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Chaperones CCS, ATOX and COXIV responses to copper supplementation in healthy adults

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Abstract Assessment of proteins in blood and other tissues has failed to identify markers of early copper effects on health. Studies in animal models show that chaperone of SOD (CCS) respond to changes of copper status. Evidence about other copper chaperones (COXIV, ATOX) is not clear. The aim of this study was to assess by means of an in vitro challenge the mRNA relative abundance of *ccs, sod1, coxIV, mtIIa and atox* in peripheral mononuclear cells (PMNCs) obtained from healthy individuals, acutely and chronically supplemented with small-to-moderate amounts of copper. Healthy participants received 8 mg Cu/d (supplemented group, SG) or placebo, (placebo group, PG) for 2 months. Biochemical indicators were assessed at basal (T0) and after 2 (T2) and 60 days (T60). At these times PMNCs were obtained, challenged with 1, 5 or 20 μM Cu-histidine for 20 h and the mRNA relative abundance of the selected genes assessed by real time PCR. The results showed that at T0, intracellular copper was not different between experimental and control groups. This increased at T2 and T60 when the copper in the media increased (two-way ANOVA, $P < 0.001$). In PG, CCS mRNA transcripts showed no significant changes (two-way ANOVA) at T2 and T60. In SG, CCS changed by treatment, time and interaction (two-way ANOVA, all $P < 0.001$). SOD, ATOX and COXIV expressions changed in both PG and SG showing various patterns of response, requiring further study. MTII responded as expected. We conclude that using healthy individuals as a human model, CCS but not SOD, ATOX or COXIV responded consistently to controlled changes of copper availability in an in vitro copper challenge.

Keywords CCS · ATOX · COXIV · Copper biomarkers · Peripheral mononuclear cells

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Introduction

Both copper deficiency and copper excess induce adverse health effects. These effects are best represented in a U shape curve where usual daily life takes place in the central area, where robust adaptive mechanisms allow a range of intakes without having health consequences; at both ends, the left and right side of the curve health effects progressively increase (copper deficiency and copper excess, respectively) (WHO 2002). Extreme conditions are represented by the genetic diseases Menkes syndrome and Wilson disease (Vulpe et al. 1993; Yamaguchi et al. 1993; Petrukhin et al. 1994; Danks 1995). However, early effects both of deficit and excess are not clear, mainly because available indicators of copper status are not sensitive enough. Lack of overt symptoms in the population, whose copper consumption is often below

the recommended dietary allowance (RDA) (WHO 2002) led to assume that in general, copper deficiency was rare (Institute of Medicine (IOM), Food and Nutrition Board 2001). Yet, recent evidence indicate that this situation is not uncommon (Weisstaub et al. 2008; Henri-Bhargava et al. 2008; Halfdanarson et al. 2009; Griffith et al. 2009); high frequency of copper deficiency has been reported in moderate and severely malnourished children, a group representing many millions of individuals in the world (Weisstaub et al. 2008); also, post bariatric cases have been recently described (Griffith et al. 2009).

