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Physiological copper exposure in Jurkat cells induces changes in the expression of genes encoding cholesterol biosynthesis proteins

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Abstract Copper is an essential micronutrient that functions as an enzymatic cofactor in a wide range of cellular processes. Although adequate Cu levels are essential for normal metabolism, excess Cu can be toxic to cells. Cellular responses to copper deficiency and overload involve changes in the expression of genes directly and indirectly involved in copper metabolism. However little is known on the effect of physiological copper concentration on gene expression changes. In the current study we aimed to establish whether the expression of genes encoding enzymes related to cholesterol (*hmgcs1*, *hmgcr*, *fdft*) and fatty acid biosynthesis and LDL receptor can be induced by an iso-physiological copper concentration. The iso-physiological copper concentration was determined as the bioavailable plasmatic copper in a healthy adult population. In doing so, two blood cell lines (Jurkat and THP-1) were exposed for 6 or 24 h to iso- or supraphysiological copper concentrations. Our results indicated that in cells exposed to an iso-physiological copper concentration the early induction of genes involved in lipid metabolism was not mediated by copper itself but by the modification of the cellular redox status. Thus our results contributed to understand the involvement of copper in the regulation of cholesterol metabolism under physiological conditions.

Keywords Gene expression · Copper · Cholesterol · ROS · Jurkat · THP-1

Introduction

Copper is an essential micronutrient in humans (Uauy et al. 1998). Its ability to accept and donate electrons

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gives copper the characteristic of being a very efficient cofactor for redox enzymes. Copper-dependent enzymes participate in diverse physiological processes, such as electron transport by cytochrome *c* oxidase in the oxidative phosphorylation, protection against free radicals such as superoxide ion through the Cu/Zn superoxide dismutase (SOD1), stabilization of extracellular matrix by cross-linking collagen and elastin through protein-lysine 6-oxidase or iron homeostasis through ferroxidase activity of ceruloplasmin, among others (Lee et al. 2001). However, excess copper potentially produces reactive oxygen species (ROS), via Fenton-like reaction, which may promote oxidative damage of many biological targets including, lipoproteins, DNA, or thiol-containing enzymes (Luza and Speisky 1996). To prevent the consequences of copper deficiency or overload, mammalian have evolved molecular mechanisms that regulate its uptake, intracellular traffic, storage, and efflux. Cellular responses to variations in copper levels included post-traductional modifications (Gupta and Lutsenko 2009) and/or changes in the gene expression patterns of the molecular components involved in copper metabolism (Cousins 1994). Regarding transcriptional mechanism, several studies in different biological models showed that transcriptional changes in response to varying copper levels include both genes directly involved in copper homeostasis (Armendariz et al. 2004; Gonzalez et al. 2008; Kelly and Palmiter 1996; Sadhu and Gedamu 1989) and genes involved in different cellular process not directly connected to copper metabolism that, however change their expression during the cellular adaptation to copper availability (Gonzalez et al. 2008; Muller et al. 2007). In this context, an interesting link between copper homeostasis and lipid metabolism has been reported in the literature (Lei 1991). For instance, in rats, copper deficiency stimulates hepatic lipogenic gene expression by increasing the hepatic translocation of mature SREBP-1 (Tang et al. 2000). Moreover, in livers of murine models of Wilson's disease, the high concentration of intracellular copper correlates with a decreased expression of genes associated with cholesterol biosynthesis and with low cholesterol concentration (Huster et al. 2007). However, the signaling pathways underlying the transcriptional regulation of cholesterol related genes by copper exposure is not known. On one hand, copper may affect intracellular redox status, which has been connected with regulatory transcriptional pathways (McElwee et al. 2009; Wang et al. 2010), on the other copper may modulated metal transcription factors response by itself (Heuchel et al. 1994; Lichtlen and Schaffner 2001; Saydam et al. 2002). Alternatively, changes of cellular copper content may alter metal-binding sites, via amine, thiolate, carboxyl, and other ligands presents in proteins, including transcription factors, following via the Irving-Williams order (Cousins 1994). In this scenario, our aim was to examine whether changes in the expression of genes encoding enzymes related to cholesterol (*hmgcs1*, *hmgcr*, *fdft*) and fatty acid (*fasn*) biosynthesis and LDL receptor (*ldlr*) can be induced by changes in the internal content of the metal or by a modification of the cellular redox status. In doing so, we use two blood cell lines (Jurkat and THP-1) (Palmer et al. 2006) maintained during 6 or 24 h at iso- or supra- physiological copper concentration (2 or 20 μM Cu-His, respectively). Our results provide experimental evidence that in an iso-physiological extracellular copper concentration the activation of cholesterol related genes correlated with a significant increment of ROS while the intracellular copper concentration was still lower than the concentration needed to induce metallothionein gene expression.

