CCS and SOD1 mRNA are reduced after copper supplementation in peripheral mononuclear cells of individuals with high serum ceruloplasmin concentration

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Abstract

The limits of copper homeostatic regulation in humans are not known, making it difficult to define the milder effects of early copper excess. Furthermore, a robust assay to facilitate the detection of early stages of copper excess is needed. To address these issues, we assessed changes in relative mRNA abundance of methallothionein 2A (MT2A), prion (PrP), amyloid precursor-like protein 2 (APLP2), Cu/Zn superoxide dismutase (SOD1) and its copper chaperone (CCS) in peripheral mononuclear cells (PMNCs) from healthy adults representing the 5% highest and lowest extremes in the distribution curve of serum ceruloplasmin (Cp) concentrations of 800 individuals. The intracellular Cu content was also determined. PMNCs were isolated from individuals before and after exposure to a single daily dose of 10 mg Cu (as $CuSO_4$) for 2 months. Results showed that although there were fluctuations in serum Cp values of the samples assessed before copper exposure, no significant differences were observed in cell copper content or in the relative abundance of MT2A, PrP and APLP2 transcripts in PMNCs. Also, these values were not modified after copper supplementation. However, CCS and SOD1 mRNA levels were reduced in PMNCs after copper supplementation in the individuals with the high Cp values, suggesting that they should be further explored as biomarkers of moderate copper overload in humans.

Keywords: CCS; SOD1; Copper status; Ceruloplasmin; Real-time PCR

1. Introduction

Although copper is clearly an essential nutrient, this metal can be toxic if allowed to accumulate in excess of cellular needs. In man, mechanisms regulate uptake, efflux, storage and utilization of Cu preventing adverse effects due to excess within a rather wide range of (dietary) exposure. Cu handling appears tightly regulated, but the upper and lower limits of its homeostatic regulation are unclear. For this reason, identifying biomarkers to detect early effects of nutrient deficiency and excess has become a pressing challenge in modern nutrition. In the case of copper excess, this is a difficult task because the biochemical markers available are not sensitive and cannot detect early effects or predict liver damage, one of the most prominent effects of copper excess [1–4]. Until now, numerous efforts have been done to define new molecular markers by measuring activities in blood of copper-related enzymes, but they have failed [4–11].

In a previous study, we measured serum Cp concentration in 800 healthy adults. We decided to explore the capacity of this protein to show differential responses to copper intake among normal individuals. We hypothesized that healthy

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individuals would respond to Cu in accordance with their place in the serum Cp distribution curve, considering a higher value as an index of long-term higher intake [5]. The groups representing the highest and lowest 5% in the Cp distribution curve proved to be significantly different [12] and exhibited differential responses to a mild-moderate increase of copper exposure [5].

In this study, we assessed the relative abundance of some transcripts encoding proteins involved in copper metabolism, by real-time PCR in peripheral mononuclear cells (PMNCs), in a subgroup of individuals that participated in the original study [5,12]. Metallothionein has been extensively described as a gene whose transcript abundance displays a high sensitivity to intracellular copper concentration [13]. Prion protein has been shown to bear a copper binding domain [14], modulating neuronal copper content [15], and some reports link its protein and mRNA abundance to changes in copper availability [16,17]. APLP2 is another protein containing a copper-binding domain that, along with the closely related APP, has been related to the regulation of copper levels in neurons [18], effect that appears to be dependent on APLP2 and APP expression [19]. On the other hand, recent reports show that CCS [20–22] responds to copper deficiency in rats and mice [23–26] and data from our laboratory showed that CCS also responds in a consistent way to copper excess (to be submitted) in healthy adult humans receiving 8 mg Cu/day for 6 months.

