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Proteomic Analyzes of Copper Metabolism in an In Vitro Model of Wilson Disease Using Surface Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectrometry

Han Roelofsen,* Reshma Balgobind, and Roel J. Vonk

Department of Pediatrics, Division of Nutrition and Metabolism, Center for Liver, Digestive, and Metabolic Diseases, University Hospital Groningen, Groningen, The Netherlands

Abstract In Wilson disease, mutations in the *ATP7B*-gene lead to hepatic accumulation of copper that becomes toxic when the hepatic binding capacity is exceeded, leading to oxidative stress and acute liver failure. Several proteins are probably involved in dealing with the excess copper and oxidative stress. As a first step towards biomarker discovery and analyzes of copper metabolism in Wilson disease patients we characterized copper-induced changes in protein expression in cell lysates and culture media from an in vitro copper-overload model using surface enhanced laser desorption/ionization (SELDI) proteomics technology. HepG2 cells were cultured for 48 h with a physiological (0.5 μM) or a pathological (100 μM) copper concentration. Samples were applied to weak cation exchange (WCX) proteinchip® arrays and chips were analyzed by time of flight (TOF)-mass spectrometry. Copper-coated IMAC chips were used to detect copper-binding proteins in cell lysate of copper depleted cells using buffers with increasing imidazole concentrations. Data from the 2 to 50 kDa range indicate that high extra-cellular copper substantially altered both intra-cellular protein expression as well as the composition of the secretome. In the lysate 15 proteins were found up-regulated, while 6 proteins were down-regulated. In culture media 21 proteins were increased while 4 proteins were decreased in abundance. Copper-coated protein chips revealed the presence of 18 high-affinity copper-binding proteins. Further identification is necessary to determine the exact cellular roles of the discovered proteins. J. Cell. Biochem. 93: 732–740, 2004.
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Key words: Wilson disease; copper metabolism; SELDI; proteomics; HepG2; liver

Copper is an essential trace element, important for proper functioning of a number of enzymes such as ceruloplasmin, lysyl oxidase, and cytochrome C oxidase [Linder and Hazegh-Azam, 1996]. Some copper-containing proteins are associated with diseases, e.g., Cu/Zn superoxide dismutase (amyotrophic lateral sclerosis), amyloid precursor protein (Alzheimer's disease), and prions (Creutzfeldt–Jakob disease) [Mercer, 2001]. Copper becomes toxic when its intracellular levels are not carefully regulated, as occurs in Wilson disease [Gollan and Gollan, 1998]. Wilson disease is caused by a mutation in a gene that encodes a copper transporting P-type ATPase named ATP7B or the Wilson disease protein. ATP7B is mainly expressed in liver and to a lesser extent in kidney, brain, and placenta [Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1993]. Affected patients accumulate large amounts of copper in their livers that ultimately leads to hepatitis and acute liver failure, probably due to oxidative stress caused by the excess copper [Sokol et al., 1994; Stohs and Bagchi, 1995; Britton, 1996]. In Wilson patients, biliary copper excretion is markedly reduced, which is considered the fundamental cause of hepatic copper accumulation [Gollan and Gollan, 1998]. Normally, ATP7B is localized to the trans-Golgi network, where it is involved in the incorporation of copper into ceruloplasmin [Hung et al., 1997; Yang et al., 1997; Schaefer et al., 1999a,b]. At increased serum copper levels, ATP7B protects the liver against copper toxicity by redistributing to the canalicular (apical) membrane of

*Correspondence to: Dr. Han Roelofsen, Department of Pediatrics, Division of Nutrition and Metabolism, University Hospital Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands. E-mail: j.roelofsen@med.rug.nl

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hepatic cells, where it mediates biliary copper excretion [Roelofsen et al., 2000].

