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Copper exposure modifies the content and distribution of trace metals in mammalian cultured cells

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Abstract

With this work, we have determined the cellular content of Cu, Fe and Zn in different cell lines, by using total reflection X-ray fluorescence spectrometry (TXRF). In addition, we examined whether cellular exposure to 100 μmoles l^{-1} of Cu-His modifies the intracellular content and distribution of these trace metals. Our results indicate that all the cell lines displayed the same pattern of relative intracellular abundance of trace metals (Cu<Fe<Zn). This pattern shifted to Fe<Zn<Cu, when extracellular copper concentration was increased. In HepG2 and Caco2 cells, copper exposure increased intracellular copper content while Fe and Zn decreased. Regarding cellular distribution of trace metals, they decreased in the cytosol fraction of HepG2 cells, after exposure to copper. Therefore, our results indicate that cellular adaptation to copper involves a new balance in the relative abundance of trace metals and induces changes in the cellular distribution of Cu, Zn and Fe.

Introduction

Copper is required for the function of several cuproenzymes, therefore the presence of the metal is essential for different physiological functions (Uauy *et al*. 1998). Copper, however, is able to generate free radicals and oxidize cellular component through its redox activity (Aust *et al.* 1985). These conflicting properties demand a close regulation of the intracellular copper level.

Cellular adaptation to high or low copper exposure depends on general mechanisms of copper management. This regulation enables the cells to control the intracellular level of this trace metal. Biochemical and molecular studies in different cell types have permitted an identification of several molecular components of copper metabolism. The mechanism of cellular Cu uptake includes the human copper transporters 1 and 2 (hCTR1/2) (Lee *et al*. 2002) and the divalent metal transporter-1 DMT1 (Gunshin *et al*. 1997). In the cytoplasm, copper is immediately transferred to GSH and metallothionein (MT) (Freedman & Peisach 1989; Steinebach & Wolterbeek 1994) that provide, along with copper chaperones, efficient and specific mechanisms for the intracellular copper transport and storage (Harrison *et al*. 2000; Palmiter 1998). Copper efflux, is mediated by P-type ATPases localized in trans-Golgi network (Mercer 2001). Therefore, Cu metabolism is a multiple step process containing several susceptible points of regulation.

An important aspect in the copper homeostasis study is the metabolic interaction of this metal with zinc and iron. Examples of this essential interplay occur in processes such as iron uptake, (de Silva *et al*. 1996) or in iron/copper activity in the regulation of ceruloplasmin, (Harris 1995) and zinc/copper activity in the regulation of metallothionein (Richards 1989; Harris 2001) and Cu/Zn superoxide dismutase (Linder & Hazegh-Azam 1996). However, not enough effort has been made to analyze the physiological interactions between these trace metals.

In this work, we have determined the cellular content of Cu, Fe and Zn in different mammalian cell lines, by using total reflection X-ray fluorescence spectrometry (TXRF). This method requires a minute amount of material and permits the simultane-

ous analysis of the different trace metals, this makes TXRF a suitable method to evaluate, with high precision and accuracy, changes in Cu, Zn and Fe content under identical biological conditions (Gonzalez *et al.* 1999). In addition, we examined whether an increased concentration of copper in the cultured medium modifies the intracellular content and distribution of these trace metals. Our results indicate that the different cell lines display the same pattern of relative intracellular abundance of these trace metals: Cu<Fe<Zn. In all the cell lines analyzed, this pattern shifts to Fe<Zn<Cu, when extracellular copper concentration is increased. In addition, a change in their distribution between soluble and particulate fractions was observed in HepG2 cell line. Regarding the net content of these metals, we observed a decreased of Fe in F805, HepG2 and Caco2 when they were exposed to high concentrations of copper.

