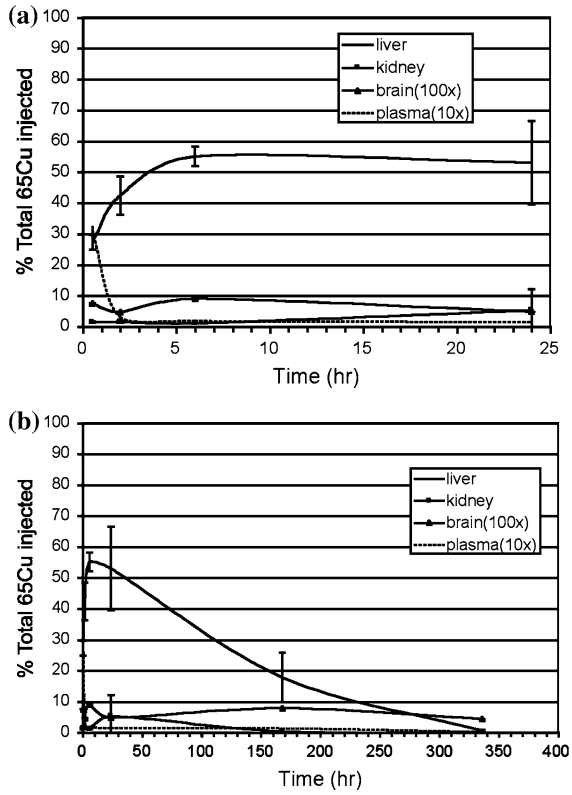

Fig. 6 Time course of distribution of extrinsic (excess) ^{65}Cu to tissues and plasma at various times after the last injection of the stable isotope (as % of dose). Error bars are average deviations of values for pairs of mice. Data for plasma and brain have been multiplied 10× and 100×, respectively, to make the changes more visible. (**a**) Results for the first 24 h; (**b**) results for the full time course of 14 days (after the last injection)

overwhelming importance of liver deposition of the injected copper is apparent, the kidney being of secondary importance, and relatively little deposition occurring in any of the other organs analyzed. Data from liver taken 30 min after the last (of 5) injection of ^{65}Cu showed that this organ contained approximately 30% of the total administered dose. By 6–24 h post-injection, over 50% of the dose was retained in the liver, after which time rapid loss was observed. Thus, from day 1 to day 7, the liver eliminated 40% of the injected copper, a further 17% being lost by the end of the second week. This indicates a highly efficient excretory mechanism, presumably involving the bile (Burstein et al. 2005; Harada et al. 2005; Linder 1991, 2002; Linder and Roboz 1986; Turnlund et al. 1989, 1998). Indeed, mass balance calculations of the total extrinsic ^{65}Cu content of the analyzed tissues at each time period showed incomplete recovery of the administered dose, with no more than 60% being accounted for at any time tested. This is consistent with either a very high initial rate of excretion (during the period of isotope administration prior to the last injection) and/or partitioning into tissues such as the bone, skin, viscera, etc. that were not analyzed.

The changes in the proportion of the total extrinsic ^{65}Cu accounted for by the cytoplasm of each organ, and temporal changes in these proportions, were also calculated (Table 1). In the case of the liver, most of the extrinsic ^{65}Cu was cytoplasmic after 24 h, and this proportion remained essentially constant for the duration of the experiment (data not shown). In the case of the kidney, just over half was initially in the cytoplasm, and this declined slowly over the

Table 1 Distribution of extrinsic ^{65}Cu among tissues and the proportion in their cytoplasms

Tissue	Total extrinsic ^{65}Cu (nmol/mg protein)	Extrinsic ^{65}Cu in Cytoplasm	
		(nmol/mg protein)	(%)
Liver (up to 24 h)	1.15 ± 0.38 (8)	0.95 ± 0.35 (8)	83
Kidney (up to 6 h)	0.19 ± 0.06 (6)	0.11 ± 0.04 (6)	55
(7–14 days)	0.039 ± 0.023 (4)	0.015 ± 0.009 (4)	38
Heart (up to 7 days)	0.092 ± 0.026 (10)	0.023 ± 0.007 (10)	25
Lung (up to 7 days)	0.084 ± 0.013 (8)	0.058 ± 0.009 (9)	69
(14 days)	0.043, 0.035 (2)	0.036, 0.033 (2)	89
Spleen (up to 24 h)	0.058 ± 0.015 (8)	0.040 ± 0.030 (4)	69
(7–14 days)	0.016 ± 0.010 (4)	0.005 ± 0.003 (4)	31
Brain (up to 24 h)	0.0085 ± 0.0027 (8)	0.0056 ± 0.0019 (7)	65
(7–14 days)	0.0072 ± 0.0019 (4)	0.0069 ± 0.0023 (4)	96