Besides ATP7B, other mechanisms are operative to prevent copper toxicity. Normally, all intracellular copper in the hepatocyte is bound to so-called copper chaperones and other metal binding proteins such as metallothioneins (MTs), leading to a free intracellular copper concentration of less than 10^{-18} M [Rae et al., 1999]. Several copper chaperones exist that, after uptake, deliver copper to a specific copper-dependent protein [Mercer, 2001]. In conditions of copper overload, as occurs in Wilson disease, several proteins are probably involved in dealing with the excess copper and the oxidative stress. Expression of copper chaperones and other copper-binding proteins such as MTs may be up-regulated, copper uptake may be down-regulated and/or secondary pathways may be activated to remove copper from the cell. Furthermore, proteins involved in the protection against oxidative stress, e.g., glutathione peroxidase and superoxide dismutase, may be up-regulated. Also, as a reaction to liver damage, expression of apoptotic and/or anti-apoptotic factors may be altered, together with factors involved in liver regeneration. We plan to characterize these changes during the development of the disease in order to discover new targets for intervention. As a first approach, described in this publication, we developed an in vitro system of copper overload using HepG2 hepatoma cells cultured with physiological and pathological copper concentrations. HepG2 cells express ATP7B [Schaefer et al., 1999; Roelofsen et al., 2000] and in this respect do not resemble the situation in Wilson disease. However, by applying a copper concentration that is 200-fold higher than the physiological concentration for 48 h, a chronic copper stress is induced that mimics conditions in hepatocytes at the early stages of the disease. We expect that the cells will counteract the copper stress by the up-regulation of several protective mechanisms. A similar approach using copper-depleted HepG2 cells and 2D gel as a proteomics method was used recently by She et al. [2003]. For proteomic analyzes, we have applied surface enhanced laser desorption/ionization (SELDI)-based proteinchip technology, developed by Ciphergen Biosystems (Fremont, CA) [Merchant and Weinberger, 2000]. This method uses ProteinChip Arrays with different affinity surfaces (e.g., weak cationic or strong anionic exchange surfaces, hydrophobic surfaces) to enhance for the detection of proteins with specific biochemical characteristics. Biofluids or lysates are spotted on absorptive target spots. After removal of unbound proteins and interfering buffer components, the proteins that are retained on the array surface are analyzed using a time of flight (TOF) mass spectrometer. Data show that both the intracellular protein profile as well as the excreted protein pattern is substantially altered in reaction to a high extra-cellular copper concentration. Further identification of these proteins is necessary to determine their role in protection against copper toxicity.

MATERIALS AND METHODS

Cell Culture

The human hepatoma cell line HepG2 was routinely cultured in DMEM (high glucose) with glutamax, supplemented with 10% fetal calf's serum (FCS), 200 U/ml penicillin G and 200 μg/ml streptomycin (all from Gibco-BRL, Breda, The Netherlands). The average copper concentration in media, as measured by atomic absorption was 0.24 μM, and was solely derived from FCS. Before the start of the experiments, cells were washed three times with media without FCS. Thereafter they were cultured for 24 h on media without copper (copper depleted cells) or for 48 h on media with 0.5 μM $CuSO_4$ (physiological concentration) and with 100 μM $CuSO_4$ (pathological concentration). The copper concentrations as well as serum deprivation did not lead to cell damage as tested by lactate dehydrogenase measurements in the media. FCS was omitted from the media to avoid interference with the measurement of excreted proteins. The experiments were performed in quadruplicate for each copper concentration.

SELDI ProteinChip Analyzes

Cell lysates (approximately 1 mg/ml total protein) were prepared in 9 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and contained protease inhibitors (Complete, Roche, Almere, The Netherlands). Lyses buffer was added to the plates and cells were scraped off and transferred to an eppendorf tube. After centrifugation (5 min at 13,000 rpm) samples were applied to weak cation exchange (WCX2) ProteinChip Arrays containing 8 spots present in a 96-well format

