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Transcriptional activation of glutathione pathways and role of glucose homeostasis during copper imbalance

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Abstract Copper is an essential micronutrient for organism health. Dietary changes or pathologies linked to this metal induce changes in intracellular glutathione concentrations. Here, we studied the transcriptional activation of glutathione pathways in Jurkat cell lines, analyzing the effect of change in glucose homeostasis during a physiological and supra-physiological copper exposure. An immortalized line of human T lymphocyte cell line (Jurkat) was exposed to different copper and glucose conditions to mimic concentrations present in human blood. We applied treatments for 6 (acute) and 24 h (sustained) to 2 μM (physiological) or 20 μM (supra-physiological, Wilson disease scenario) of $CuSO_4$ in combination with 25 mg/dL (hypoglycemia), 100 mg/dL (normal) and 200 mg/dL (hyperglycemia, diabetes scenario) of glucose. The results indicate that a physiological concentration of copper exposure does not induce transcriptional changes in the glutathione synthesis pathway after 6 or 24 h. The G6PDH gene (regeneration pathway), however, is induced during a supra-physiological copper condition. This data was correlated with the viability assays, where fluctuation in both glucose conditions (hypo and hyperglycemia scenario) affected Jurkat proliferation when 20 μM of $CuSO_4$ was added to the culture media. Under a copper overload condition, the transcription of a component of glutathione regeneration pathway (G6PDH gene) is activated in cells chronically exposed to a hyperglycemia scenario, indicating that fluctuations in glucose concentration impact the resistance against

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the metal. Our findings illustrate the importance of glucose homeostasis during copper excess.

Keywords Copper · Glutathione · Glucose · Metabolism · Nutrigenomic

Abbreviations

Cu	Copper
GSH/GSSG	Glutathione (reduced/oxidized)
GGCS	γ-Glutamyl cysteine synthetase
GS	Glutathione synthase
GLUT1	Glucose transporter 1
G6PDH	Glucose-6-phosphate dehydrogenase
GR	Glutathione reductase
NADPH/ $NADP^+$	Nicotinamide adenine dinucleotide phosphate (reduced/oxidized)
ROS	Reactive oxygen species
ATP	Adenosine triphosphate
OD	Optical density

Introduction

It is recognized that copper (Cu) is an essential micronutrient for human health, it is a part of prosthetic group of enzymes that participate in primarily redox processes vital to the cell, such as cell respiration, free radical detoxification, biosynthesis of connective tissue, cellular iron metabolism and synthesis of neurotransmitters (Harris 2000; Kim et al. 2008). Cu deficiency can cause severe problems during embryonic and fetal development, and in later life, is accompanied by normocytic or macrocytic anemia, as well as altered lipid metabolism and (in severe cases) myelopathy (Jaiser and Winston 2010; Linder and Hazegh-Azam 1996; Medici 2013). Due to the inherent reactivity of Cu, excess Cu has toxic effects on human health, and is able to produce genome-wide damage of proteins and membranes (Linder and Hazegh-Azam 1996).

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Two diseases related to imbalances of Cu as a result of defective transport have been studied during the last 30 years. Menkes syndrome is an X-linked disease, which produces Cu deficiency causing progressive mental deterioration, hypothermia, connective tissue abnormalities, eventually leading to death in early childhood (Mercer 1998). Wilson's disease is autosomal pathology and is characterized by defective biliary excretion of Cu and hepatic pathology, followed by neurological, psychiatric, renal, hematologic and endocrine disorders (Bingham et al. 1998).

In recent years, genome-scale expression analysis has allowed the identification of the global transcriptional response of different cellular models to changes in Cu availability (Gonzalez et al. 2008; Muller et al. 2007; Song et al. 2009). Interestingly, besides genes involved in Cu homeostasis mechanisms, other metabolic process processes related to oxidative stress responses, protein synthesis, energy generation and primary glucose metabolism cover more than 90 % of the total transcriptional changes. These mechanisms describe a complex integrative response during Cu exposure, which involves other metabolic adjustments in what we understand today as the nutrigenomics of Cu (Panagiotou and Nielsen 2009). In particular, several studies indicate the importance of glutathione (GSH) during Cu imbalance (Cater et al. 2014; Jimenez et al. 2002; Jomova and Valko 2011). GSH is one of the principal antioxidant molecules generated by the organism involved in protection against oxidative stress, which itself can be markedly influenced by diet, principally by the bioavailability of glucose (Beutler 1989). The correct intracellular amount of GSH is controlled mainly by two independent metabolic processes (Lu 2009), (i) *de novo* synthesis, where two enzymes γ-glutamyl cysteine synthetase (GGCS) and glutathione synthase (GS), which binds glutamate, cysteine and glycine to generate the GSH molecule and ii) the GSH regeneration pathway, in which the oxidized GSH (GSSG) is reduced by the enzyme glutathione reductase (GR) to recover the molecule. This process requires the reducing power provided by the NADPH coenzyme principally produced by glucose metabolism through the enzymes glucose-6-phosphate dehydrogenase (G6PDH) in the pentose phosphate pathway.