Values are Means ± SD (N) for samples taken at different times after the last injection of ^{65}Cu

following 7–14 days. In this respect, it is of interest to note that the kidney remained enriched with ^{65}Cu longer than the liver (Fig. 6b), and that most of the extrinsic ^{65}Cu remaining at 7 and 14 days was not in the cytoplasm (Table 1). Similarly, the spleen showed a marked reduction in the proportion of extrinsic ^{65}Cu retained in the cytoplasm. In contrast, the cytoplasmic content of extrinsic ^{65}Cu in the brain and lung increased from initial values of about 65% to account for more than 89% of the retained dose by 7–14 days. However in heart cytoplasm, the proportion stayed relatively low and constant.

To detect and compare the changes in enrichment of ^{65}Cu in the cytoplasmic copper binding proteins, samples from the liver (Fig. 7a), kidney (Fig. 7b) and brain (Fig. 7c) 2 h, 6 h and 14 days after the last ^{65}Cu injection were fractionated on the Biosep 4000 column. The relative degree of Cu enrichment of each protein was determined by comparing the increases in the absolute intensities of the ^{65}Cu signal and the ^{63}Cu/^{65}Cu ratios at the peak centroid. In the untreated mice, the isotopic ratios of Cu ^{63}Cu/^{65}Cu were invariably lower than the expected natural geological abundance, presumably due to interferences caused by the buffer, averaging 1.70 ± 0.13 (Mean ± SD; $N = 9$). Nevertheless, following injection of ^{65}Cu, a pronounced decrease in the ^{63}Cu/^{65}Cu ratios was noted in all the liver and kidney components (Fig. 7a, b). In contrast, the ^{63}Cu/^{65}Cu ratios in the brain showed little change over the treatment period (Fig. 7c) indicating minimal accumulation of ^{65}Cu in the protein components of this organ.

In general, the elution profiles and patterns of turnover of ^{65}Cu in the fractionated cytoplasmic proteins of liver and kidney (Fig. 7a, b) were very similar. In addition to having elevated ^{65}Cu in the void volume and 110 kDa components (peaks 1 and 2), and in the dominant 45 kDa peak (No. 3) seen in untreated mouse cytoplasm (Fig. 4a), most of the administered extrinsic ^{65}Cu 2 h post injection was bound by peaks 4 and 5 (Mr about 28 and 11 k). Neither peak 4 or 5 bound significant amounts of Cu in control animals (Fig. 4a, 7a, b, dashed line). After the ^{65}Cu treatment, the general background signal (between 12 and 17 min) of ^{65}Cu in the chromatogram, increased markedly as well. In the liver (Fig. 7a), there was considerably less copper at 6 h than 2 h, with ^{65}Cu being eliminated most rapidly from the largest and the 28 kDa component and least rapidly from the 11 kDa moiety. Similar patterns of ^{65}Cu depletion were observed in the cytoplasmic components of the kidney (Fig. 7b), although significantly less ^{65}Cu was associated and consequently lost from the void volume peak. Turnover of the metal in peak 3 was significantly more rapid in the liver than the kidney. There was almost complete turnover of metal (or specific loss of the injected ^{65}Cu) in the former (but not the latter) by day 14. By 14 days post injection, much of the injected copper had been eliminated from the liver and kidney cytosol, although the isotopic ratios clearly indicated long-term retention of a proportion of the administered ^{65}Cu. At this time, the profiles of ^{63}Cu and ^{65}Cu on the various peaks were closely aligned, implying exchange and equilibration of the intrinsic and extrinsic Cu between the cytoplasmic proteins in these organs (Fig. 7a, b). Unlike the liver and kidney, the ratios of ^{63}Cu/^{65}Cu in the cytosolic components in

the brain did not change significantly from that of the untreated mice, even 30 min (data not shown) and 2 h post injection. The pool of copper in this organ was thus essentially pharmacokinetically isolated from the administered injection of ^{63}Cu.