bioprocessor (both from Ciphergen Biosystems). Samples were diluted 10-fold in binding buffer (100 mM NH_4-acetate + 0.05% Triton-X-100 pH 4.0). Culture media were directly applied to the WCX chip, diluted 2-fold with binding buffer. After binding for 30 min at room temperature, the different spots were washed three times with binding buffer and once with Milli-Q ultra-pure water (Millipore B.V., Amsterdam, The Netherlands). After drying, 3×0.5 µl of an energy absorbing molecule solution was added. Both a saturated solution of sinapinic acid (SPA) and a 5-fold diluted saturated solution of (α-cyano-4-hydroxycinnamic acid) (CHCA) in 50% acetonitrile (v/v/), 0.5% trifluoroacetic acid (v/v) were used. After drying the proteinchips were measured in a SELDI-TOF mass spectrometer (PBS II, Ciphergen Biosystems). In addition copper-coated IMAC chips (Ciphergen Biosystems) were used to detect copper-binding proteins in lysates from copper-depleted cells. The spots on the IMAC chips were coated with copper by incubating them for 5 min with a 100 mM $CuSO_4$ solution. The excess copper was removed by a brief wash with Milli-Q ultra pure water followed by a wash with 100 mM NH_4-acetate pH 4.0 followed by again a brief wash with MilliQ water. Thereafter the spots were washed twice with binding buffer (0.5 M NaCl in phosphate buffered saline (PBS) + 0.1% Triton). Next, the spots were incubated for 30 min with a 10-fold dilution of cell lysate from copper-depleted cells. After removal of the cell lysate each spot was washed three times with binding buffer with an increasing concentration of a competitor for copper-binding, imidazole (0–250 mM), followed by a water wash. After drying, 2×1 µl of a saturated solution of SPA in 50% acetonitrile (v/v/), 0.5% trifluoroacetic acid (v/v) was added. After drying the proteinchips were measured in a SELDI-TOF mass spectrometer according to an automated data collection protocol using proteinchip software 3.1. Before the measurements, the machine was calibrated using the All-in-1 Peptide Standard for the mass range lower than 20 kDa and the protein molecular weight calibrant kit for the mass range higher than 20 kDa (both from Ciphergen Biosystems). In total four samples for each condition (0.5 and 100 µM copper) were measured on two different WCX chips. This resulted in eight protein profiles for each condition which were analyzed for differences in expression using proteinchip software 3.1 with the integrated Biomarker Wizard™ cluster analyzes software (Ciphergen Biosystems) [Fung and Enderwick, 2002]. First peaks with a signal to noise ratio higher than 5 were selected which were clustered with peaks with similar masses in other profiles with signal to noise ratios higher than 2. Before cluster analyzes the baseline was subtracted and profiles were normalized using total ion current. Significant differences ($P < 0.05$) in peak height of particular masses between the two conditions (0.5 and 100 µM copper) were calculated by the software package using a Mann–Whitney non-parametrical test.

The coefficient of variation (CV) of individual peaks in replicate spectra was calculated by dividing the standard deviation by the mean peak height multiplied by 100%.

RESULTS

Profiling of Cell Lysate

To determine the effect of high copper concentrations on protein expression in liver cells as well as on protein excretion of liver cells, cell lysates, and media of HepG2 hepatoma cells, cultured in the presence of a physiological copper concentration (0.5 µM) Cu^{2+} and a pathological copper concentration (100 µM) were compared. Changes in protein expression were measured using proteinchip technology. For this purpose, WCX chips with a pH 4.0 binding buffer were used since initial experiments showed that these chips gave the best results in terms of peak number and background noise levels (not shown). Measurements were performed in the 2–50 kDa mass range where the sensitivity and resolution of the apparatus is the highest. In cell lysate 21 proteins were found that had a significant change in expression due to the increase in the extra-cellular copper concentration (Fig. 1B). Six proteins were down-regulated while 15 proteins were found up-regulated with increases in expression of 1.5 to more than 8-fold. Coefficients of variation ranged from 12 to 144% depending on the abundance of the particular protein and the sensitivity of the measurement. Interestingly, the mass range from 5,500 to 6,500 Da showed a number of quite strongly up-regulated proteins as depicted in Figure 1A. For easy comparison, in this figure the protein profiles of the four samples of each condition are depicted as gel views. In the gel view mode the peak height is

translated to the blackness level of a band. A search in the Swiss-prot database (www.expasy.org), using the tagident tool with a pI of 7 ± 10, indicated that the masses 6,036; 6,064; 6,092; and 6,164 Da probably are MTs considering their almost identical masses to known MTs and the fact that they can be induced by copper [Kagi and Schaffer, 1988]. The peptide with mass of 6,036 Da could be MT1H, MT1I, or MT2 (database mass 6,039; 6,040; and 6,042 Da, respectively). The peptide with mass of 6,064 Da is probably MT1G, MT1L, or MT1R (database mass 6,070; 6,068; and 6,062 Da, respectively). The peptide with mass 6,092 Da is probably MT1F (database mass 6,086 Da) and the peptide with mass 6,164 Da is likely to be MT1K (database mass 6,141). Previously it has been shown that MTIF, MT1G, and MT2 are differentially regulated by copper [Sadhu and Gedamu, 1988]. The other masses in Figure 1B did not lead to meaningful hits in the Swiss-prot search.