This result supposes a putative influence of sugar availability over Cu homeostasis through the production of GSH, suggesting a molecular connection

between these two nutrients. Here we select a human blood cell line (Jurkat) to examine the metabolic regulation of this metal on the expression of the genes encoding the key enzymes involved in GSH synthesis and regeneration pathways, illustrating for the first time the importance of glucose homeostasis in Cu tolerance.

Methods

Cell line and reagents

The human leukemia T cell line: Jurkat Clone E6-1 (ATCC_ Number: TIB-152TM was acquired from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium (Gibco, Life Technology, Carlsbad, CA) supplemented with 10 % fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C and 5 % CO_2. Cells were then cultured at a density of 7.5×10^5 cells/well (8×10^4 cell/cm^2) in 6-well plates for 6 and 24 h to the 70–80 % confluence.

Cu and glucose treatments

Cu was supplemented to the culture medium as Cu-His complex (1:10 ratio) (Tapia et al. 2003), to simulate iso- (2 μM) or supra-physiological (20 μM) concentration for 6 or 24 h (acute or chronic sustained treatment, respectively) (Gutierrez-Garcia et al. 2013; Lech and Sadlik 2007; Linder and Hazegh-Azam 1996; Suazo et al. 2008). For glucose treatment, RPMI 1640 was supplemented with glucose to a final concentration of 25 mg/dL (hypoglycemia), 100 mg/dL (normal), 200 mg/dL hyperglycemia (untreated diabetes) (Expert Committee on the and Classification of Diabetes 2003). Relative survival of cells was evaluated by using MTT (trypan blue and 3-(4,5-105 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay as described previously (Tapia et al. 2003). The viability was above 85 % in all treatments with Cu–His. All analyses were performed in triplicate.

Intracellular Cu content

For Cu content quantification, cells were processed as described in Tapia et al. (2003). Briefly, cells were digested in nitric acid (JT Baker, Dublin, Ireland) in a vortex and diluted with distilled deionized water. Samples were incubated at 60 °C overnight. The Cu concentration was determined by means of a graphite furnace atomic adsorption spectrophotometer (AAS, Perkin Elmer; SIMMA 6100). Calibration was against a standard curve prepared using dilutions of a Cu standard (J.T. Baker), and the sample values were normalized to the total protein content (Bradford method) (Bradford 1976).

RNA extraction and cDNA synthesis

Total RNA extraction from cells was carried out using Tri-reagent (Ambion-Life Technologies, Carlsbad, CA), following manufacturer recommendations. RNA quantity was determined through an MBA 2000 (Perkin Elmer, Waltham, MA) at 260 nm and RNA purity was determined from optical density ratio at 260/280 nm. The total RNA extraction was treated with DNAse (DNAse I) and the integrity was checked by denaturing gel. 1.5 μg of RNA was used to synthesize single-strand cDNA with reverse transcriptase (M-MLV) (Promega, Madison, WI), plus RNAse inhibitor (RNAsin) according to manufacturer recommendations.

Real-time RT-PCR

Reactions were carried out in a LightCycler System 1.5 (Roche Diagnostics, Indianapolis, IN) using Platinum SYBR Green qPCRSuperMix-UDG (Applied Biosystems, Waltham, MA). Primers were designed using Primer3plus software: G6PDH (sense CGGC AACAGATACAAGAACG, antisense AGCAGTG GGGTGAAAATACG), 6PGDH (sense ATGCCCT GTTTTACCACTGC, antisense GATAAACTGCCC TGGTTTGG), GGCS (sense TCAGTGGGCACAGG TAAAAC, antisense CAGTCAAATCTGGTGGCA TC), GS (sense TGTGCAGATGGACTTCAACC, antisense CATCCTGTTTGATGGTGCTG), GR (sense TGCAGTTGGGGATGTATGTG, antisense TGAAGACCACAGTTGGGATG) and GLUT1 (sense AGCTGCCCTGTGTTCATTTC, antisense CT GGAAATTCAGGGGTGAAG). Reactions were performed in a volume of 10 μl containing 2 mM $MgCl_2$, 5 pmol of each primer and cDNA (1:15 dilution). Efficiency was calculated for each sample and gene by LinRegPCR v7.5 using data obtained from