2. Materials and methods

2.1. Design and study groups

We measured the levels of expression of MT2A, SOD1, CCS, PrP, APLP2 and copper content in PMNCs obtained from individuals who participated in the larger study previously mentioned, which assessed the response to copper exposure of several biochemical and urinary potential indicators of copper status [5]. Details of the design and sampling protocol were published elsewhere [5]; briefly, individuals received a single daily dose of 10 mg of Cu, as copper sulfate, [27] for 60 days. Blood samples to isolate PMNCs for gene expression and cellular copper content analyses were obtained prior to and immediately after copper supplementation in 40 of the 82 healthy individuals who participated in the larger study (20 Cp low and 20 Cp high). These individuals (19–55 years old) represented the highest and lowest Cp serum concentrations in the distribution curve, maintaining a similar proportion of men and women in both groups; participants remained healthy throughout the study period. Serum copper was measured by atomic absorption spectrometry (Perkin Elmer Model 2280, Norwalk, CT, USA) and Cp protein by nephelometry (Array Protein System, Beckman Instruments, Inc., Brea, CA, USA). Non-Cp copper was calculated assuming that ceruloplasmin molecular weight is 132 kDa and that each molecule binds six copper atoms [28]. Commercial kits were used to measure the activity of the serum liver aminotransferases glutamic–oxaloacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT) and γ-glutamyltransferase (GGT) (Boehringer Mannheim, Mannheim, Germany).

2.2. Isolation of PMNCs

PMNCs were obtained on a Histopaque gradient following the protocol described by Muñoz et al. [29]. Briefly, blood was diluted 1:1 with sterile phosphate buffered saline (PBS, Gibco, Invitrogen Life Technology, Carlsbad, CA, USA), layered onto Histopaque (Histopaque 1077, density: 1.119, Sigma Diagnostic, St. Louis, MO, USA) in a 4:3 ratio, and centrifuged at 1800 rpm for 35 min at room temperature. The mononuclear layer (buffy coat) was removed and washed twice in PBS at 1200 rpm for 10 min each, following the same wash protocol with RPMI 1640 medium (Gibco, Invitrogen Life Technology). Cells were resuspended in 1 ml of RPMI 1640 culture medium with gentamycin (Gibco, Invitrogen Life Technology).

2.3. Cellular Cu content

For total metal content quantification, PMNCs were processed as described in Ref. [30]. Briefly, cells were disrupted in concentrated suprapure nitric acid (Merck, Chemical Co., Darmstadt, Germany) in a vortex and diluted with distilled deionized water. Samples were digested at 60°C overnight. Final nitric acid concentration was 5%. Cu determination was made by means of a graphite furnace atomic absorption spectrophotometer (Perkin Elmer, SIMMA 6100, Shelton, CT, USA). Calibration was against a Cu standard curve (J T Baker, Phyllisburg, NJ, USA) and Cu content values were expressed per 10^6 cells.

2.4. RNA extraction and cDNA synthesis

Total RNA extraction from PMNCs was carried out using RNAWIZ reagent (Ambion, Austin, TX, USA), following manufacturer recommendations. RNA quantity was determined through an MBA 2000 (Perkin Elmer, Norwalk, CT, USA) at 260 nm and RNA purity was ascertained from optical density ratio at 260 and 280 nm. Two micrograms of RNA was used to synthesize single-strand cDNA with Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA), according to manufacturer recommendations. The resulting cDNA was either immediately used for PCR or stored at −20°C until use. As a control of integrity, cDNA was amplified using primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCCTGTTGCTGTA-3′). Primers were synthesized by Alpha DNA (Montreal, Quebec), and PCR amplification was performed in a 25-μl volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP Mix, 1 U of Taq DNA Polymerase (Invitrogen, San Diego, CA, USA), 20 pmol of each primer and 200 ng of cDNA. The standard program comprised 30 s at 94°C, 30 s at 56°C and 2 min at 62°C

for 30 cycles. The PCR products were analyzed in a 1% agarose gel.