Profiling of Culture Media

Figure 2B shows differences in abundance of proteins detected in the media of cells cultured with 0.5 and 100 μM copper. In total 25 different proteins showed a significant change in abundance. Twenty one proteins were increased while only 4 proteins decreased. Coefficients of variation (CV's) ranged from 6 to 182%. Two proteins with masses of 6,071 and 6,099 Da showed the highest increase with factors 3.5 and almost 6, respectively. A Swiss-prot database search with these masses indicated that they are most likely MTs. The peptide with mass 6,071 Da is probably MT1L or R (database mass 6,068 and 6,062 Da, respectively). The peptide with mass 6,099 Da is probably MT1F (database

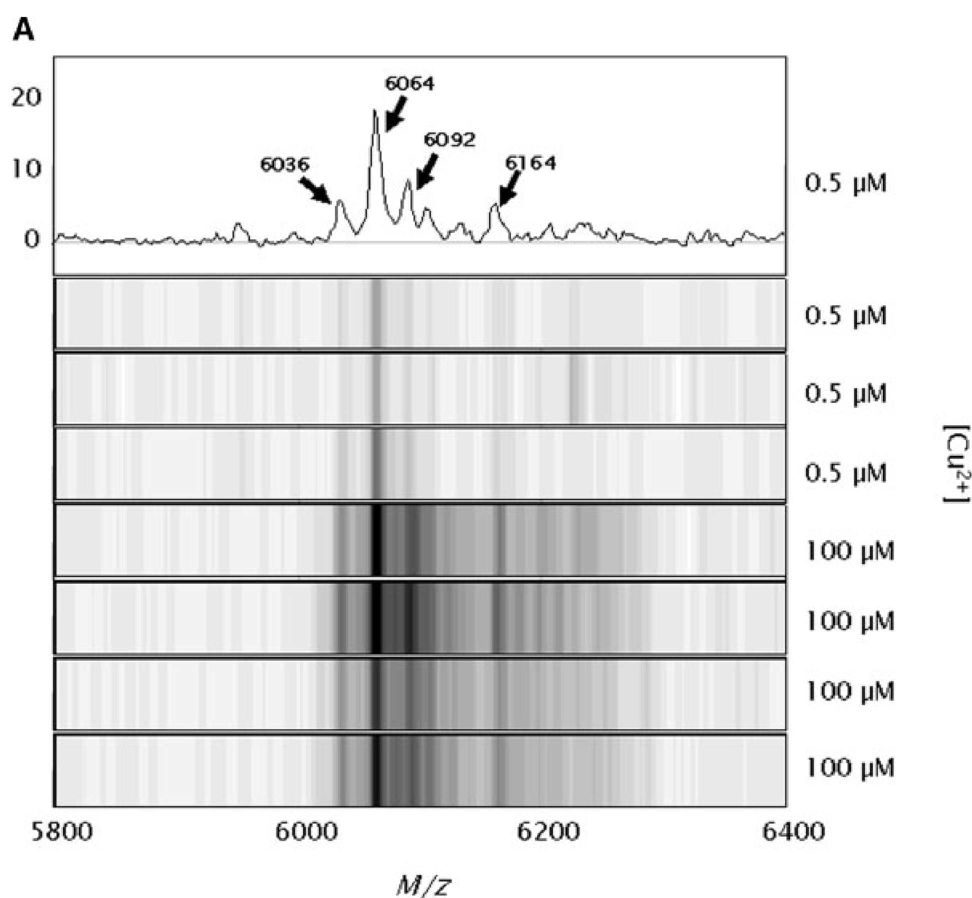

Fig. 1. Differences in protein abundance in cell lysates of HepG2 cells incubated with 0.5 and 100 μM $CuSO_4$. **A**: Differential display comparing cell lysates of HepG2 cells grown in 0.5 μM copper (**upper four traces**) or in the presence of 100 μM copper (**lower four traces**) on a weak cation exchange (WCX) ProteinChip Array. Mass range displayed 5,800–6,400 Da. Arrows show four proteins that are induced with the high copper concentration. Data were normalized for total ion current. Some of the traces are shown in gel view for easy comparison (see text). **B**: Overview of all significant differences ($P < 0.05$) in the 2–50 kDa mass range between the two conditions. Significance was calculated with a non-parametrical Mann–Whitney test.