Many cuproenzymes have been evaluated as potential new copper markers. Ceruloplasmin (Cp) is the most frequently used in the medical field for diagnosing Menkes syndrome and Wilson disease, but because it is an acute phase protein and rises during inflammation is not a reliable indicator in less intense conditions (Bertinato and Zouzoulas 2009). Extra cellular superoxide dismutase (SOD3) also rises during inflammation and responds to zinc supplementation, being of little use in real life situations. Cu–Zn-superoxide dismutase (SOD1) activity has been described to decrease in copper deficiency in animal models and humans, but data on marginal changes of copper status in humans are not clear (Prohaska 1991; Bertinato et al. 2003; Weisstaub et al. 2008). In platelets of adult females, cytochrome C oxidase (CCO) responded to dietary copper restriction (Milne and Johnson 1993) while SOD1 activity did not; although this may seem promising, more data is clearly necessary. Studies measuring the chaperone of superoxide dismutase (CCS) in different tissues have yielded reproducible results in animal (mice and rats) (Bertinato et al. 2003; Prohaska et al. 2003a, b; West and Prohaska 2004; Iskandar et al. 2005); in humans, supplementation with 10 mg of copper/day for 2 months in adult women and men resulted in concurrent changes of CCS mRNA transcripts (Suazo et al. 2008). A clear bias in the evidence originated in most of the animal studies is that the copper deficient states assessed are intense (rats, mice and cows (Bertinato et al. 2003; Prohaska et al. 2003b; West and Prohaska 2004; Hepburn et al. 2009). Performance of CCS in milder situations is crucial to decide whether larger, population based studies—necessary to establish a marker—are justified. Recent work by Prohaska et al. (2003a) reported changes of protein levels of mitochondrial CCO subunits in copper deficient rats. Previous studies on heart tissue reported lower content of certain subunits of the CCO complex after dietary copper deficiency in rats (Zhou et al. 2009; Zen et al. 2007). In copper deficient rodents, CCO subunit IV (COX IV) was consistently lower in choroids plexus and cerebellum (Broderius and Prohaska 2009) and also in white cells and platelets (WHO 2001) These are valuable data suggesting another potential copper marker. Unfortunately, there are no studies of COX IV in humans. Among other chaperones, ATOX1 has been assessed in Wilson disease but not in other conditions (Simon et al. 2008).

In this study, we further assessed mRNA relative abundance of chaperones *ccs, coxIV* and *atox* in peripheral mononuclear cells (PMNCs). We chose these cells because they respond to copper supplementation (Suazo et al. 2008) and also, because they are easily accessed, a requisite to be used as an indicator in population studies. In addition to these chaperones we also assessed *sod* and *mtIIa* to draw a parallel with the changes observed, together with a set of blood biochemical indicators. We modified the original protocol measuring the mRNA abundance of transcripts prior to, after 2 days (acute changes) and after 60 days (chronic changes) copper supplementation, in PMNCs that were copper challenged in vitro. This latter was incorporated to clarify whether these cells respond differently to extra copper after challenge, suggesting pre adaptation.

Methods and procedures

Study groups

1,200 healthy women and men were interviewed, received detailed explanation about the protocol and those that fulfilled the inclusion criteria and signed an informed consent were assigned randomly to the experimental or placebo group (PG). Inclusion criteria were being apparently healthy women and men aged 20 and 50 years, with no significant clinical history, not obese (BMI < 30), not anemic ($\geq$13 g/dl in men and $\geq$12 g/dl in women) (WHO 2001), not receiving multiple medications, vitamins or mineral supplements, with liver GOT, GPT and GGT activities within normal limits (St. Louis 1991), C Reactive Protein (CRP) below 8 (cut off set by the manufacturer), serum copper concentration at or above the cut off (70 and 80 μg/dl in men and women, respectively)

Table 1 Biochemical blood indicators in SG and PG during the two-month follow up (mean ± SD)

	T0		T2		T60		ANOVA (*P*)*		
	SG $N = 26$	PG $N = 27$	SG $N = 26$	PG $N = 27$	SG $N = 26$	PG $N = 27$	Group	Time	Interaction
Hb (g/l)	144 ± 12	145 ± 16	141 ± 11	143 ± 14	147 ± 14	146 ± 15	0.839	0.002[a]	0.307
MVC (fl)	87 ± 3	87 ± 5	87 ± 4	88 ± 5	88 ± 4	88 ± 5	0.802	0.104	0.420
Znpp (μg/dl RBC)	63 ± 13	67 ± 16	62 ± 14	65 ± 16	63 ± 14	68 ± 16	0.285	0.082	0.765
SF (μg/l)**	36 (17–77)	35 (16–75)	35 (17-73)	33 (14–77)	30 (15–60)	32 (14–70)	0.913	0.058	0.465
GOT (U/l)	48 ± 15	50 ± 16	50 ± 20	53 ± 17	52 ± 16	56 ± 19	0.771	0.028[b]	0.456
GPT (U/l)	48 ± 17	48 ± 24	51 ± 25	48 ± 21	49 ± 24	55 ± 22	0.785	0.029[c]	0.247
GGT (U/l)	26 ± 17	33 ± 29	28 ± 19	34 ± 30	30 ± 18	36 ± 32	0.965	0.171	0.841
CRP	8.2 ± 7.7	7.1 ± 4.9	8.2 ± 7.5	8.3 ± 6.5	7.5 ± 6.2	9.9 ± 9.5	0.413	0.416	0.200
Cp	26 ± 13	24 ± 14	28 ± 14	26 ± 10	24 ± 10	23 ± 9	0.970	0.057	0.275
sFe (μg/dl)	124 ± 35	135 ± 45	136 ± 34	134 ± 40	126 ± 34	137 ± 48	0.044	0.568	0.690
sCu (μg/dl)	112 ± 43	114 ± 41	115 ± 41	118 ± 42	109 ± 39	116 ± 41	0.921	0.066	0.174
sZn (μg/dl)	77 ± 14	80 ± 16	78 ± 17	80 ± 17	78 ± 10	77 ± 15	0.695	0.178	0.906