The response of the blood plasma proteins to the excess copper was also examined. Figure 8a–e show that 30 min to 6 h post injection, most of the extrinsic ^{65}Cu was associated with albumin (peak 3, at 18.7 min). In comparison, ceruloplasmin and macroglobulin (peaks 1 and 2, at 15.5 and 16.8 min) bound much less ^{65}Cu, although their isotopic ratios still indicated a substantial enrichment with ^{65}Cu. Plasma concentrations of ^{65}Cu diminished rapidly, as shown by the increase in the ^{63}Cu/^{65}Cu peak ratios from 30 min to 2 h after the last isotope injection (Fig. 8a, b), and subsequently between 6 h and 14 days after injection (Fig. 8c, d). It should be noted that except right after injection, when albumin was primarily responsible for binding the excess copper, enrichment of the three main copper components (macroglobulin, ceruloplasmin and albumin) appeared to be about the same, consistent with the rapid entry, turnover and equilibration of the copper in these components.

Discussion

Our analyses of copper in the plasma/serum and tissues indicates that its distribution in the mouse is similar to the human and rat but also differs significantly in some aspects. The most striking difference is that mice have less than half as much copper in the circulating blood plasma as humans and

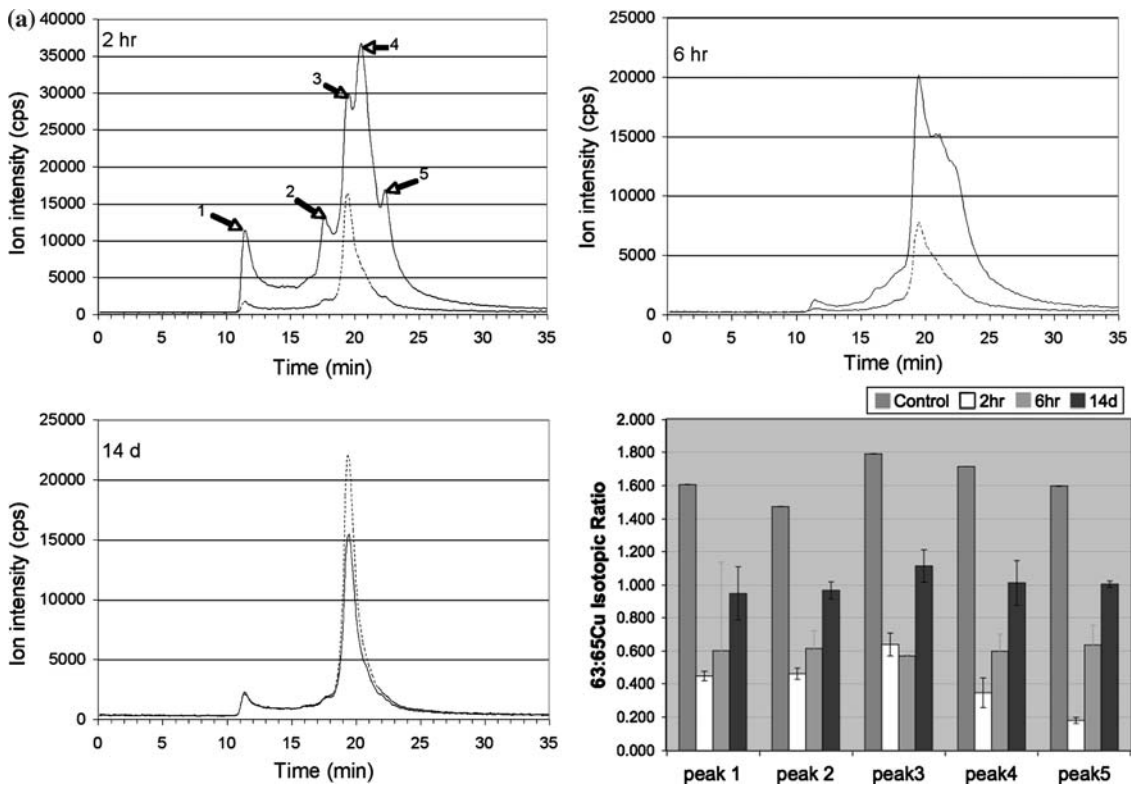

Fig. 7 Distribution of excess copper among components of tissue cytoplasm at various times after the final injection of ^{65}Cu. Profiles of total ^{63}Cu (dashed line) and total ^{65}Cu (solid line) associated with copper binding components of the cytoplasm, 2 h, 6 h and 14 days after the last ^{65}Cu injection, separated by size exclusion HPLC on the large-pore Biosep 4000 column. Elution time (min) on the *x*-axis is plotted against the ion intensity, which indicates copper quantitation. Also shown for each tissue (at bottom) is the ratio of ^{63}Cu/^{65}Cu for individual peaks, at the times indicated (control, 0-time ratios being from un-injected mice). Error bars represent average deviations for values from pairs of mice. Results are for liver (**a**), kidney (**b**) and brain (**c**). Copper peaks/shoulders 1–5 were calculated to have Mr values of >1000, 110, 45, 28 and 11 k, respectively