Materials and methods

Cell line and reagents

The human leukemia T cell line: Jurkat Clone E6-1 (ATCC® Number: TIB-152TM) and human leukemia monocyte: THP-1 (ATCC® Number: TIB-202TM) were acquired from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium (Gibko, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C and 5 % CO_2. These cells were 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-His treatments

Copper was supplemented to the culture medium, as Cu-His complex (1:10 ratio) (Tapia et al. 2003), to

reach iso- or supra-physiological concentration (2 or 20 μM) for 6 or 24 h (Linder and Hazegh-Azam 1996; Suazo et al. 2008). For copper depletion, cells were incubated 48 h previous to copper treatment in medium supplemented with 200 μM of bathocuproine disulfonic acid (BCS), and then washed three times with PBS-EDTA. 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 samples.

Intracellular copper content

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

ROS determination

Intracellular ROS was determined using 2′,7′-dichlorodihydro fluorescein diacetate (H_2DCF-DA) (Sigma-Aldrich Corp., St. Louis, MO, USA) as described (Steinbrenner et al. 2005). Cells were exposed to Cu-His (2 and 20 μM) for the indicated times (6 and 24 h) and then stained with H_2DCF-DA (10 μM) for 30 min at 37 °C and 5 % CO_2 in the dark. After that, cells washed twice with PBS and measured fluorescence (excitation: 485 nm, emission: 545 nm). The mean data was normalized to the total protein content.

RNA extraction and cDNA synthesis

Total RNA extraction from cells was carried out using Tri-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 260/280 nm. The total RNA extraction was treated with DNAse (DNAse I) (Ambion, Austin, TX, USA) and the integrity was checked by denaturant gel. 1.5 μg of RNA was used to synthesize single-strand cDNA with reverse transcriptase (M-MLV) (Promega, Madison, WI, USA), plus RNAse inhibitor (RNAsin) (Promega, Madison, WI, USA) according to manufacturer recommendations.

Real-time RT-PCR

Reactions were carried out in a LightCycler System 1.5 (Roche Diagnostics, GmbH, Rotkreuz, Switzerland) using Platinum SYBR Green qPCR SuperMix-UDG (Applied Biosystems, CA, USA). Primers were designed using Perlprimer software (Marshall 2004) and are described in Table 1S (see supplementary material). Reaction mixes were performed in a volume of 15 μl containing 2 mM $MgCl_2$, 5 pmol of each primer and 12 ng of cDNA (1:15 dilution). Efficiency was determined 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 (de Cremoux et al. 2004) was used as gene reference 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. mRNA abundance data for each gene are expressed as fold change according Pfaff method (Pfaffl 2001).

Statistical and data analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Differences between multiple groups were assessed by ANOVA using Tukey's post-test. A mix model two ways ANOVA was used in Fig. 1. Statistical significance was defined as $p < 0.05$. The data of Fig. 2 was fitted to four parameters logistic (4PL) equation using non-linear regression analysis.