Hb hemoglobin, *MVC* media volume corpuscular, *Znpp* erythrocyte zinc protoporphyrin, *SF* serum ferritin, *GGT* gamma-glutamyl transpeptidase, *GOT* glutamic–oxaloacetic transaminase, *GPT* glutamic–pyruvic transaminase, *CRP* C reactive protein, *Cp* ceruloplasmin, *sFe* serum iron, *sCu* serum copper, *sZn* serum zinc

* Two-way ANOVA repeated measures was applied for analysis of all two groups (SG and PG) at the three times of study

** Geometric mean and 1 SD range

Scheffé Post hoc test, [a] T2 versus T60: $P = 0.003$; [b] T0 versus T60: $P = 0.028$; [c] T0 versus T60: $P = 0.030$

and serum zinc concentrations at or above 80 μg/dl. Results of this initial assessment appear in Table 1. 53/1200 individuals fulfilled the inclusion criteria, provided complete information and were analyzed divided into Supplemented group (SG, $n = 26$) and PG ($n = 27$) (see algorithm in Fig. 1). The protocol was approved by the IRB of the Institute of Nutrition and Food Technology (INTA), University of Chile.

Copper loading and follow up

Previous studies showed that individuals living in the area where our participants originated have a customary mean copper intake of ~0.9 mg Cu/day, with little variations along the last 10 years (unpublished). In these groups, adults copper consumption is below the estimated adequate intake (EAR) in 33% of women and 16% of men (Olivares et al. 2004); therefore, we estimated that copper status in the study groups could be marginally low. Eight milligram Cu/day (as copper sulfate) or placebo (in a capsule of similar color and aspect) was administered daily to participants, under direct supervision. They maintained their usual everyday jobs, health events were recorded daily and those

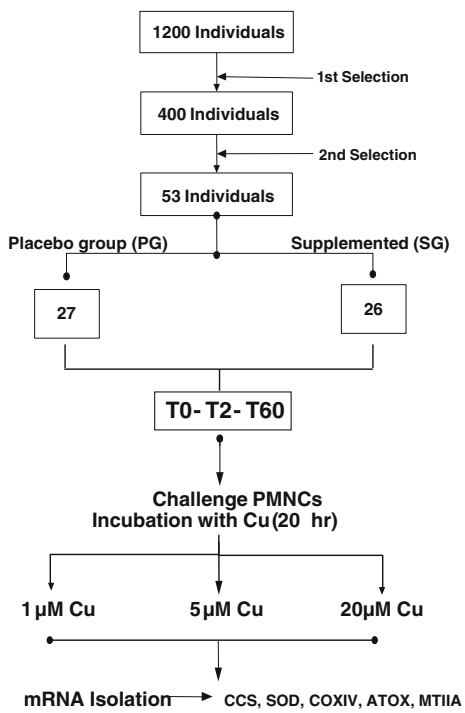

Fig. 1 Algorithm

needing medical care were first seen by a physician that belonged to the research team; when necessary, he/she was advised to consult to their usual medical service.