Materials and methods

Cell culture

The experiments were performed using cell lines from different species, their selection was based on the fact that they have been used in several studies of trace metal homeostasis. Clonal human cell lines were HepG2 (from hepatoblastoma), Caco-2 (from a primary colonic tumor), and HeLa (from adenocarcinoma of cervix). Clonal cell lines from mice were NIH 3T3 (from embryonic fibroblasts), and N2A (from neuroblastoma). The F805 cell line was originated by tripsinization of mouse embryos from day 11 of gestation and immortalized with SV40 virus (Kelly & Palmiter 1996). Clonal cell lines from rats were B12 (from glioblastoma) and fibroblast NRK (from normal rat kidney). All cell lines were incubated at 37 °C in a 5% CO_2 atmosphere and grown in plastic cell culture flasks (Nalge Nunc Int. Corp.) containing Dulbecco's modification of Eagle's medium (DMEM) and 10% fetal bovine serum (SFB). The concentrations of elements in this basal culture medium were Cu 0.4; Fe 2.7, and Zn 3.8 μmoles l^{-1}.

Copper treatments

HepG2, Caco2 and F805 cells were treated with an excess of copper (100 μmoles l^{-1}) in the culture medium. Copper was supplemented as Cu-His complex (1:10 molar ratio) (Gonzalez *et al.* 1999). After 48 h of copper exposure, control and treated cells were subjected or not to subcellular fractionation before quantification of trace metals (see next section). Relative survival of the cells exposed to copper was evaluated using trypan blue and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assays as described (Denizot & Lang 1986).

Quantification of intracellular elements

The cells were processed as described (Gonzalez *et al.* 1999). Briefly, cells at 80% of confluence were washed twice with phosphate buffered saline (PBS) and harvested using trypsin-EDTA (5.3 mM), trypsinization was stopped by dilution with 10 volumes of PBS. Immediately cells were sedimented by centrifugation at 3,500 rpm in a Beckman GS-15R centrifuge for 5 min, resuspended in 1 ml of PBS, and re-pelleted. For total content determination, 1–2 $\times$ 10^6 cells were disrupted by incubation with 0.2 ml of PBS containing 0.1 N NaOH (PBS-NaOH) to yield an H fraction (Homogenate). In some experiments, the harvested cells were washed once again with PBS-EDTA (10 mM). For subcellular distribution analysis, a pellet of 4–6 $\times$ 10^6 HepG2 cells was lysed by 3 freeze and thaw cycles in 0.5 ml of PBS. These lysates were then centrifuged at 100,000 *g* for 60 min at 4 °C to yield soluble (S) and particulate (P) fractions. The latter fraction was resuspended in 0.5 ml of PBS-NaOH. Aliquots of H, S and P were kept for protein determination by Bradford assay (Bradford 1976) and the remainder of each fraction was mixed (1:1, v/v) with nitric acid (Merck suprapure) and digested at room temperature for 48 h.

The analysis by TXRF was performed using a Seifert EXTRA-II spectrometer (Rich. Seifert & Co.). Spectra were acquired at 50 kV and 20 mA for 1000 s and a dead time of 30%. For each case, one fifth of the digested sample volumes was standardized with 1 μg ml^{-1} of selenium standard solution (Merck). An aliquot of 5 μl was then taken and deposited on previously cleaned quartz made reflector mounted on a vitro-ceramic plate, at a temperature of 50 °C (Gonzalez *et al.* 1999).

Statistical analyses

In all of the experiments variables were tested in triplicate samples and most of the time the analyses of each sample was repeated at least twice. One-way ANOVA (Zar 1984) was used to test differences in means, and post-hoc *t*-test was used for comparisons using STATA 6.0 (Stata Statistical Software).

Results

Cellular adaptation to fluctuations in copper exposure is based on homeostatic mechanisms that regulate uptake, storage and efflux of copper. These are responsible for the control of intracellular levels of the metal under excess and deficit of copper. This study, based in the analysis of different cell lines, shows that extracellular copper fluctuations in turn modify intracellular copper content and modulate the content of Zn and Fe. These fluctuations also affect the cellular distribution of these trace metals.