exponential phase of each individual amplification plot (Ramakers et al. 2003). Human RPLP0 gene (de Cremoux et al. 2004) was used to normalize the expression levels between the samples (Dheda et al. 2004). The following standard thermal profile was used: 2 min at 50 °C, 2 min at 95 °C, 40 repetitions of 5 s at 95 °C and 15 s at 60 °C, and a final stage of 15 s at 72 °C. The mRNA abundance data for each gene are expressed as fold change according Pfaffl method (Pfaffl 2001). Three technical replicates were done for each combination of cDNA and primer pair, and the quality of the PCR reactions was checked through analysis of the dissociation and amplification curves. The products were resolved by 3 % agarose gel electrophoresis to confirm the DNA fragments of expected size.

Statistical and data analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Differences were assessed by Student's *t* test $p < 0.05$.

Results

Excess Cu activates the glutathione regeneration pathway

Figure 1 shows qPCR expression changes of the principal genes involved in the metabolism of GHS (*de novo* synthesis and regeneration pathways) in Jurkat cells, we include the glucose transporter 1 (GLUT1) as a putative indicator of sugar intake expressed in this cell line (Caro et al. 1998). The results indicate that the physiological Cu concentration (2 μM) does not induce changes into the GSH synthesis metabolic gene pathways, even after longer times of Cu exposure. On the contrary, the regeneration pathway, in particular G6PDH gene was induced during a supra-physiological Cu condition (20 μM during 24 h).

Cellular Cu content as determined under the same culture conditions as in the gene expression analysis is shown in Fig. 2. The results indicate that in all cases, cells gradually accumulate Cu in relation to the increase in the supply of metal, where the maximum concentration of the micronutrient (three-fold increase above the baseline) is reached at 24 h of treatment with 20 μM of Cu.

Glucose modulates cell viability under excess Cu conditions

Figure 3 illustrates cellular viability during Cu exposure of Jurkat cells lines under different concentrations of glucose added in the media that emulate three blood sugar scenarios. During 6 h of Cu treatment no changes were observed in viability in comparison to the normal condition, correlating this data with the absence of expression changes showed in the qPCR assays. The low glucose concentration (hypoglycemia) during 24 h of exposure to 20 μM of Cu produced a small but significant decrease in cellular viability relative to the normal blood glucose state. Addition of glucose at 100 mg/dL with the 20 μM Cu treatment recovered viability at 24 h compared to the 25 mg/dL (hypoglycemic) state; while cells exposed to 20 μM Cu and 200 mg/dL of glucose (hyperglycemia) for 24 h, showed enhanced cellular viability with an increase of 20 %, indicating condition protection against the metal 1overload.

Discussion

Given the broad biochemical requirements of Cu for humans, the correct nutrition of sufficient levels of this metal for growth, proliferation and development is fundamental. Conversely, the potential to generate oxidative stress via production of reactive oxygen species (ROS) not only demands a tight homeostatic regulation of Cu acquisition, distribution and use, but also requires a complete cellular metabolic adjustment (Kim et al. 2008).

Currently, the availability of global scale experiments has allowed for the determination that this metal has a global metabolic effect over the organism (nutrigenomics of Cu), where the micronutrient activates several metabolic processes in order to maintain global homeostasis and avoid damage generated by ROS. In this context, it has been shown that genes involved in glucose metabolism, in particular the pentose phosphate pathway, can be activated as a potential requirement to prevent ROS damage through the participation of GSH (Armendariz et al. 2004; Freedman et al. 1989; Ralser et al. 2007).

Our transcriptional approach in the Jurkat cell line showed that the exposure to a supra-physiological concentration of Cu (emulating a Wilson disease