Procedures

A 30 ml blood sample was obtained between 8 and 9 a.m., after overnight fasting, from the arm anticubital vein at incorporation to the protocol (T0), after 2 days (T2) and 60 days (T60) copper supplementation. Hematological indicators included total blood count (erythrocytes, white cells, hemoglobin, MCV, formula), serum ferritin (SF) by ELISA and erythrocyte zinc free erythroporfirin (FEP) (hematofluorimeter, Aviv Model 206D). Cp ferroxidase activity was measured following the *p*-phenylendiamine oxidase technique (Sunderman 1970). Commercial kits were used to measure CRP (Orion Diagnostica Oy, Espoo, Finland), following the manufacturer's instruction (cut off: <8 mg/l); proteins (Bio-Rad Laboratories, Life Science Group, Hercules, California) using albumin as standard and gamma-glutamyl transpeptidase (GGT), glutamic–oxaloacetic transaminase (GOT) and glutamic–pyruvic transaminase (GPT) activities (Química Clínica Aplicada SA, Amposta, Spain). Serum iron (sFe), copper (sCu) and zinc (sZn) were measured by atomic absorption spectrophotometry (Perkin Elmer, Model 2800, The Perkin-Elmer Corporation, Norwalk, CT, USA).

The second phase of the study consisted in preparing the PMNCs obtained at T0, T2 and T60 to undergo the in vitro *challenges*, separating them by Ficoll–Histopaque gradient sedimentation (1.119: density, Sigma, St. Louis, MO). The mononuclear layer was removed and washed twice in PBS and then adjusted to 40×16^6 PMNCs/ml using RPMI-1640 with gentamicin. PMNCs were then incubated in a six well plate with RPMI-1640 media, exposing cells to 1; 5 and 20 μM Cu-Histidine (1:10) for 20 h. Cells were centrifuged to $400 \times g$ for 5 min and then washed in PBS; aliquots were separated for metal quantification (by AAS spectrophotometry with graphite furnace (Perkin Elmer Simaa 6100), protein determination (Lowry et al. 1951) and total RNA isolation, and stored at −80°C.

RT-PCR

RNA was extracted from PMNCs using TRIzol Reagent according to the manufacturer's protocol (Invitrogen). One microgram of RNA was reverse transcribed using Affinity Script cDNA Synthesis Kit (Stratagene). Real-time PCR was performed using Brilliant II SYBR® Green QPCR Master Mix (Stratagene) on a LightCycler™ system 1.5 (Roche Diagnostics, GmbH, Rotkreuz, Switzerland). Relative mRNA levels were determined according to Pfaffl (2001). A*ctin* and *hprt1*mRNA levels were used for normalization.

Primer sequences were: CCS-s: GCAACAGCT GTGGGAATCACTT, CCS-a: ATAATCAGGGTCC GGCCAATCA; SOD-s: ACTGAAGGCCTGCATGG ATT, SOD-a: ACATCGGCCACACCACTCTT; MT IIA-s: CTCTTCAGCACGCCATGGAT; MTIIA-a: CCCTTTGCAGATGCAGCCTT; COXIV-s: AGCC AGAAGGCACTGAAGGA; COXIV-a: AGCCCCT GTTCATCTCAGCA; and Actin-s: TGGCACCCAG CACAATGAAGA; Actin-a: GAAGCATTTGCGGT GGACGAT; HPRT1-s: TCTGTGGCCATCTGCT TAGT and HPRT1-a: AACAATCCGCCCAAAGG GAA. The amplification protocol consisted of an initial denaturation step at 95°C for 10 min followed by 50 amplification cycles at 95°C for 5 s, annealing at 60°C for 15 s, and elongation at 72°C for 15 s.

Variables and sample size

The independent variable was daily copper dose. Dependent variables were defined as *sod, ccs, atox, coxIV* and *mtIIa* mRNA relative abundance measured in PMNCs; *sod* and *mtIIa* mRNA abundance were measured to assess whether they changed concurrently with CCS and ATOX. Results were normalized by actin. Sample size was calculated to detect a significant difference when a delta of 1 SD occurred in serum Cp; using a power value of 90% and alpha value of 0.05, the sample size was 13 individuals for group. For each protein, analyses of PMNCs mRNA transcripts originated from SG and PG were analyzed in two ways: (i) the three curves sets (after incubation with 1, 5 and 20 μM Cu) at the three times of study (T0, T2 and T60) by two-way ANOVA); (ii) the curves obtained after incubation with 1, 5 and 20 μM Cu, at each time of assessment (one-way ANOVA).