B

M (Da)	0.5 μM Cu		100 μM Cu		fold change
	Mean	SD	Mean	SD	
4265	10.93	2.50	6.95	0.95	-1.6
4920	52.85	29.29	18.13	5.84	-2.9
4965	19.02	5.68	9.76	1.40	-2.0
4984	16.77	3.53	8.95	1.62	-1.9
5741	1.18	1.04	4.03	1.08	3.4
6036	2.71	1.06	7.00	2.17	2.6
6064	10.06	3.43	19.30	4.56	1.9
6092	3.86	1.65	11.28	2.41	2.9
6164	1.94	1.01	5.95	1.21	3.1
6203	0.28	0.19	2.25	1.16	8.2
6227	1.41	1.38	2.55	1.20	1.8
7998	0.37	0.29	1.27	0.58	3.4
10659	7.70	0.96	3.01	0.47	-2.6
12395	1.27	0.68	3.50	0.74	2.7
12466	0.93	0.40	3.08	0.68	3.3
12519	0.79	0.36	3.04	0.74	3.9
13752	6.34	3.67	9.43	2.79	1.5
14644	0.71	0.46	1.75	0.83	2.5
20131	1.57	2.25	0.59	0.43	-2.6
39610	0.49	0.30	0.77	0.28	1.6
41765	0.34	0.20	0.56	0.24	1.7

Fig. 1. (*Continued*)

mass 6,086 Da). Both peptides were also found up-regulated in cell lysate (Fig. 1B, masses 6,064 and 6,092 Da) suggesting that these MTs are excreted by the cell to bind copper in the media or excreted as such as a protective mechanism against copper toxicity. However, release of MTs by damaged cells cannot be excluded. Another interesting mass region in the culture media profiles is shown in Figure 2A. Four peptides are shown with masses of 7,420; 7,917; 7,988; and 8,177 Da that all are approximately 2-fold up-regulated (see Fig. 2B). However, a Swiss-prot database search did not lead to significant hits. The mass of 7,988 Da in the media profile is likely to correspond to the mass of 7,998 Da that was found up-regulated in the cell lysate (Fig. 2B) suggesting that this peptide is secreted after synthesis. Further identification is necessary to determine their cellular roles.

Discovery of Copper-Binding Proteins

We also explored the possibility to use IMAC ProteinChip Arrays to detect copper-binding proteins. These arrays are able to bind metals and were preloaded with 100 mM $CuSO_4$. Lysate from cells cultured in the absence of copper was applied directly to the copper-coated spots on an IMAC array. We assume that copper-depletion results in unoccupied copper-binding sites of proteins that will facilitate binding of these proteins to the copper-IMAC array. After binding, each spot on the array is washed with a buffer containing an increasing concentration of imidazole, a competitor for copper binding. As shown in Figure 3A, the increased stringency wash resulted in the disappearance of peptides with low affinity for the copper surface at imidazole concentrations as low as 10 mM (e.g., peptides of 6,667 and

6,711 Da). Others remain bound to the chip surface at concentrations of 250 mM imidazole and even became better detectable due to the lower complexity of the bound sample at this concentration of competitor (see, e.g., peptides 6,242; 6,285 Da). In total 18 peptides (see Fig. 3B) in the range of 2–50 kDa remained bound to the chip at the highest stringency wash (250 mM imidazole) indicating their ability to bind copper with high affinity. A Swiss-prot database search with the found masses did not lead to any relevant hits except for the 15,685 Da peptide that can be identified as the copper transporter hCTR2 (database mass 15,681). CTR2 has been shown to be expressed in the liver [Zhou and Gischier, 1997]. Two proteins with masses 13,742 and 41,719 Da listed in Figure 3B are also listed in Figure 1B (13,752 and 41,765 Da, respectively), considering a mass uncertainty of 0.1%, and are probably identical. This suggests that these two proteins function as copper-binding proteins and can be induced by high extra-cellular copper concentrations.