Copper, iron and zinc content in mammalian cell lines

We used TXRF method to determine the contents of Cu, Fe and Zn in different mammalian cell lines, which were maintained in a basal culture medium (see Materials and Methods). The results, which are summarized in Figure 1, show that the order of the relative abundance for the different elements was Cu<Fe<Zn in all the cell lines examined. A comparative analysis of the relative content of each trace metal in the different cell lines indicated that Zn/Fe ratios fluctuated between 1.4 and 2.4; Zn/Cu ratios between 5.5 and 21.1 and Fe/Cu ratios between 3.0 and 12.3. Among cell lines, the values of the contents for each trace metal were similar, except for Caco-2 and HepG2 cells, which showed a significantly higher amount of copper ($P < 0.05$) and F805 cells with a higher amount of zinc and iron ($P < 0.05$) (Figure 1).

Metal content in cell lines exposed to copper

In order to evaluate whether an increased amount of extracellular copper modifies the intracellular concentrations of trace elements, Caco2, HepG2 and F805 cells were grown in basal culture medium, which contains 0.4 μmoles l^{-1} copper, and then transferred to a medium supplemented with 100 μmoles l^{-1} Cu-His. After 48 h of treatment, TXRF analysis showed that in comparison with control cells (Figure 2A), the intracellular copper content of treated cells increased 13.8, 28.1 and 2.5-fold, respectively (Figure 2B). We have calculated that up to 10% of total cellular copper corresponds to a pool of copper associated to plasma membrane surface that can be removed by using the metal chelator EDTA (data not shown). The relative survival of the cells exposed to 100 μmoles l^{-1} Cu-His did not change, as determined by trypan blue and MTT reduction assay (data not shown). Regarding the other elements, we observed a significant decrease of

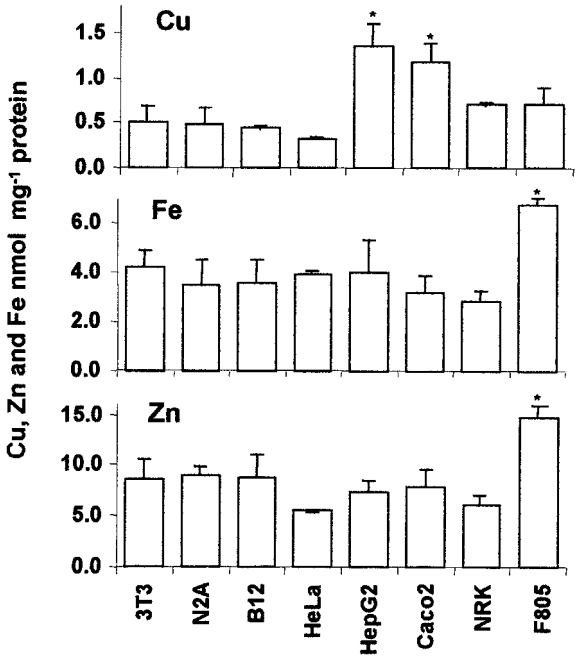

Fig. 1. Total content of copper, iron and zinc in different mammalian cell lines. Cell were grown in basal culture medium, containing 0.4 nmoles l^{-1} of copper. Trace metal contents were measured by TXRF. The values correspond to the average of three different samples. $P < 0.05$ (*).

Fe content in the three cell lines, while Zn content decreased in HepG2 and Caco2 cells (Figure 3). Interestingly, in comparison with Zn/Cu or Fe/Cu, the Zn/Fe ratio for these cells was practically not affected, indicating that the major element affected by the rise of extracellular copper, is copper itself.