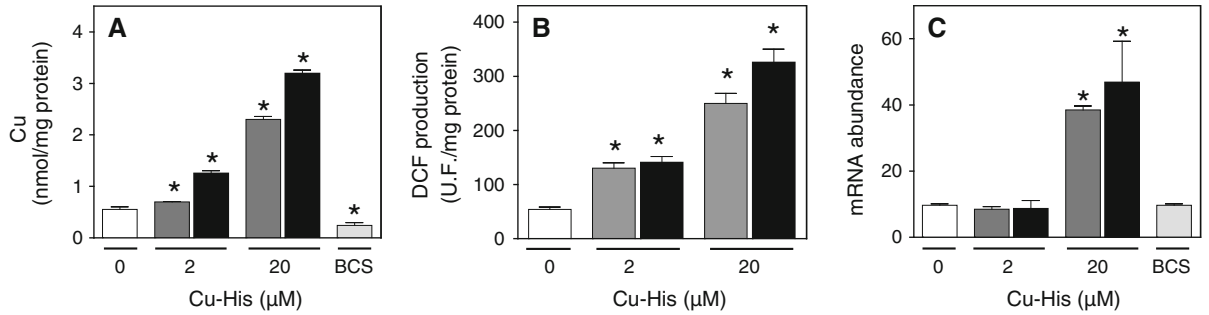

Fig. 1 Copper and ROS contents in Jurkat cells. **a** Intracellular copper content was measured by AAS in Jurkat cells supplemented with 2 and 20 μM of Cu-His for 6 (*gray bars*) and 24 h (*black bars*) and 24 h without supplementation as a control. (*white bars*). Cells treated with BCS (200 μM) for 48 h (*light gray bars*). **b** ROS production was measured by fluorescent detection of DCF (excitation: 485 nm, emission: 545 nm) in cells treated as described in (**a**) and values are expressed as mean ± SEM from $n = 6$. **c** *mt2a* transcript abundance was quantified by real time RT-PCR and normalized with the values of the housekeeping gene (*rplp0*) in cells treated as was described in (**a**). Values for (**a**, **b** and **c**) are expressed as mean ± SEM from $n = 5$. *Asterisks* indicate significant difference (*$p < 0.05$, ANOVA, Tukey post-hoc test)

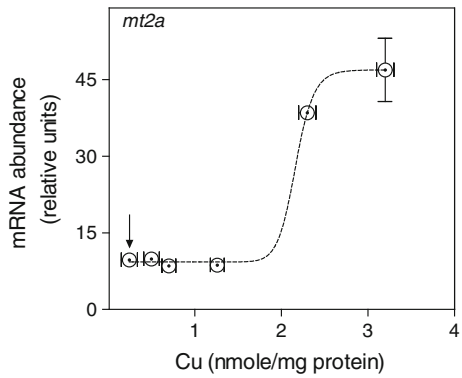

Fig. 2 Correlation between intracellular copper content and *mt2a* transcript levels when cells were exposure to graded concentrations of the metal (data from Fig. 1a, b). Values are expressed as mean ± SEM ($n = 5$). The data were fitted to a logistic function with r^2: 0.91. *Arrow* indicate data from BCS treatment

Results

Copper and ROS contents in Jurkat cells exposed to iso- and supra-physiological copper concentrations