DISCUSSION

The data presented in this publication illustrate the possibilities of the SELDI proteinchip technology in studies on copper metabolism and oxidative stress. The data show that copper has profound effects on protein expression and protein excretion of liver cells. In total 21 proteins in cell lysate and 25 proteins in the culture media were found to react to a high extracellular copper concentration. In the 6,000–6,500 Da range of lysate and culture media, expression of several peptides was induced 2- to

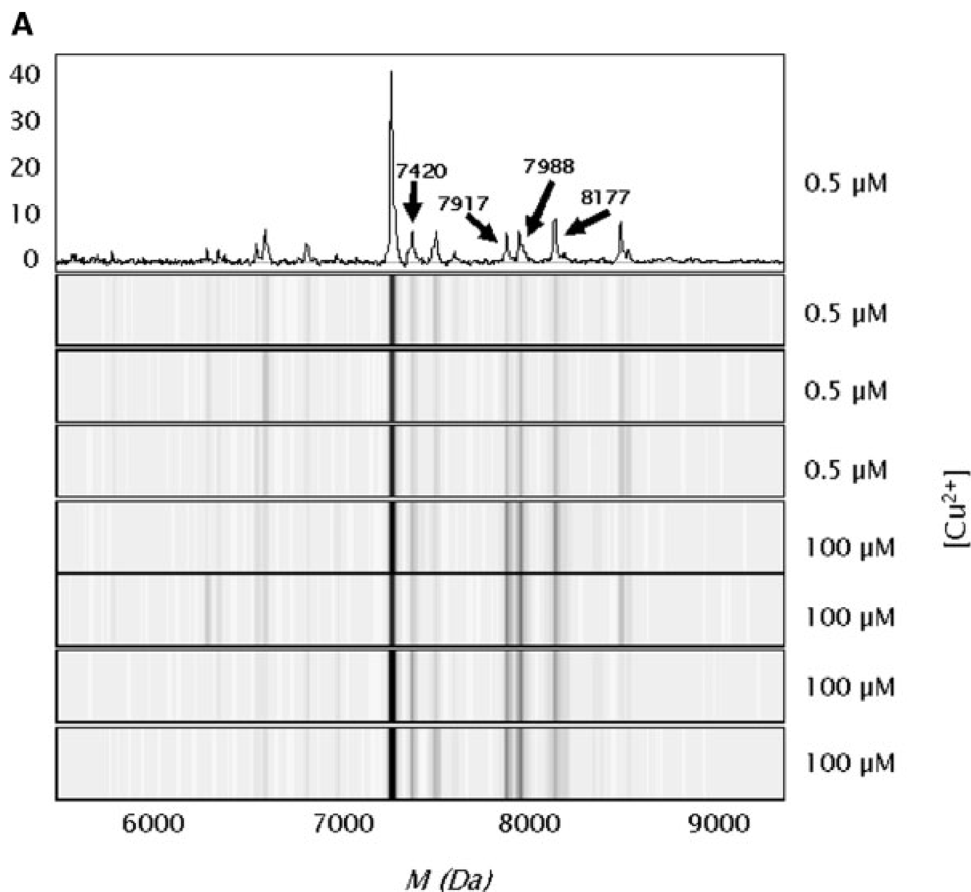

Fig. 2. Differences in protein expression in culture media of HepG2 cells incubated with 0.5 and 100 μM $CuSO_4$. **A**: Differential display comparing culture media of HepG2 cells grown in 0.5 μM copper (**upper four traces**) or in the presence of 100 μM copper (**lower four traces**) on a WCX ProteinChip Array. Mass range displayed 5,500–9,500 Da. Arrows indicate four proteins that are induced with 100 μM copper. Data were normalized for total ion current. Some of the traces are shown in gel view for easy comparison (see text). **B**: Overview of proteins in the 2–50 kDa mass range that show a significant difference ($P < 0.05$) between the two conditions. Significance was calculated using a non-parametrical Mann–Whitney test.