2.5. Real-time RT-PCR

Real-time RT-PCR reactions were carried out on a LightCycler System 1.5 (Roche Diagnostics, GmbH, Rotkreuz, Switzerland) using the LightCycler-FastStart DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany). Primer sequences, annealing temperatures and amplicon lengths are given in Table 1. All primers but the pairs for SOD1 [31] and Rplp0 [32] were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Alpha DNA. Primer pairs used to amplify the genes of interest through real-time RT-PCR (Table 1) were designed to avoid cross-amplification with other similar genes, such as MT2A and the other members of the MT family. Reaction mixes had a final volume of 10 μl and contained 2 mM $MgCl_2$, 10 pmol of each primer and 100 ng of cDNA. A standard curve was plotted for each primer set with isolated PCR products obtained from amplification of pooled PMNC cDNAs. Human RPLP0 (large ribosomal protein) was used as reference to normalize the expression levels between the samples [33]. The following protocol was used for all genes: *denaturation*: 10 min — 95°C; *amplification and fluorescence acquisition* (40 cycles): 15 s — 95°C, 10 s annealing temperature (Table 1); 10 s — 72°C with a single fluorescence measurement; *melting curve*: from annealing temperature to 99°C, 0.1°C/s heating rate and continuous fluorescence measurement. Abundance data for each gene are expressed as fold change.

2.6. Immunoblot analyses

Selected samples of PMNCs were subjected to Western immunoblot analyses as previously described [34]. An equal amount of 20 μg protein was applied to 15% SDS-PAGE gels and transferred to nitrocellulose membranes after electrophoresis. Loading was verified by Ponceau S staining. Both CCS and SOD1 antibodies reacted strongly with human white blood cell proteins. Immunoblotting was carried out using rabbit anti-bovine SOD1 as primary antibody (AB 1237, Chemicon International Inc., Temecula, CA, USA) followed by the donkey anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (Amersham NA 934, Amersham Pharmacia, Piscataway, NJ, USA). For CCS, primary antibody was affinity purified anti-human CCS as detailed previously [25]. Membranes were reprobed for actin. Membranes were processed for chemiluminescence detection (SuperSignal Pierce) and the images were directly captured using the FluorChem system. Size of the immunoreactive bands was estimated from regression analysis using standard peptides (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

Comparisons were made by Wilcoxon matched pairs test and Mann–Whitney *U* test. Correlations were performed by Spearman rank order. Differences were considered significant at $P<.05$. Data are presented as means and standard error (S.E.). All statistical analysis was performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA) and SYSTAT 11.0 (SYSTAT, Inc., Evanston, IL, USA) software. Differences that were not statistically significant are referred to as NS.

3. Results

Determination of the relative abundance of transcripts encoding proteins assessed was performed by real-time PCR, in PMNCs obtained from healthy adults representing the 5% lower and 5% higher values in the serum Cp distribution curve of a group of 800 individuals [5]. We hypothesized that PMNCs from individuals supplemented with copper during 60 days may exhibit transcriptional changes in response to chronic copper exposure and that this response should depend on the position of individuals in the Cp distribution curve (high or low Cp).

3.1. Effect of copper supplementation in serum and PMNC copper content

Serum copper and Cp concentrations in the high- and low-Cp groups appear in Table 2. As expected, there was a significant correlation between serum copper and Cp ($P < .05$, Spearman rank order correlations) in both groups, which were not affected by copper supplement. Copper supplementation modified Cp concentration and liver enzyme activities (GOT, GGT and GPT) in both Cp groups, while hemoglobin remained constant (Table 2). Amino-transferases activities remained well within the normal range

Table 1
Primers and conditions used for quantification of gene expression by real-time PCR

Gene	Forward primer	Reverse primer	Length (bp)	Annealing (°C)
MT2A	ATGGATCCCAACTGCTCCTGCG	AGGGCTGTCCCAACATCAGGC	200	68
SOD1	CTGAAGGCCTGCATGGATTC	CCAAGTCTCCAACATGCCTCTC	138	65
APLP2	GTGGAATAGGGAACTGTAAT	GGGGAAGTGAACGGTAAAA	315	56
RPLPO	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC	149	64
CCS	ACAGCTGACCCCTGAGCG	ACAGAGCCAAGGTGAGGTC	531	60
PrP	GCTTGAGGGAGGCGGTAT	CCTGGCAGAAATGTTGTCG	423	58