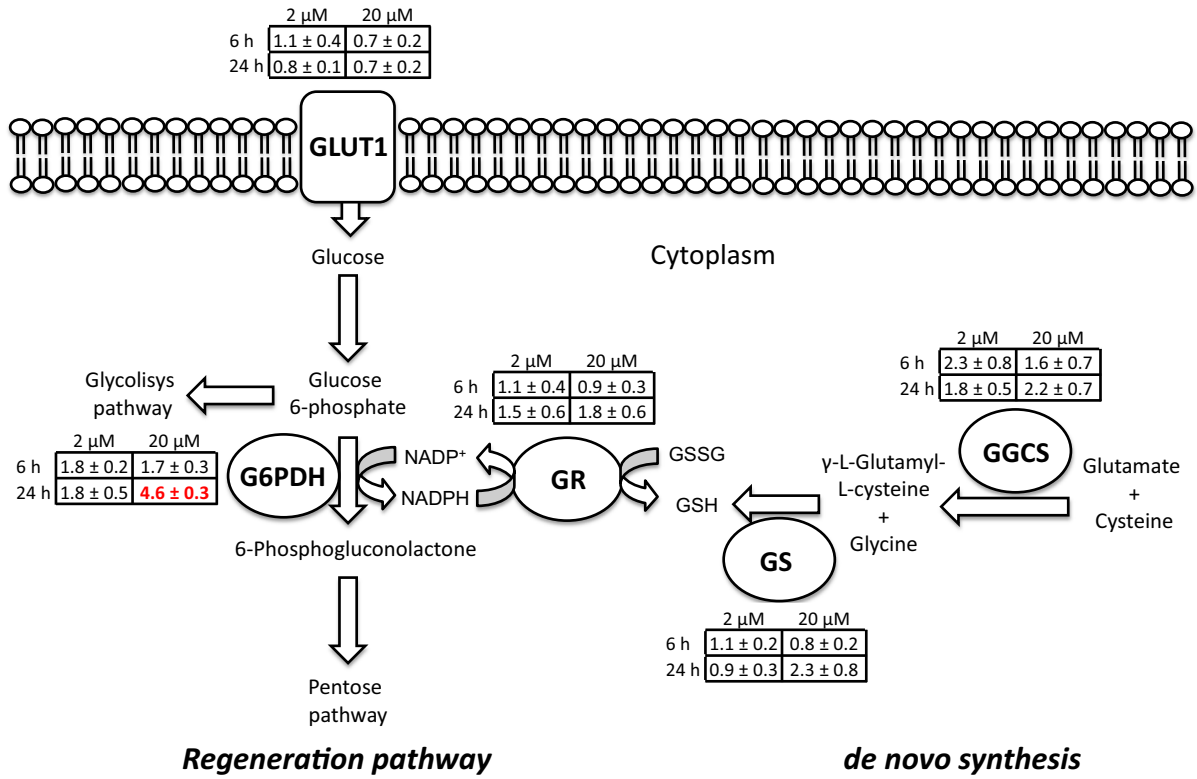

Fig. 1 Changes in the relative abundance of synthesis *de novo* and regeneration of GSH genes during different scenarios of Cu excess. Values in tables denote the fold change (ratio) between the Cu treatment and the basal status for each time and concentration determined by qPCR. Red number indicates significant differences between Cu treatment and the basal status at the same exposure time (Student's t test $p < 0.05$). (Color figure online)

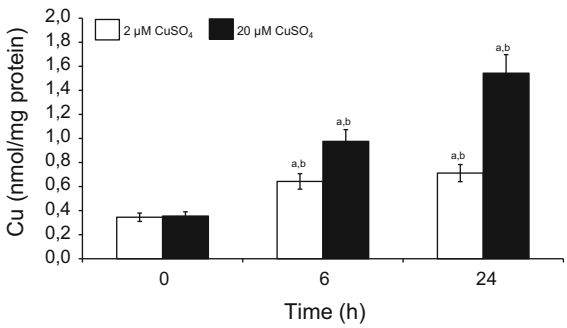

Fig. 2 Time course of intracellular Cu content exposed to physiological and supra-physiological levels of Cu. All values are expressed as the direct Cu content normalized by the total protein quantification. Letters indicate significant differences (student test $p < 0.05$), **a** between the basal status and the Cu, **b** between Cu treatments at the same time

scenario) activates the expression of G6PDH gene. This enzyme controls carbon flow through the pentose phosphate pathway, generating the reducing power (NADPH) used during the regeneration of GSH (Salvemini et al. 1999), whose activity also is directly enhanced by dietary carbohydrates and is inhibited by dietary polyunsaturated fats (Salati and Amir-Ahmady 2001).

The observation that the activation of G6PDG gene in a supra-physiological Cu condition is correlated with the highest intracellular Cu content suggests that during chronic Cu exposure, the transcriptional activation of the rate-limiting regeneration pathway component is a mechanism to avoid damage produced by excess of the metal, probably related with the generation of ROS. Likewise, the non-activation of the synthesis *de novo* indicates that the cells already contain the necessary amount of total GSH forms (GSH and GSSG include) to handle Cu toxicity. This remains true even under the extreme supra-physiological metal concentration exposure treatment. Similarly, the absence of change in GLUT1 transcription at the physiological glucose concentration indicates that glucose is not limiting in this scenario.