Results

Participants in both groups remained asymptomatic during the study period. Biochemical indicators

showed no statistical differences between SG and PG at the three times of study (T0, T2 and T60, two-way ANOVA) or between groups at each time of assessment (*T* test) (Table 1).

In the challenge tests, cell copper concentration increased as there was more copper in the culture medium, at all times of study (Table 2). Only at T0 differences between PG and SG were not significant (*T* test). When treatment groups (PG or SG) are analyzed at the three copper doses (1, 5 and 20 μM) and at the three times of study (T0, T2 and T60), intracellular copper concentration changes were significant by two-way ANOVA ($P < 0.001$). Also, values tended to be greater in SG than in placebos (Table 2).

In PG, CCS mRNA transcripts showed no significant changes by two-way ANOVA or by one-way ANOVA (Fig. 2). Instead, in SG CCS changed significantly by treatment, time and interaction (all $P < 0.001$, two-way ANOVA, Fig. 2). Comparing the expression at each time of assessment, mRNA expression decreased when copper concentration increased in the medium (one-way ANOVA, $P < 0.001$).

Relative abundance of SOD mRNA also showed different patterns of response in SG as compared with PG (Fig. 3). Two-way ANOVA yielded significant differences by time, treatment and interaction ($P < 0.001$). In the one-way ANOVA analysis, PG showed a clear trend to increase SOD mRNA relative abundance as extra cellular copper concentration increased in the medium ($P < 0.001$). In SG there was a different pattern of response depending on extra cellular copper concentration, appearing an opposite response at 20 μM Cu when compared with 5 μM Cu; the highest SOD mRNA expression was at 5 μM of extra cellular Cu at all times, reaching significance both at T2 and T60 (one-way ANOVA, $P < 0.001$).

Patterns observed in ATOX mRNA relative abundance differed in PG and SG (Fig. 4), not always being consistent. In PG differences were significant by time, treatment and interaction (two-way ANOVA, $P < 0.02$). Instead, in SG differences were significant only by time ($P < 0.02$, two-way ANOVA). Also, in PG the lowest expression was observed at 5 μM of extra cellular Cu at all times (one-way ANOVA; $P < 0.01$ between 1/5 μM and 5/20 μM), whereas SG exhibited the highest expression at 5 μM extra cellular Cu concentration (Fig. 4).

COXIV mRNA relative abundance is shown in Fig. 5. In PG, there was a significant difference by treatment but not by time or interaction (two-way ANOVA, $P < 0.001$). Also, COXIV expression decreased when Cu concentration increased in the media (one-way ANOVA, $P < 0.05$ between 1 and 5 μM and $P < 0.01$ between 1 and 20 μM). In SG, COXIV expression increased at T2 and T60, suggesting time- and concentration- dependence. Differences were found between 1 and 5 μM and 1 and 20 μM (one-way ANOVA, both $P < 0.05$). In SG, COXIV expression was difference by two-way ANOVA, by time ($P < 0.0001$), treatment ($P < 0.04$) and interaction ($P < 0.03$).

Trends in MT2 mRNA relative abundance were as expected and similar in PG and SG (Fig. 6), with a clear trend to increase as copper concentration in the medium increased; in both groups differences were significant by time, treatment and interaction (two-way ANOVA, $P < 0.001$).

Table 2 Intracellular total copper in PMNCs challenged with different copper concentration

Total Cu (nmole/mg protein)	Placebo	Supplemented	*T* test *P*
T0*			
1 μM	6.38 ± 0.83	8.79 ± 1.09	NS
5 μM	8.32 ± 1.02	10.67 ± 1.12	NS
20 μM	11.27 ± 1.15	11.40 ± 0.96	NS
T2*			
1 μM	4.35 ± 0.44	5.70 ± 0.64	NS
5 μM	5.20 ± 0.29	6.66 ± 0.50	<0.018
20 μM	7.26 ± 0.42	9.66 ± 0.62	<0.003
T60*			
1 μM	4.33 ± 0.47	6.49 ± 0.64	<0.010
5 μM	6.42 ± 0.56	8.91 ± 0.61	<0.004
20 μM	8.97 ± 0.75	12.97 ± 0.81	<0.001