Table 2
Effect of copper supplementation in low- and high-Cp groups

	Low Cp		High Cp	
	Baseline	60 days	Baseline	60 days
n	18	18	22	22
Cp (μmol/L)	2.0±0.1[a]	1.7±0.1[b]	3.6±0.2[c]	3.2±0.2[d]
Serum copper (μmol/L)	13.1±0.5[a]	13.5±0.6[a]	23.0±1.5[b]	24.2±1.4[b]
PMNC copper (pmol/10^6 cells)	54.8±11.8	28.3±9.4	29.6±8.4	30.8±4.9
Non-Cp Cu (μmol/L)	1.1±0.5[a]	3.0±0.7[b]	1.8±1.4[a]	4.8±0.7[b]
Hemoglobin (g/L)	14.7±0.3	14.9±0.3	14.6±0.2	14.3±0.2
GOT (UI/L)	13.8±1.5[a]	23.5±1.9[b]	15.5±1.9[a]	21.3±1.5[b]
GPT (UI/L)	16.0±2.3[a]	29.5±2.7[b]	16.1±2.5[a]	25.4±1.8[b]
GGT (UI/L)	12.9±2.7[a]	19.8±3.5[b]	17.5±2.8[a]	22.4±3.2[b]

Data are expressed as means±S.E. Values within a line with different superscript letters are significantly different at $P<.05$ (Wilcoxon matched pairs test or Mann–Whitney *U* test).

and the increases found are of no clinical significance. At baseline, PMNC copper content did not differ between groups (NS, Mann–Whitney *U* test). After copper supplementation, these values did not change significantly in either group (NS, Wilcoxon matched pairs test). Regarding non-Cp copper, we observed a significant increase in both groups after copper supplementation, reaching a major concentration in the high-Cp group.

3.2. CCS, SOD1, MT2A, PrP and APLP2 expression

In search of genes involved in the putative adaptive response, we used real-time RT-PCR to assess the levels of several genes that are associated with copper metabolism. Relative abundance values for CCS, SOD1, MT2A, PrP and APLP2 transcripts were compared before and after copper supplementation (Fig. 1). In these experiments, normalization was performed on the basis of RPLP0 (large ribosomal protein) [33]. Changes in MT2A, PrP and

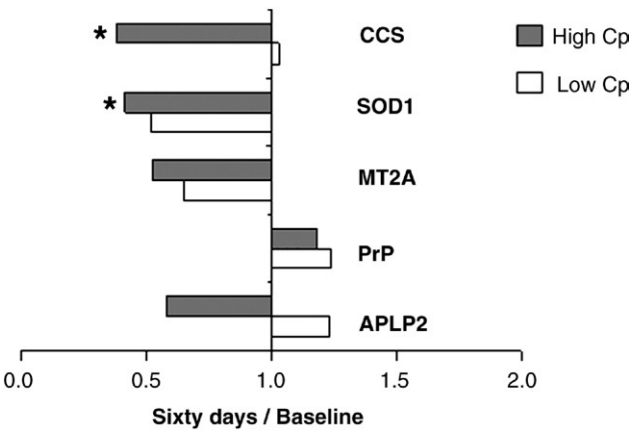

Fig. 1. Ratios of MT2A, APLP2, PrP, SOD1 and CCS expression between 60 days and baseline from high Cp and low Cp. Relative abundance of gene expression changes was assessed by quantitative real-time PCR. Relative expression calculations used RPLP0 as the endogenous normalization control. Data are expressed as ratio between 60 days and baseline relative abundance means (*$P<.05$; Wilcoxon matched pairs test, $n=10$).

Table 3
Relative abundance of CCS and SOD1 in PMNCs from low- and high-Cp groups

	Low Cp (relative abundance)		High Cp (relative abundance)	
	Baseline	60 days	Baseline	60 days
CCS (mRNA)	0.028±0.007	0.029±0.007	0.057±0.015	0.022[a]±0.004
SOD1 (mRNA)	0.288±0.113	0.123±0.058	0.202±0.064	0.084[a]±0.030
CCS (protein)	1.823±0.343	1.992±0.439	1.729±0.348	1.676±0.277
SOD1 (protein)	5.370±1.053	5.610±1.561	8.274±2.503	11.730±1.561

Relative gene expression using RPLP0 as the endogenous normalization control, $n=10$ and $n=12$ from low Cp and high Cp, respectively. Protein relative abundance was normalized against actin ($n=5$). Data are expressed as means±S.E.