Copper, iron and zinc distribution in HepG2 cell line

In addition, we wished to test whether cellular adaptation to copper excess might involve changes in the localization of Cu, Zn and Fe, since during storage and transport processes, these elements can interact with metal-proteins in different cellular compartments. Therefore, intracellular trace metal distribution was examined in HepG2 cells, after 48 h of exposure to 0.4 or 100 μmoles l^{-1} copper, by quantifying the metal content in the soluble (S) and particulate fractions (P) (Table 1). Values were normalized to 100% for total metal content in the cellular homogenate (H). The results indicate that copper and zinc were differentially distributed in S and P fractions. Thus, in control cells, Cu and Zn were mainly found in S. When HepG2 cells were supplemented with 100 μmoles l^{-1} of Cu-His, the percentage of each trace metal de-

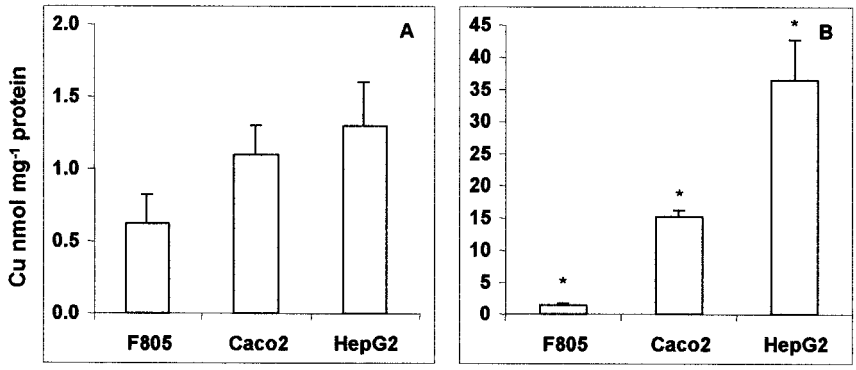

Fig. 2. Copper content in F805, Caco2, HepG2 cells exposed to 0 (A) and 100 μmol l^{-1} (B) of Cu-His for 48 h. The cellular content of copper in treated and control cells was determined by TXRF. The values correspond to the average of three different samples. $P < 0.05$ (*).

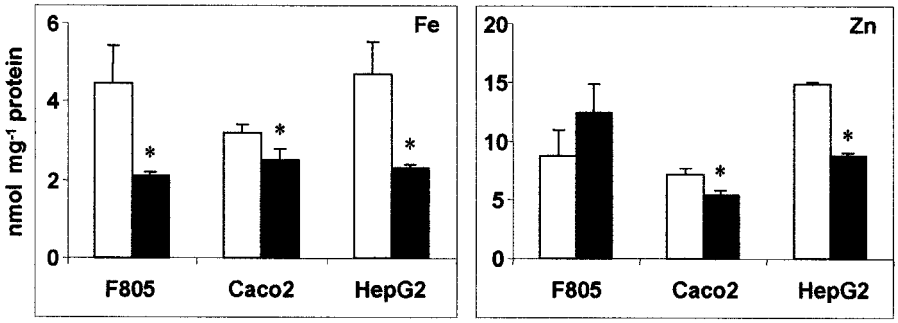

Fig. 3. Iron and Zinc content in F805, Caco2 and HepG2 cells exposed to 0 (white bars) and 100 μmol l^{-1} (black bars) of Cu-His for 48 h. $P < 0.05$ (*).

creased in S and increased in P fraction, as compared with the control cells (Table 1). Under this condition, the concentration of copper had a significant increase ($P < 0.05$) in S (from 13.1 ± 1.2 to 400.4 ± 69.4 nmoles mg^{-1} protein) and P fractions (from 0.3 ± 0.04 to 16.4 ± 0.89 nmoles mg^{-1} protein). These results indicate that exposure to excess of copper induce changes in cellular distribution of Cu, Zn and Fe.

Table 1. Total content (H) and distribution of Cu, Zn and Fe between soluble (S) and particulate (P) fractions of HepG2 cells.