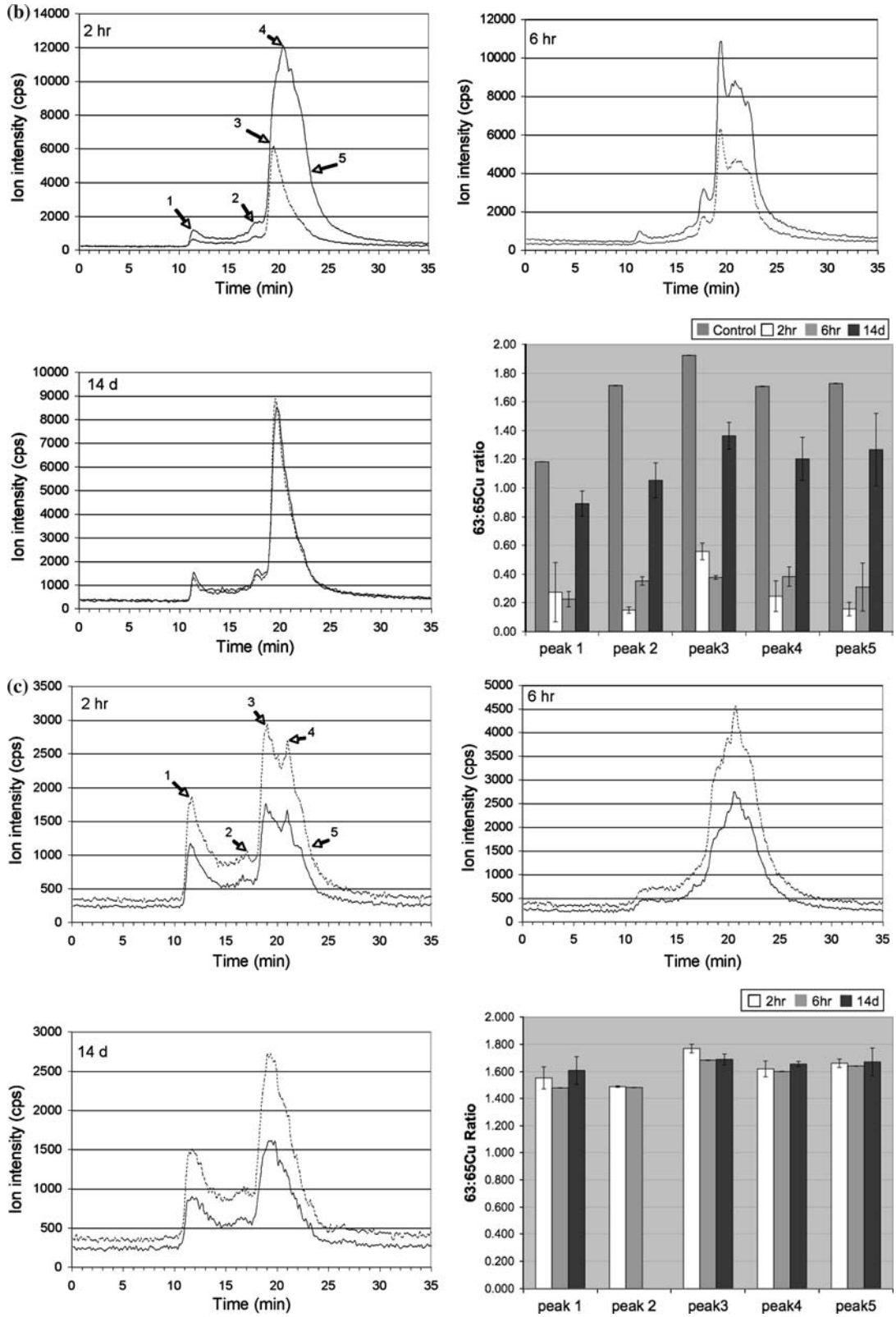

Fig. 7 continued

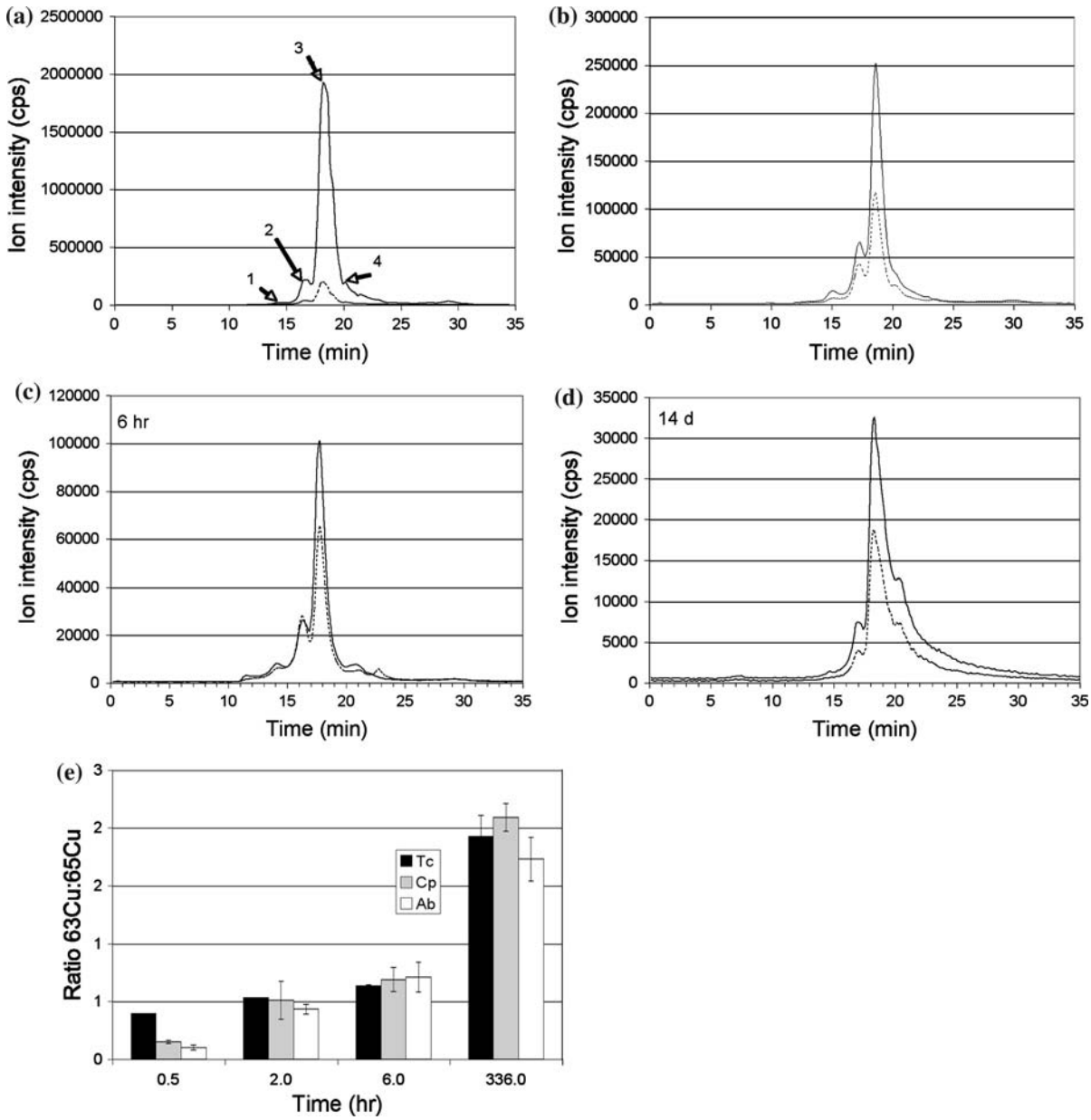

Fig. 8 Distribution of excess copper to components of blood plasma at various times after the final injection of ^{65}Cu. Profiles of total ^{63}Cu (dashed line) and total ^{65}Cu (solid line) associated with copper binding components 30 min, 2 h, 6 h, and 14 days after the last ^{65}Cu injection (**a**–**d**), as separated by size exclusion HPLC on the large-pore Biosep 4000 column (as in Fig. 7). (**e**) Ratios of ^{63}Cu/^{65}Cu for peaks 2, 3, 4 over time, the error bars indicating average deviations for values from pairs of mice. Copper peaks/shoulders 1–4 were calculated to have Mr values of about 450, 190, 78 and 45 k, respectively. [The numbering of peaks is not identical to that in the untreated mice (Fig. 2), where an additional component (peak 1) was detectable. Here, peak 2 (rather than 3) is ceruloplasmin]

rats. This is consistent with our earlier findings (Montaser et al. 1992) and is also in accord with the observation that mice also have less copper in their livers and kidneys. Thus, Meyer et al. (2001) reported liver concentrations of 2.6 ± 0.2 μg/g for 10–12 week old mice of the same strain used here, which is significantly lower than the values of 4.6 ± 1.1 and 6.2 ± 0.8 reported for rats and humans, respectively (Means ± SD, $N = 9$–23; Linder 1991. Similarly, mouse kidney values of