[a] $P<.05$ between before and after supplementation (Wilcoxon matched pairs test).

APLP2 mRNA expression values were not significant ($P>.05$, Wilcoxon matched pairs test). APLP2 expression increased 1.23-fold after copper supplementation in the low-Cp group, while it decreased 1.72-fold in the high-Cp group. PrP expression increased 1.24- and 1.18-fold, and MT2A decreased 1.53- and 1.92-fold in the low- and high-Cp groups, respectively. SOD1 transcript decreased significantly 2.41-fold in the high-Cp groups ($P<.05$, Wilcoxon matched pairs test). CCS expression did not change in the low-Cp group after copper supplementation, but it decreased (2.59-fold) significantly in the high-Cp group ($P<.05$, Wilcoxon matched pairs test), indicating that in PMNCs the CCS and SOD1 transcript abundance were dependent on both copper supplementation and the Cp values. In contrast, CCS and SOD1 protein levels did not differ between baseline and 60 days in either of the groups (Table 3).

4. Discussion

Results support the hypothesis that individuals with high or low serum Cp concentrations would respond differently to mild excess of copper exposure. As expected by the criteria used to select the participants, groups had significantly different serum Cp and copper concentrations, but within the physiological range. Changes in liver aminotransferases after 60 days of copper supplementation remained in the range observed in normal population, below the cut-off used on clinical grounds to diagnose hepatic damage. Within this "normal" range of variations of liver enzyme activities, they have been described to change along time (seasonal variations) and also depending on lifestyles [35].

Results of quantitative RT-PCR amplification of candidate genes showed that there was a significant reduction in the relative abundance of SOD1 and CCS mRNA after increasing copper intake for 6 months. These changes were not confirmed at the protein level. In copper-deficient mice and rats, the protein levels of both genes have been reported to change in several tissues including blood [23–26,35,36]. CCS was higher and SOD1 lower following copper

deficiency. This has been interpreted as likely due to a copper-dependent post-transcriptional mechanism since CCS and SOD1 mRNA were not altered [24,25,35,36]. In this human study, CCS and SOD1 gene expression decreased only in the high Cp group, in which we previously reported an increment in GSH level [5]. At the same time, there were no changes in intracellular copper levels, which correlated with the absence of changes in the abundance of transcripts coding for MT2A, APLP2 and PrP. Thus, our results support that transcriptional changes in transcripts encoding for CCS and SOD1 in PMNCs might be a marker of early changes in copper status and deserve further investigation. However, it remains an open question whether the changes observed in CCS and SOD1 represent a direct response of PMNCs to variation in copper status, or they are a consequence of regulatory mechanisms that sense and respond to this micronutrient. It is important to emphasize that the changes observed in rats and mice models result from drastic changes in dietary copper in contrast with the studies in humans, derived from modifying copper intake within the range efficiently handled by physiological mechanisms. Considering that both groups (high or low Cp serum values) exhibited similar levels of copper content in PMNCs, without significant changes after the period of copper supplementation, we believe that differentially expressed genes are likely to represent the specific adaptation of cells to serum features of individuals with high Cp levels when exposed to a mild excess of copper. In this study, non-Cp copper showed a significant increment after supplementation, which was considerably greater in the high-Cp group, suggesting that the increment in copper bioavailability may induce a change in the CCS and SOD1 mRNA abundance in the PMNCs, this possibility will be assessed in the near future.

In summary, we confirmed that individuals with serum Cp concentrations representing the highest and lowest values observed in normal population respond differently to controlled mild excess copper exposure. Results showed that CCS and SOD1 mRNA responded specifically to copper dosing and they should be further assessed as potential biomarkers of moderate copper overload in humans.

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