	Cu-His μmol l^{-1}	H nmol mg^{-1} prot	S (%)	P (%)
Cu	0	1.3 ± 0.5	81.7	18.3
	100	36.5[2] ± 6.3	56.9	43.1
Fe	0	4.7 ± 0.8	48.5	51.5
	100	2.3[2] ± 0.1	35.8	64.2
Zn	0	14.9 ± 0.1	77.8	22.2
	100	8.8[2] ± 0.2	65.3	34.7

[1] Values are mean ±SD, $n = 3$.
[2] $P < 0.05$

Discussion

The current report represents an effort to define, under similar culture conditions, the content of trace metals in several mammalian cell lines. These cell lines were selected based on the fact that they are commonly used in cellular and molecular studies of Cu, Zn and Fe metabolism. In this work, we took advantage of the small sample size required for TXRF analysis, which was compatible with standard cell culture conditions. This method has been use in the analysis of different kinds of samples, including water, plants, animal tissues and cell lines (Gunther & von Bohlen; 1990, Klockenkämper & von Bohlen, 1996; Brown *et al.* 1997; Gonzalez *et al.* 1999). Here, the multi-element analysis by TXRF was particularly useful in the determination of cellular trace metal content, and permitted an evaluation of their modification in response to copper exposure.

The intracellular trace metal content of the different cell lines analyzed in this work presented a

constant pattern in their relative abundance that was not dependent on the type of cells (neuronal, hepatic, glial, etc.), neither on the species that they came from (mouse, rat or human). In agreement with our observation, the same pattern (Cu<Fe<Zn) was determined in the human breast carcinoma cell line MCF-7 (Verhaegh *et al*. 1997) using atomic absorption spectrometry (AAS). Considering that each element is involved in essential biological functions, the existence of this intracellular pattern of relative abundance suggests that a conservative mechanism had been selected to maintain the intracellular concentration of trace metals within a certain range.

The content of these trace metals in the eight cell lines analyzed in this work indicated that the averages of the intracellular concentration of Zn, Fe and Cu were 8.6 ± 2.8, 4.0 ± 1.2 and 0.7 ± 0.4 nmoles mg^{-1} protein, respectively. Despite their variability, the values of trace metals are in strong agreement with the concentration reported for MCF-7 (Verhaegh *et al*. 1997) and for Caco2 cells (Arredondo *et al*. 2000). Particularly for copper, similar values have been reported in human fibroblasts culture cells (Goka *et al*. 1976) MCF-7 cells (Verhaegh *et al.* 1997) and hepatic cells (Stockert *et al*. 1986; Foster *et al*. 1991; Schilsky *et al*. 1998). Two of the cell lines, HepG2 and Caco2 are cellular models for hepatic and intestinal tissues, respectively. In agreement with the essential roles of these tissues in the control of storage and absorption of copper, these cells contained a significantly higher amount of this metal as compared with the others cell lines, supporting their use in copper metabolism studies.

When we increased the extracellular concentration of copper in the culture medium from 0.4 μmoles l^{-1} to 100 μmoles l^{-1}, which represent a 225 fold increment, the intracellular concentration of this metal increased up to 28-fold. This indicates that the analyzed cells were able to control their intracellular content of the metal by regulating the storage through copper uptake, efflux or both. We have measured similar increments for Caco2 (Arredondo *et al*. 2000) and HepG2 cells (our unpublished results) exposed to a lower concentration of copper (10 μmoles l^{-1}). This suggests that the capacity of cells to accumulate copper was saturated near the physiological concentration of the metal (Linder & Hazegh-Azam 1996). Since the relative survival of the cells exposed to 100 μmoles l^{-1} copper concentration did not change, appears that under higher copper exposure, uptake/efflux adaptive processes and not storage capacity protect cells from intracellular copper excess and its toxicity.

To our knowledge this is the first report that describes a decreasing in Fe content of cells exposed to copper. In Caco2, HepG2 and F805 cells exposed to 100 μM of copper the decreasing in iron content may be the consequence of a competence with copper for DMT1 mediate uptake, since DMT1 is able to transport several divalent cations. Interestingly, DMT1 can be up-regulated by an iron deficiency (Tandy *et al*. 2000; Yeh *et al*. 2000), through an IRE-dependent mechanism (Fleming *et al.* 1999). Recently, an increment of copper content (10-fold) in Caco2 cells, after copper exposure, was associated to an increase of ^{55}Fe uptake/transport as well as to the up-regulation of DMT1 (Han & Wessling-Resnick 2002). Thus our data is in agreement with a model where extracellular copper modulates cellular content of iron, which induce the expression of DMT1.