Values are average ± SEM

* Two-way ANOVA: treatment; groups and interaction $P < 0.001$

Discussion

The protocol sought to shed light on the cellular capacity to respond to small and to moderate copper oral supplementation, mimicking situations likely to occur in real life. Results show that—as expected—

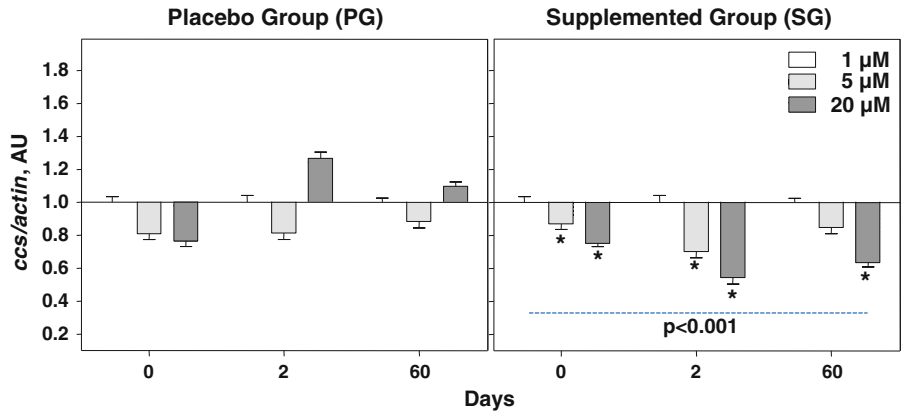

Fig. 2 *ccs* mRNA relative abundance in PMNCs obtained in PG and SG individuals. PMNCs were incubated with three copper concentrations (1, 5 and 20 µM) for 20 h, at three times (T0, T2 and T60). *Significant differences within a given time of experiment (one-way ANOVA, $P < 0.02$). *Dotted line* indicates significant differences when curves built at the three times (T0, T2 and T60) were compared after incubation treatment (1, 5 and 20 µM Cu) (two-way ANOVA, $P < 0.001$)

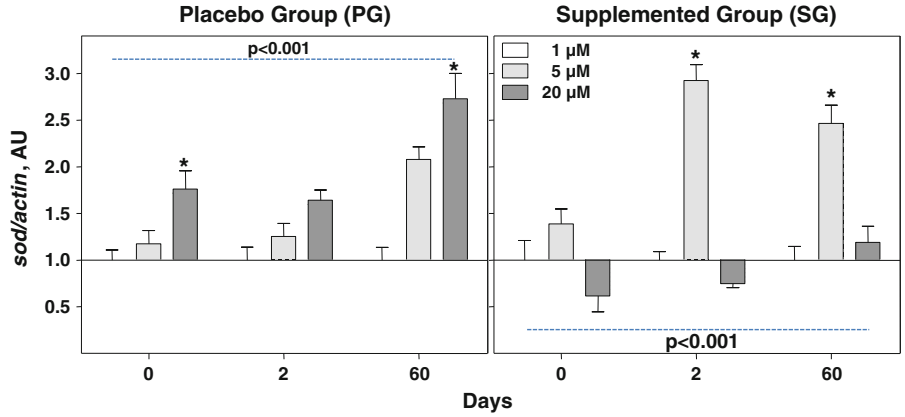

Fig. 3 *sod* mRNA relative abundance in PMNCs obtained in PG and SG. PMNCs were incubated with three different copper concentrations (1, 5 and 20 µM) for 20 h, at the three times (T0, T2 and T60). *Significant differences within a given time of experiment (one-way ANOVA, $P < 0.001$). *Dotted line* indicates significant differences when curves built at the three times (T0, T2 and T60) were compared after incubation treatment (1, 5 and 20 µM Cu) (two-way ANOVA, $P < 0.001$)

there were no relevant differences between biochemical indicators in the two cohorts at all times assessed (Table 1). However, by in vitro challenging PMNCs different patterns of response of the genes studied were made evident. Results after 2 days (representing acute changes) and 60 days (representing chronic changes) showed that the relative abundance of mRNAs assayed changed, but only CCS did so in a consistent manner. Testing CCS in these conditions adds relevant information considering that it has been proposed as a potential copper marker (Bertinato et al. 2003; Prohaska et al. 2003b).