3.8 ± 0.1 (Meyer et al. 2001) are significantly less than the values of 7.9 and 12 for rats and humans, respectively (Linder 1991). Heart and brain did not show marked interspecies differences.

As concerns the copper binding components of plasma and serum, we report here for the first time the distribution of copper in the circulation of the mouse together with a refined picture for the human and rat. The current analyses confirm our previous studies and those of others that ceruloplasmin contains approximately two-thirds of the total copper in human and rat blood plasma (Barrow and Tanner 1988; Gless et al. 1992; Weiss and Linder 1985; Wirth and Linder 1985). This is contrary to the 95% still often quoted from the old and outdated literature (see Weiss and Linder 1985). Approximately 15% of the plasma copper from humans and rats eluted in the position of albumin, which is known to bind one copper ion very tightly at the N-terminus (Linder 1991; Masuoka et al. 1993); and 5–10% eluted with components of Mr consistent with the transcupreins alpha-2-macroglobulin (in humans) or alpha-1-inhibitor-3 (in rats) which bind copper even more tightly than albumin (Linder 1991, 2002; Liu et al. 2007; Masuoka et al. 1993). Our values for unclotted human blood plasma differ from those of Gless et al. (1992) for *serum* who reported 2.3%, 84% and 14% of total copper co-eluting with alpha-2-macroglobulin, ceruloplasmin and albumin, respectively. This difference may relate to the observation that a substantial proportion of the large (750 kDa) alpha-2-macroglobulin can become trapped in the clots removed when serum is harvested from whole blood (Salvatore Pizzo personal communication). The current studies indicate the mouse has a significantly smaller proportion of its plasma copper (about half) associated with the ceruloplasmin peak than humans and rats, and more associated with the macroglobulin/transcuprein and albumin peaks (about 10% and 25%, respectively).

Using size-exclusion HPLC-ICP-MS, we detected additional copper binding components in plasma, not previously reported. The first (Mr 15 k) was present in humans and mice and might be metallothionein, which is known to occur in serum (Garvey and Chang 1981; Mehra and Bremner 1983) and to elute as a seemingly larger protein (Kagi and Nordberg 1979). (Reliable antibodies were not available to verify the identity of this component.) A second in mice and humans was larger than transcuprein, eluting close to the void volume. A third (~45 kDa) was evident in plasma from all three species. It is noteworthy that no very low molecular weight copper components were detected. Thus, ceruloplasmin is clearly only one of a number of proteins in the blood fluid that bind quantitatively significant amounts of copper.