Regarding the change observed in the content of zinc, after copper exposure this trace metal decreased in HepG2 and Caco2 cells, but not in F805 cells. It will be interesting to analyze zinc contents under cellular exposure to different trace metals in order to determine whether its variation may be explained by competence for common molecular components involved in uptake, storage or efflux processes. Alternatively, zinc content might be the consequence of the differential expression of components that contribute to zinc homeostasis in different cell lines.

Cellular distribution and storage of trace metals can be envisioned as a highly regulated process that involves the binding of these elements to several components. Our result indicated that close to 80% of Cu and Zn and 50% of Fe are found in the cytoplasm, probably bound to MT and GSH (Cu and Zn) (Linder & Hazegh-Azam 1996) or to ferritin (Fe) (Garate & Nuñez 2000). After exposure to an excess of copper, the percentage of Cu, Fe and Zn in the cytosol fraction decreased, thus the increment of intracellular copper seems to promote interactions of trace metals with metallo-proteins present in the non-soluble fraction. In particular for copper, the observation that copper-resistant hepatic cell lines show a decreased content of cytosol copper, along with a threefold increase in copper transporter ATP7B protein (Schilsky *et al*. 1998), suggests that this protein may facilitate the exit of metal from the cytosol to the lumen of Golgi or directly to the extracellular medium (Mercer 2001). Therefore, the regulation of intracellular distribution of trace metals, as a function of cop-

per exposure, depends on the relative contributions of several components located at different cellular compartments.

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References

Aust SD, Morehouse LA, Thomas CE. 1985 Role of metals in oxygen radical reactions. *J Free Radic Biol Med* **1**, 3–25.

Arredondo M, Uauy R, Gonzalez M. 2000 Regulation of copper uptake and transport in intestinal cell monolayers by acute and chronic copper exposure. *Biochim Biophys Acta* **1474**, 169–176.

Bradford MM. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle dye binding. *Anal Biochem* **72**, 248–254.

Brown DR, Qin K, Herms JW, Madlung A, Manson J, Strome R, Fraser PE, Kruck T, von Bohlen A, Schulz-Schaeffer W, Giese A, Westaway D, Kretzschmar H. 1997 The cellular prion protein binds copper *in vivo. Nature* **390**, 684–687.

Denizot F, Lang R. 1986 Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Inmunol Meth* **89**, 271–277.

de Silva DM, Askwith CC, Kaplan J. 1996 Molecular mechanisms of iron uptake in eukaryotes. *Physiol Rev* **76**, 31–47.

Fleming RE, Migas MC, Zhou X, Jiang J, Britton RS, Brunt EM, Tomatsu S, Waheed A, Bacon BR, Sly WS. 1999 Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. *Proc Natl Acad Sci USA* **96**, 3143–3148.

Foster R, Jahroudi N, Gedamu L. 1991 5-Azacytidine increases the total cellular copper content and basal level metallothionein mRNA accumulation of human Hep G2 cells. *Biochim Biophys Acta* **1088**, 373–379.

Freedman JH, Peisach J. 1989 Intracellular copper transport in cultured hepatoma cells. *Biochem Biophys Res Commun* **164**, 134–40.

Gárate MA, Núñez T. 2000 Overexpression of ferritinIron-responsive element decrease the labile iron pool and abolishes the regulation of iron absorption by intestinal epithelial (Caco2) cells. *J Biol Chem* **275**, 1651–1655.

Goka TJ, Stevenson RE, Hefferan PM, Howell RR. 1976 Menkes disease: a biochemical abnormality in cultured human fibroblasts. *Proc Natl Acad Sci USA* **73**, 604–606.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S. 1997 Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482–488.