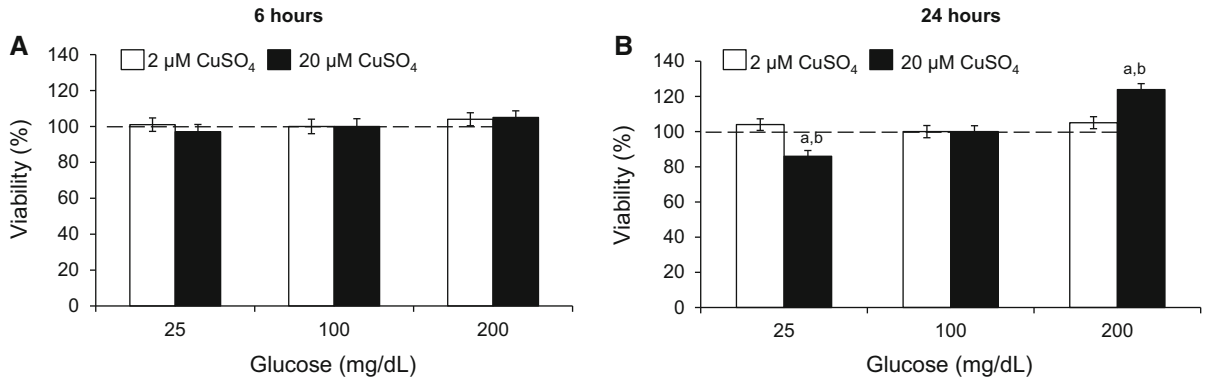

Fig. 3 Cellular viability in different glucose conditions under physiological and supra-physiological levels of Cu. Values expressed as the percent OD change between the glucose treatment against the normal glucose condition (100 mg/dL) at two times of Cu exposure 6 (**a**) and 24 h (**b**) respectively. Each value represents the average of three biological replicates with three technical replicates. Letters indicate significant differences (student test $p < 0.05$), **a** between the basal status and the Cu or glucose concentration, **b** between Cu treatments at the same time or glucose concentration

We also evaluated whether changes in glucose status could impact the viability of the cells during the imbalance of Cu. At 20 μM of extracellular Cu, the hypoglycemia condition significantly reduced the viability of the Jurkat cells, relative to normal glucose status, while hyperglycemia increased cellular viability (a phenotype observed previously in prokaryotic organisms) (Latorre et al. 2014). One explanation for these effects on cell viability may lie in variation in GSH regeneration via the pentose phosphate pathway, which as has been reported as a functional capacity dependent of glucose availability (Ralser et al. 2007). While 200 mg/dL generated protection against Cu toxicity, this concentration of blood glucose can only be present or observed in untreated diabetic patients (Home et al. 2008). Several studies indicate a close relationship between diabetes and Cu metabolic disorders (Basaki et al. 2012; Uriu-Adams et al. 2005; Zheng et al. 2008). A high intake of glucose is not recommended for human health, however, even in Wilson patients, highlighting the importance of maintaining appropriate concentrations of glucose in the blood.

Conclusion

The nutrigenomics of Cu can be understood as the metabolic interactions between the metal dietary fluctuations and the genome as well as the resulting changes in gene expression, proteins and other metabolites. The data generated here, demonstrated that under physiological conditions, Jurkat cells (a model of human blood cell line) could accommodate variation of Cu and glucose and maintain cellular homeostasis. However, in a scenario similar to Wilson disease, the complete transcriptional analysis of GSH pathways showed that the regeneration of GSH is activated as a response to Cu overload. Within the same conditions, fluctuation in glucose can impact tolerance to the metal, reaffirming the crucial importance to maintain glucose homeostasis in the organism and avoid the serious nutritional problems generated by the imbalance in both micronutrients (McGuinness 2005). In addition to its direct effects over the GSH regeneration pathway, glucose participates in other metabolic processes involved in Cu cellular protection, including energy production (ATP) via glycolysis (Bundy et al. 2008), and nucleoside triphosphates used by Cu-ATPases during the detoxification of the metal (Southon et al. 2010), opening a completely new line of investigation in terms of which other metabolic pathways related to glucose also are activated during Cu imbalance.

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Conflict interest All the authors of this work declare that they have no conflict of interest.

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