It is interesting that ATOX (Simon et al. 2008), a copper chaperone that had not been evaluated in humans as in this study, changed differently in placebo and supplemented individuals, showing that increased oral copper led PMNCs to decrease their ATOX mRNA transcripts when in vitro copper challenged with 5 µM Cu. This chaperone response should be further assessed.

In PG, incubation with different copper concentrations allowed evaluating cells that have not been previously exposed to extra copper. It is interesting that the four genes assessed showed different patterns of response. While CCS mRNA transcripts did not change, SOD mRNA transcripts showed a clear trend to increase when Cu concentrations in the medium increased (and this pattern was maintained along

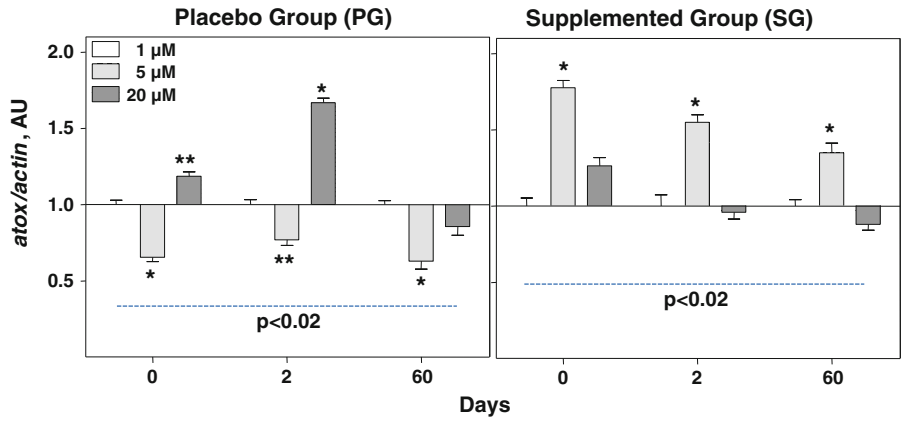

Fig. 4 *atox* mRNA relative abundance in PMNCs obtained in placebo and supplemented individuals. PMNCs were incubated with three different copper concentrations (1, 5 and 20 µM) for 20 h, at three times (T0, T2 and T60). *, **Significant differences within a given time of experiment (one-way ANOVA, $P < 0.001$ and $P < 0.01$, respectively). *Dotted line* indicates significant differences when curves built at the three times of study (T0, T2 and T60) were compared after incubation treatment with 1, 5 and 20 µM Cu (two-way ANOVA)

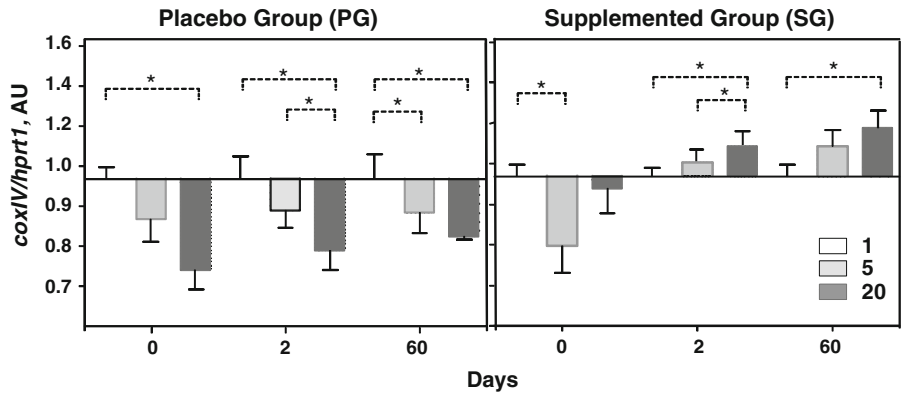

Fig. 5 *coxIV* mRNA relative abundance in PMNCs obtained in PG and SG. PMNCs were incubated with three different copper concentrations (1, 5 and 20 µM) for 20 h, at three times (T0, T2 and T60). *, **Significant differences within a given time of experiment (one-way ANOVA, $P < 0.05$ and $P < 0.01$, respectively). *Dotted line* indicates significant differences when curves built at the three times of study (T0, T2 and T60) were compared after incubation treatment with 1, 5 and 20 µM Cu (two-way ANOVA)

time). ATOX responded differently depending on whether previous copper ingestion was acute (T2) or chronic (T60). MT2 responded as expected, increasing the mRNA transcripts as copper increased in the medium, strongly suggesting that the copper dosing used was—as planned—within the range handled by physiological adaptive mechanisms.