First, we evaluated the effect of copper supply on the uptake of copper in the Jurkat cell model. For this, cells were exposed to copper in the form of Cu-His complex (1:10) to improve the uptake of metal because this form is more similar to the copper that is available in the plasma (Deschamps et al. 2004; Kreuder et al. 1993). Cells were supplemented with Cu-His (2 μM) during 6 and 24 h. This copper concentration is within the iso-physiological range of bioavailable copper (Suazo et al. 2008), as calculated from plasmatic levels of non-ceruloplasmin copper data obtained from healthy adults that represented the 5 % highest and lowest extremes in the distribution curve of serum ceruloplasmin concentrations of 800 individuals (Suazo et al. 2008). Therefore, a supplement of 20 μM of Cu–His was used as a supra-physiological copper exposure (Linder & Hazegh-Azam 1996). In addition, BCS a specific copper chelator, was used to evaluate the effect of the copper depletion. In the control condition, the unsupplemented medium contained 0.4 μM of copper according to Tapia et al. 2004. The results showed that both copper exposure and BCS treatment modified the intracellular concentration of the metal (Fig. 1a). The variations in copper contents were both dose (2–20 μM of Cu–His) and time-dependent (6–24 h) and a two way ANOVA mixed model (dose x time) revealed the interaction of both variables. However, 90 % of the variation was dependent on the dose, with a maximum of 3.2 nmol Cu/mg protein after 24 h of exposure to 20 μM (Fig. 1a). Given that, increase of intracellular copper can lead to ROS (Gaetke and Chow 2003; Kehrer 2000), we analyzed whether intracellular ROS level was affected in Jurkat cells exposed to copper (Fig. 1b). ROS increased significantly after 6 and 24 h in the cells exposed to iso-physiological copper concentration, however more ROS was accumulated in the cells treated with 20 μM of Cu–His compared with 2 μM Cu–His treatment (Fig. 1b).

Induction of metallothionein gene expression in Jurkat cells is controlled by the level of intracellular copper

We evaluated the transcriptional response *mt2a* gene under the same conditions of copper exposure described above. This gene that encodes the metallothionein 2A protein has been reported to be responsive to copper in a wide range of conditions (Pauwels et al. 1994; Sadhu and Gedamu 1989; Tapia et al. 2004; Thiele 1992). A significant increase in *mt2a* transcript abundance was detected after 6 and 24 h of exposure to 20 μM of Cu–His (Fig. 1c). To analyze the correlation between mRNA abundance of *mt2a* and intracellular copper content, we re-assessed the expression data with respect to the intracellular copper content in the Jurkat cells exposed to different copper concentrations for 6 and 24 h, as presented in Fig. 2. The results indicated a positive correlation between the internal contents of copper and the mRNA abundance of *mt2a*, which fits with a logistic regression model (see "Materials and methods" section). Thus below 2 nmol Cu/mg protein, a basal expression level of *mt2a* was measured, however when the content exceeded this level, *mt2a* abundance increased more than five times (Fig. 2).

Physiological copper concentration induces the expression of cholesterol and fatty acid genes

To assess whether iso- or supra-physiological extracellular copper were able to induce the expression of cholesterol genes we measured transcript abundance of key genes associated with lipid metabolism, such as *hmgcs1, hmgcr* and *fdft1* genes that encode enzymes involved in the biosynthesis cholesterol from acetyl-CoA. In addition we quantified the mRNA abundance of *ldlr*, an important receptor in the incorporation of exogenous cholesterol and that of *fasn*, a key enzyme in the biosynthesis of fatty acids from malonyl-CoA (Fig. 3). All these genes showed a significantly increment in their abundance after 6 h of exposure to 2 and 20 μM copper, similar results were observed for *hmgcs1*, *fasn* and *ldlr* when the monocyte line cell THP-1 was used (see Fig. 1S). For *fdft1* increase in transcript abundance was still observed after 24 h of exposure to 2 and 20 μM of extracellular Cu–His, whereas in the case of *hmgcs1* increased levels of transcripts were detected only after 24 h of exposure to 20 μM of Cu–His (Fig. 3). The other three transcripts (*hmgcr*, *ldlr* and *fasn*) showed a transient increment in abundance reaching a maximum at 6 h of Cu–His exposure (2 and 20 μM) and decreasing after that to the level measured in cells non-exposed to copper (white bar, Fig. 3). Similar temporal gene expression pattern was observed in THP-1 cells for *hmgcs1*, *ldlr* and *fasn* (Fig. 1S). However the BCS abolished the early transcriptional response of *ldlr* and *fasn* at iso and supra-physiological copper concentration (right panel, Fig. 3), suggesting that this response is sensitive to decrease a basal intracellular copper content. These results obtained with peripheral blood mononuclear cell lines indicated that transcripts level of genes that encoded protein involved in cholesterol and fatty acid metabolism were modified early (6 h) in response to an iso-physiological copper exposure.