Although albumin normally accounts for only 15–25% of the copper in human, rat and mouse blood plasma, it has the potential for binding a great deal more. Indeed, calculations indicate that the albumin in 1 ml of plasma can theoretically bind more than 600 μg of copper with high affinity. This is many orders of magnitude more than that normally bound [about 100 ng/ml in mouse and 120–200 ng/ml in human plasma (Barrow and Tanner 1988; Linder 1991)]. This available binding capacity is clearly illustrated in the current study where approximately 28 μg ^{65}Cu was sequestered by the circulating albumin of one ml of mouse plasma within 30 min of the last injection (one ml being about equivalent to the total plasma volume of the mouse). Thus, albumin may be viewed as an important component of the buffering system of most mammals that acts in concert with other transfer and transport proteins to protect against acute copper toxicity that could otherwise be induced by free copper ions (Linder 2002; Rae et al. 1999).

The cytoplasmic distribution of copper in all of the major organs examined in the mouse showed its consistent association with 2 or 3 major components that were separable by size exclusion HPLC. This finding is comparable to earlier reports for Cu distribution in a more limited number of *rat* organs. Thus, rat liver, kidney (and intestinal) cytosols fractionated on Sephadex G75 or 100 showed three consistent components, one eluting in the void volume, one at about 35 kDa and another at about 15 kDa (Linder 1991; Norton and Heaton 1980; Sharma and McQueen 1981; Terao and Owen 1973). It has generally been accepted that the second and third peaks contain Cu/Zn superoxide dismutase and metallothionein (MT), respectively (Linder 1991). However, these reports preceded awareness of several other proteins, including the copper "chaperones", ATOX1 and COX17, presumed to be present in most cells, and which transport copper to the TransGolgi network and mitochondria, respectively. Since these are very similar to MT in apparent size (Linder 2002), they will contribute to copper in the 15 kDa peak

range. These proteins and MT did not separate very well from Cu/Zn SOD on the larger pore columns used in this study, generally eluting together in a broad (and large) peak. SOD and the others were best differentiated by comparing the heart (which has mainly the reputed MT/ATOX1/COX17 peak) with the kidney (which has mainly the SOD peak). The leading edge of the latter also co-eluted with a major Zn peak, consistent with Cu/Zn SOD. However, the SOD chaperone, CCS, is about the same size as SOD (Huffman and O'Halloran 2001) and would also elute there. A monomeric (48 kDa) form of S-adenosyl homocysteine hydrolase (Bethin et al. 1995), which also binds copper tightly and accounts for a significant portion of liver copper in the cytoplasm, should elute just ahead of SOD and would contribute to this peak as well. A dimer of the hydrolase might also account for the 110 kDa component in the various cytosols, since it binds copper as well, at least in liver, kidney and brain (to decreasing degree). Aggregates of the 23 kDa COMMD proteins [which include MURR1/COMMD1 implicated in biliary excretion and defective in the Bedlington terrier; Burstein et al. 2005)] also probably contribute to the copper components eluting in the 20–50 kDa range, as should the dimeric "S100b" protein (Nishikawa et al. 1997) particularly prevalent in brain. Thus, the large and broad copper peak in the 20–50 kDa region is presumably comprised not just of SOD and MT but several other proteins. Clearly, further studies involving more refined and powerful separatory techniques, such as multidimensional HPLC together with confirmatory molecular identification by techniques such as MALDI-TOF are necessary to resolve the specific molecules involved.

The identity of the largest copper binding component is still a complete mystery. Although a number of new, larger copper binding proteins have recently been identified in mammalian cells, these are bound to intracellular membranes or the plasma membrane (ATP7A and B; hephaestin; DMT1; CTR1; CTR2) (Arredondo and Nunez 2005; Linder 2002; Puig and Thiele 2002; van den Berghe et al. 2007). Prior studies had indicated it was larger than 100 kDa. Our current work using a larger pore column shows it to be a great deal larger (>1000 kDa). The absence of very small copper components may again be noted. Although evidence from cell culture has shown that copper can bind the tripeptide, glutathione, when given in high doses and preserved under anaerobic conditions, it is questionable whether this happens in vivo, since others have shown that cytoplasmic proteins can successfully out-compete GSH for Cu (Ohta et al. 2001).