Analysis of the SG should keep in mind that the individuals tested were healthy by strict criteria and initially had normal or marginally low copper status. This clearly differs from data available in the literature, obtained in animal models with intense copper deficiency. Changes observed should indicate adaptive responses, representing a relevant piece of information in the process of characterizing CCS and other proteins as potential copper markers. It is interesting that CCS mRNA transcripts abundance decreased both after 2 days and after 60 days copper supplementation (Fig. 2), supporting the idea that CCS responds to copper availability in the range of the copper dosing used. Although speculative, we would like to interpret this finding as representing the physiological response to the extra copper "seen" by these cells during the supplementation period. After longer periods of exposure the response tended to diminish, suggesting adaptation (two-way ANOVA,

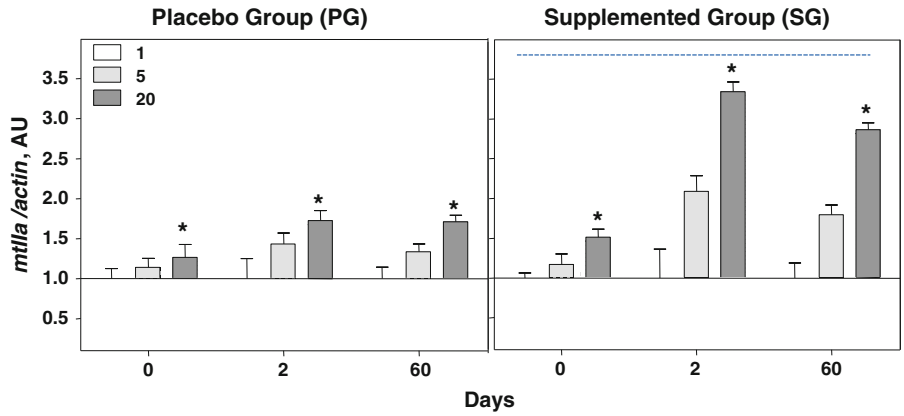

Fig. 6 *mt2* mRNA relative abundance in PMNCs obtained in PG and SG. PMNCs were incubated with three different copper concentrations (1, 5 and 20 μM) for 20 h, at three times (T0, T2 and T60). *, **Significant differences within a given time of experiment (one-way ANOVA, $P < 0.01$ and $P < 0.001$, respectively). *Dotted line* indicates significant differences when curves built at the three times of study (T0, T2 and T60) were compared after incubation treatment with 1, 5 and 20 μM Cu (two-way ANOVA $P < 0.001$)

time, treatment and interaction $P < 0.001$). Most previous studies have been conducted measuring CCS protein (Bertinato et al. 2003; Prohaska et al. 2003a; West and Prohaska 2004; Hepburn et al. 2009); in defining CCS as potential copper marker is important that other groups confirm that mRNA transcripts respond as found in this study.

Responses of SOD and ATOX mRNA transcripts were highest at 5 μM copper in the medium at the three times of study, suggesting that for these proteins regulation may differ at 5 and 20 μM copper in the medium; why mRNA transcripts of these two proteins are of a lesser magnitude at higher copper concentrations in the medium deserves further investigation. We may speculate that supplemented individuals may be conditioned after 60 days but not 48 h. Changes of Atox mRNA transcripts relative abundance is intriguing; there were clear trends in PG to decrease their expression at 5 μM Cu in the medium but the relation in SG was opposite.

In summary, in humans minor-to-moderate copper supplementation induced changes of CCS mRNA transcripts measured in PMNCs. These were as expected and in accordance to data obtained in protocols of severe copper deficiency. These results further support the idea that CCS may be a copper marker.

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