B

M (Da)	0.5 μM Cu		100 μM Cu		fold change
	Mean	SD	Mean	SD	
3977	4.51	0.71	5.98	1.09	1.3
4011	4.03	0.86	6.12	1.18	1.5
4578	5.13	1.27	6.71	1.83	1.3
6071	1.74	1.18	6.08	2.88	3.5
6099	1.05	0.74	6.00	3.44	5.7
6642	7.94	1.56	5.56	2.12	-1.4
6900	7.00	1.64	4.11	0.89	-1.7
7420	6.13	0.37	9.77	2.74	1.6
7917	7.96	3.34	16.85	6.94	2.1
7988	9.27	3.82	19.45	7.88	2.1
8025	14.74	4.03	19.57	1.26	1.3
8177	8.33	1.50	14.08	3.18	1.7
8225	2.62	1.09	3.85	1.58	1.5
11308	2.32	0.75	1.23	0.32	-1.9
12845	0.93	0.48	1.87	0.65	1.6
13787	4.99	1.16	3.90	0.37	-1.3
14064	3.69	1.43	5.14	2.07	1.4
14852	17.41	4.20	27.70	7.92	1.6
15100	5.98	7.47	20.12	13.79	3.4
18134	0.50	0.12	1.27	0.58	2.6
25534	0.96	0.39	1.52	0.55	1.6
28028	1.74	0.87	3.79	1.43	2.2
28196	1.91	0.72	4.52	2.67	2.4
29365	4.53	1.97	10.06	6.23	2.2
44065	0.39	0.11	1.01	0.51	2.6

Fig. 2. (*Continued*)

8-fold. A database search with these protein masses showed that they are most likely belonging to the MT family. MTs are key compounds involved in the intracellular handling of a variety of essential and non-essential post-transition metal ions [Kagi and Schaffer, 1988] and therefore they have an important role in the protection against copper toxicity. The findings indicates that MT synthesis is induced and MTs are possibly secreted in response to high copper concentrations probably to detoxify the metal by sequestering copper in the cytosol and the medium. Some publications indicate that MTs can be secreted by the liver [Evering et al., 1991] and can be found in blood [Garvey and Chang, 1981; Mulder et al., 1991]. However, more research is needed to substantiate this finding. It cannot be excluded that MTs in the media were derived from damaged cells although the lactate dehydrogenase measurements in the media did not indicate increased cell damage with the copper concentrations used. Interestingly, if it can be established that these MTs are also excreted by livers of Wilson disease patients they may possibly be candidate biomarkers for early diagnosis. The functions of the other proteins that reacted to increased copper levels remain unclear thus far but may provide new targets for clinical intervention.

In addition, it proved possible to use the proteinchip technology to search for proteins based on function. Using copper-coated IMAC chips we were able to find 18 high affinity copper-binding proteins present in cells under copper depleted conditions. One of them may be the copper uptake transport hCTR2. Proper identification and more research are needed to determine whether the discovered proteins also function as copper-binding proteins in vivo. Certainly these experiments have shown the

potential of the copper-coated IMAC chips in the discovery of copper-binding proteins.

The strongpoint of the proteinchip technology is that it allows fast, high throughput, analyzes with high reproducibility and sensitivity, especially in the low molecular weight range, which is essential for biomedical research and cannot be achieved by conventional 2D-PAGE. While the SELDI method is a very rapid method for protein profiling, it does not allow direct identification. Identification needs purification of each of the discovered proteins followed by peptide fingerprinting of tryptic fragments and possibly amino acid sequencing using MS/MS which can be a time-consuming process. The selective surfaces of the proteinchips can help with the purification process by providing information on biochemical properties of proteins. The data indicate that SELDI is a useful tool for future biomarker discovery and ana-