González M, Tapia L, Alavarado M, Tornero JD, Fernández R. 1999 Intracellular determination of trace elements by total-reflection X-ray fluorescence spectrometry in mammalian cultured cells. *J Anal At Spectrom* **14**, 885–888.

Gunther K, von Bohlen A. 1990 Simultaneous multielement determination in vegetable foodstuffs and their respective cell fractions by total-reflection X-ray fluorescence (TXRF). *Z Lebensm Unters Forsch* **190**, 331–335.

Han OM, Wessling-Resnick, M. 2002 Copper repletion enhances apical iron uptake and transepithelial iron transport by Caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* **282**, G527–G533.

Harris ED. 1995 The iron-copper connection: the link to ceruloplasmin grows stronger. *Nutr Rev* **53**, 170–173.

Harris ED. 2001 Zinc and copper: evidence for interdependence, not antagonism. *Nutr Rev* **59**, 281–285.

Harrison MD, Jones CE, Solioz M, Dameron CT. 2000 Intracellular copper routing: the role of copper chaperones. *Trends Biochem Sci* **25**, 29–32.

Kelly EJ, Palmiter, RD. 1996 A murine model of Menkes disease reveals a physiological function of metallothionein. *Nat Genet* **13**, 219–222.

Klockenkämper R, von Bohlen A. 1996 Elemental analysis of environmental samples by total reflection X-Ray fluorescence: a review. *X-Ray spectrom* **25**, 156–162.

Lee J, Pena MM, Nose Y, Thiele DJ. 2002 Biochemical characterization of the human copper transporter ctr1. *J Biol Chem* **277**, 4380–4387.

Linder MC, Hazegh-Azam M. 1996 Copper biochemistry and molecular biology. *Am J Clin Nutr* **63**, 797S–811S.

Mercer J. 2001 The molecular basis of copper-transport diseases. *Trends Mol Med* **7**, 64–69.

Palmiter RD. 1998. The elusive function of metallothioneins. *Proc Natl Acad Sci USA* **95**, 8428–8430.

Richards MP. 1989 Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism. *J Nutr* **119**, 1062–1670.

Schilsky ML, Stockert RJ, Kesner A, Gorla GR, Gagliardi GS, Terada K, Miura M, Czaja MJ. 1998 Copper resistant human hepatoblastoma mutant cell lines without metallothionein induction over-express ATP7B. *J Hepatol* **28**, 1347–1356.

Steinebach OM, Wolterbeek HT. 1994 Role of cytosolic copper, metallothionein and glutathione in copper toxicity in rat hepatoma tissue culture cells. *Toxicology* **92**, 75–90.

Stockert RJ, Grushoff PS, Morell AG, Bentley GE, O'Brien HA, Scheinberg IH, Sternlieb I. 1986 Transport and intracellular distribution of copper in a human hepatoblastoma cell line, HepG2. *Hepatology* **6**, 60–64.

Tandy S, Williams M, Leggett A, Lopez-Jimenez M, Dedes M, Ranesh B, Srai SK, Sharp P. 2000 Nramp2 expression is associated with pH dependent iron uptake across the apical membrane of human intestinal CaCo cells. *J Biol Chem* **275**, 1023–1029.

Uauy R, Olivares M, González M. 1998 Essentiality of copper in humans. *Am J Clin Nutr* **67**, 952S–959S.

Verhaegh GW, Richard M, Hainaut P. 1997 Regulation of p53 by metal ions and by antioxidants: dithiocarbamate down-regulates p53 DNA-binding activity by increasing the intracellular level of copper. *Mol Cell Biol* **17**, 5699–5706.

Yeh KY, Yeh M, Watkins JA, Rodriguez-Paris J, Glass J. 2000 Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression. *Am J Physiol Gastrointest Liver Physiol* **279**, G1070–G1079.

Zar JH. 1984 in: Biostatistical Analysis. 2nd ed. Prentice-Hall, Englewood, Cliffs, New York: 162–205.