Discussion

In this paper, we evaluated the transcriptional consequences of intracellular copper increment and/or ROS accumulation in peripheral blood mononuclear cell lines exposed to iso- and supra-physiological extracellular copper. We aimed to assess whether the expression of genes that are not directly associated with copper metabolism (cholesterol and fatty acid genes) was affected in this range of copper exposure. As a first step, we defined cell culture conditions that increase intracellular copper content and expression of *mt2a* gene. Our results indicated that in Jurkat cells, the *mt2a* was only induced when intracellular copper reached a threshold level of 2 nmol Cu/mg protein after either 6 or 24 h of exposure to supra-physiological concentration of extracellular copper. These results are in agreement with the metallothionein gene expression is controlled by the level of intracellular copper (Huster et al. 2007; Sadhu and Gedamu 1989; Tapia et al. 2004). In contrast, the expression of genes involved in lipid metabolism, was induced by iso-physiological concentration of extracellular copper and, thus the intracellular copper concentration was below the experimental threshold level calculated for *mt2a* induction. These results suggest that lipid metabolism genes were induced by a mechanism that probably depend on copper exposure but it did not require a significant increment of intracellular copper.

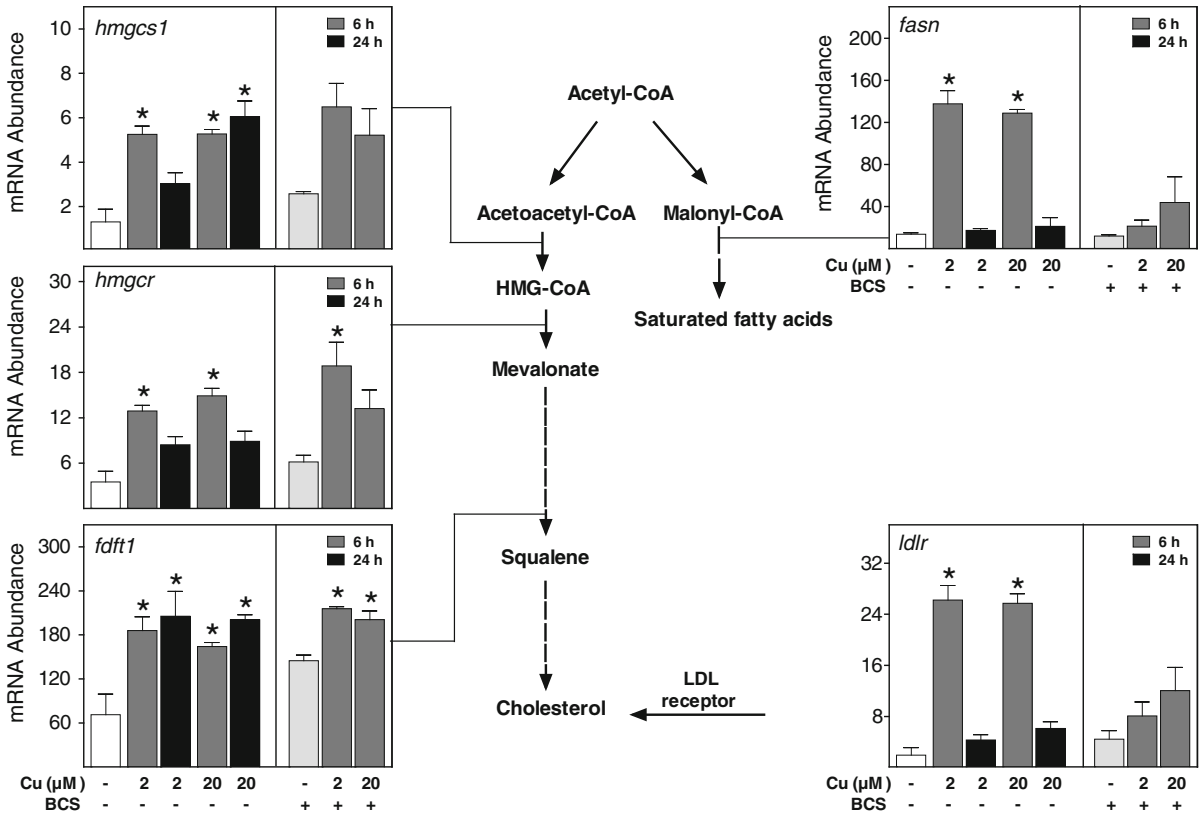

Fig. 3 Relative transcript abundance of lipid metabolism genes in cells exposed to copper. The diagram key genes in cholesterol biosynthesis (*hmgcs1*, *hmgcr* and *fdft1*) of fatty acids (*fasn*) and uptake of cholesterol (*ldlr*). Jurkat cells were supplemented with 0, 2 and 20 μM of Cu-His for 6 h (*gray bars*) and 24 h (*black bars*) of exposure to the metal Relative transcript levels were analyzed by real time RT-PCR (*left panel*). Cells treatment with 200 μM of BCS (light gray bars) for 48 h and 2 and 20 μM of Cu-His for 6 h (gray bars, right panel). Data shown are transcript abundance relative to gene reference (*prlp0*). Values are expressed as mean ± SEM in triplicate and *asterisks* indicate significant difference ($*p < 0.05$, ANOVA, Tukey post-hoc test)