Our observations that mice were able to tolerate the potentially toxic effects of large doses of copper and were able to rapidly eliminate the metal is consistent with previous observations made in humans and rats (Linder and Roboz 1986; Turnlund et al. 1989). The level of tolerance exhibited by the mice in the current study was particularly impressive. Based on the copper concentrations of various organs and tissues in mice and rats, we estimate that an adult mouse of 25–35 g contains a total of 35–50 μg copper. In this study we injected of 25–30 μg copper 5× over 14 h, for a total of about 141 μg, a dosage three times greater than the amounts of copper already present. This was not only tolerated (the mice did not alter their behavior after an injection) but our analyses of the amounts of copper remaining in the animals at various times after the last injection indicated that elimination was very rapid. Consistent with the well-established observation that the liver and kidney take up most of the new copper entering the circulation (Linder 1991), these organs were the only ones examined that responded to the copper treatment with large increases in tissue copper accumulation. Thirty minutes after the last injection, liver concentrations of copper (most of which was extrinsic ^{65}Cu) were 8x higher than normal, while those in the kidney were 2× the normal. The liver accounted for 30–55% of the total ^{65}Cu administered in the first hours after the last injection, the kidney only about 5%. Mass balance calculations indicated only 65% of the injected dose could be accounted for in the organs examined. This implied that either a considerable portion of the dose may have been already excreted during the 14 h injection period, or that the ^{65}Cu was sequestered in tissues not analyzed, such as the skin, viscera and skeleton. In humans, the bones are known to account for 40% of the total body copper (Linder 1991), and the release of newly absorbed ^{65}Cu from these compartments back into the blood would explain the continuing buildup of ^{65}Cu noted in the liver up to 6 h after the last injection. This buildup could not be reconciled with the fall in blood plasma ^{65}Cu alone, which could account for only 10–20% of that copper.

Over the following 2 weeks, the liver copper content showed an exponential decline, with a half-life of about 4 days. Except for the kidney, which remained enriched longer due to ^{65}Cu retained in non-cytoplasmic components (Table 1), similar rates of decline were observed in other organs, with only about 20% of the injected ^{65}Cu dose remaining after 7 days and approximately 5% after 14 days. This is similar to our previous findings for whole body copper in rats where we followed the loss of ng quantities of injected ^{67}Cu for 2 weeks (Linder and Roboz 1986). Most of the radioisotope turned over with a half-life of 2.5–3 days; a second compartment had a much longer half-life (8–10 days). Prior injection of 25 μg of copper (about 10% of the estimated total in a 150 g rat) increased liver copper concentrations about 50% but did not alter the turnover rate. The same and a previous study (Owen et al. 1975) indicated that 10–14% of the injected copper was lost in the bile and feces (and about 2% in the urine) over the first 24 h. All of this is consistent with the established concepts that (a) copper homeostasis is mainly controlled at the level of excretion, and mainly by the liver, which eliminates most of the excess through the bile (Linder 1991); (b) with the exception of dogs and sheep and individuals with specific genetic defects, such as in ATP7B in Wilson disease, most mammals are capable of dealing with unexpectedly large intakes of copper.

Finally, the current studies have shown the utility of stable isotopic Cu to study the long-term metabolism of the metal over physiologically relevant time scales beyond those feasible with radioisotopic forms of the element. Relatively small doses of the stable copper isotope, ^{65}Cu, have been used successfully in humans to study whole body intestinal absorption, transport and excretion kinetics, with the finding that long term retention and excretion of copper are inversely related to copper status and intake, those with low intakes retaining more and excreting less and vice versa (Turnlund et al. 1989, 1998). Studies in humans are obviously more limiting, however, and do not allow us to follow the turnover of copper in specific organs and their proteins, which is the advantage of using experimental animals. The current feasibility studies have shown that stable isotopic copper ^{65}Cu administered i.p. to mice results in the detectable enrichment of specific copper proteins in all organs but the brain, and particularly so in the liver and kidney. Although large doses of ^{65}Cu were administered, the data indicate that physiologically relevant doses of stable isotopic copper could be used in conjunction with HPLC-quadrupole ICP-MS (or more preferably, multicollector magnetic sector instruments) to successfully model the long-term homeostatic capabilities and turnover of copper in the individual binding components. This would be particularly possible in the tissues most actively involved in the metabolism and processing of this essential but potentially toxic trace element.

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