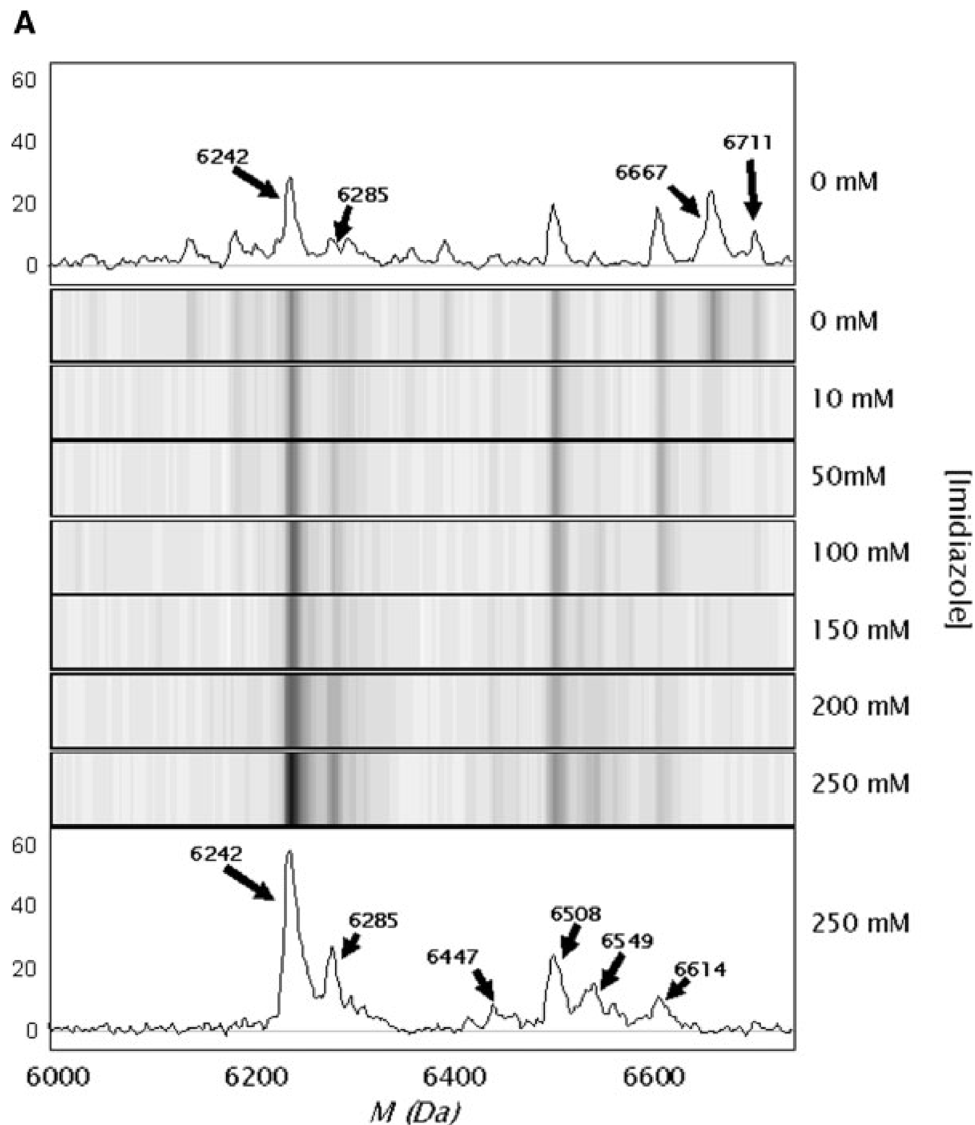

Fig. 3. Detection of high affinity copper-binding proteins using copper-coated IMAC ProteinChip Arrays and a high stringency imidazole wash. **A**: Differential display comparing lysate from copper depleted cells bound to a copper-coated IMAC ProteinChip Array and washed with increasing concentrations of the competitor for copper binding imidazole. The increased stringency washes resulted in the disappearance of peptides with low copper affinity at imidazole concentrations as low as 10 mM (e.g., peptides of 6,667 and 6,711 Da). Others remain bound to the chip surface at concentrations as high as 250 mM imidazole and were more clearly detected due to the lower complexity of the bound sample at this concentration (see, e.g., peptides 6,242; 6,285 Da). **B**: Overview of all high affinity copper-binding proteins bound to the copper-coated IMAC ProteinChip Array at 250 mM imidazole in the 2–50 kDa mass range.

B

6242	13966
6285	15434
6446	15685
6508	17585
6549	24027
6614	27740
6771	29474
11269	32673
13742	41719

(Mass values (Da))

Fig. 3. (*Continued*)

lyzes of copper metabolism in Wilson disease patients.

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