Interestingly, it has been shown that null or marginal increments of intracellular copper may affect the redox status of cells (Gaetke and Chow 2003; Kehrer 2000), which in turn can induce transcriptional changes in a range of minutes to hours (Sekiya et al. 2008, 2004; Svensson et al. 2003). In support of this possibility, a previous work has showed that human macrophages maintained for 6 h in a medium supplemented with a low concentration of copper (0.4 μM of $CuSO_4$) increased the expression of *ldlr* and *hmgcr* (Svensson et al. 2003). Considering that the level of non-Cp copper observed in healthy adults, with low level of ceruloplasmin, is 1.1 ± 0.5 μmol/L (Suazo et al. 2008), supplements of 0.4 μM of $CuSO_4$ may be considered as an iso-physiological plasmatic copper concentration. Moreover, transcriptional changes of genes encoding proteins involved in fatty acid metabolism have been induced in cells treated with H_2O_2 (Sekiya et al. 2008), suggesting that a small increment of intracellular copper may induce expression of genes associated with lipid metabolism through a copper dependent change of cellular redox status. In this work, we showed a direct correlation between extracellular level of copper and ROS accumulation in Jurkat cells, suggesting that the early induction of the lipid genes in cells exposed to an iso-physiological copper concentration may be mediated by the early accumulation of ROS.

In this context we propose a two-step signaling pathway activated by an iso-physiological plasmatic copper level: 1) a slight increment of intracellular copper modifies the redox status of cells, and 2) changes of redox status may promote the activity of transcription factors, for example SREBP-1a, -1c

and -2 belonging transcription factor SREBPs family (Amemiya-Kudo et al. 2002; Burkhead et al. 2011). However, the strongest up-regulation of *ldlr* and *fasn* after 6 h of exposure to 2 μM copper seemed to depend on a basal intracellular copper content, which was necessary to elicit gene activation under iso-physiological conditions. Even though further investigation is required to elucidate the molecular bases of the signaling pathway activated by iso-physiological copper level, our results suggest that variations in copper bioavailable even in a physiological range, might be affect cellular processes linked to lipid metabolism, among them cell–cell interaction, cell signaling through lipid rafts or just *de novo* cholesterol biosynthesis. However, further investigation is required to elucidate the molecular bases of the signaling pathway activated by iso-physiological copper level.

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