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Copper depletion increases the mitochondrial-associated SOD1 in neuronal cells

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Abstract The role of copper in the toxicity of mutant copper-dependent enzyme superoxide dismutase (SOD1) found in patients affected with the familial form of amyotrophic lateral sclerosis (fALS) is widely debated. Here we report that treatment of human neuroblastoma cells SH-SY5Y with a specific copper chelator, triethylene tetramine (Trien) induces the decrease of intracellular copper level, paralleled by decreased activity of SOD1. A comparable effect is observed in mouse NSC-34-derived cells, a motoneuronal model, transfected for the inducible expression of either wild-type or G93A mutant human SOD1, one of the mutations associated with fALS. In both cell types, the drop of SOD1 activity is not paralleled by the same extent of decrease in SOD1 protein content. This discrepancy can be explained by the occurrence of a fraction of copper-free SOD1 upon copper depletion, which is demonstrated by the partial recovery of the enzyme activity after the addition of copper sulphate to homogenates of SH-SY5Y cells. Furthermore, copper depletion produces the enrichment of the physiological mitochondrial fraction of SOD1 protein, in both cells models. However, increasing the fraction of mitochondrial, possibly copper-free, mutant human SOD1 does not further alter mitochondrial morphology in NSC-34-derived cells. Thus, copper deficiency is not a factor which may worsen mitochondrial damage, which is one of the earliest events in fALS associated with mutant SOD1.

Keywords Copper · Mitochondria · SOD1 · SH-SY5Y · NSC-34 · ALS

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It is established that copper deficiency can cause impairment of brain development and neurodegeneration through mitochondrial damage, energy depletion, oxidative stress, and eventually neuronal cell

death (Prohaska and Smith 1982; Lombardo et al. 2003), and much evidence converges on the alteration of copper homeostasis as one of the possible factors involved in neurodegenerative processes, like those involved in Alzheimer's disease (Barnham and Bush 2008; Zheng et al. 2010). Furthermore, patients presenting pathological features typical of amyotrophic lateral sclerosis (ALS), a lethal disease accompanied by selective and progressive degeneration of motor neurons and by mitochondrial impairment (Valentine et al. 2005; Cozzolino et al. 2008), were in fact affected by copper deficiency (Weihl and Lopate 2006).

Twenty percent of the cases of the familial form of ALS (fALS) are linked to mutations in the gene coding for SOD1, that turn SOD1 into a cytotoxic agent, independently from the enzymatic activity (which is fully retained by many of the mutated forms) possibly because of its propensity to aggregate, both in the cytosol and in the mitochondria (Deng et al. 2006; Cozzolino et al. 2009).

Copper homeostasis in mammalian cells is under the control of a well coordinated network of proteins (called "copper chaperones") which regulate its uptake and extrusion, and selectively escort the metal to the different subcellular districts (Huffman and O'Halloran 2001; van den Berghe and Klomp 2010). It has been recently shown, in the spinal cord of transgenic mice, that a mutant SOD1 associated with fALS (namely G93A) affects the expression of copper chaperones, also sequestering some of them in aggregates, thus disrupting intracellular copper homeostasis (Tokuda et al. 2009).

Several studies on different mutant SOD1s reported that these proteins preferentially associate to motor neuron mitochondria (reviewed by Cozzolino et al. 2008). For a long time, SOD1 was considered to be exclusively a cytosolic enzyme, until a small fraction was identified in the intermembrane space (IMS) of mitochondria (Okado-Matsumoto and Fridovich 2001; Sturtz et al. 2001). As demonstrated in yeast, SOD1 enters the outer mitochondrial membrane in the form of non-metallated protein and in disulfide reduced state; copper insertion, mediated by the protein chaperone CCS (Copper Chaperone for Superoxide dismutase), then entraps the protein in the IMS (Field et al. 2003). We have previously demonstrated, in both cell and animal models, that under depletion of copper content, SOD1 is inactive but remains partially as copper-free protein (Rossi et al. 1994, 1997; Lombardo et al. 2003); therefore, it is conceivable that, upon shortness of the metal, the amount of SOD1 associated with mitochondria might increase.

In order to investigate the effect of low levels of copper on the amount of mitochondria-associated SOD1 and on mitochondria integrity, we induced copper depletion in human neuroblastoma cells SH-SY5Y or in mouse motoneuronal cells NSC-34, the latter transfected for the inducible expression of wild-type or of one of the mutant SOD1 associated with fALS, namely G93A, which is a "wild-type-like" protein, retaining almost full SOD1 activity (Ferri et al. 2006). Our results demonstrate that copper deficiency, in both experimental models, causes an increase of the mitochondrial content of SOD1 that, however, in NSC-34-derived cells, does not alter mitochondrial morphology and does not worsen SOD1 mutant G93A-induced mitochondrial impairment.

Materials and methods

Cell lines

Human neuroblastoma cells SH-SY5Y were purchased from the European Collection of Cell Culture (Salisbury, UK) and were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Sigma, St Louis, MO, USA).

Mouse cell line NSC-34, a hybrid neuroblastoma × spinal cord-34 cells, considered the best motoneuronal cell line model system available, was originally donated by Dr. N.R. Cashman, University of Toronto, ON, Canada (Cashman et al. 1992). Cells were stably transfected for the tetracycline-inducible expression the cDNA encoding human SOD1 (hSOD1), either the wild-type (Wt) or the mutant G93A (G93A) form (for details see Ferri et al. 2006). NSC-34-derived cells were grown in Dulbecco's modified Eagle's/F-12 medium (Sigma, St Louis, MO, USA) supplemented with 10% tetracycline-free fetal calf serum (Cambrex, East Rutherford, NJ, USA).

Both cell lines were maintained at 37°C in an atmosphere of 5% CO_2 in air.

For all experiments, cells were plated at the density of 2×10^5 cell/ml of culture medium.

Treatments

To achieve copper depletion, SH-SY5Y cells or NSC-34-derived cells were incubated with the specific copper chelator triethylene tetramine tetrahydrochloride (Trien) (Sigma, St Louis, MO, USA), at the final concentration of 125 μM, from the day after plating up to 3 days later as previously described (Lombardo et al. 2003). In NSC-34-derived cells, expression of exogenous hSOD1 was induced by adding 2 μg/ml doxycycline in the last 24 h of Trien treatment.

Immunocytochemistry

After Trien and doxycycline treatment, NSC-34-derived cells were fixed as described (Arciello et al. 2010) and incubated with primary rabbit polyclonal antibody against SOD2 (a specific mitochondrial matrix enzyme) (Upstate Biotechnology, Whaltam, MA, USA) followed by anti-rabbit secondary antibody conjugated with tetramethyl rhodamine isothiocyanate (TRITC, red) (Molecular Probes, Eugene, OR, USA) and examined under a fluorescence microscope (Nikon-Eclipse TE 200; Nikon Instruments Inc., Melville, NY, USA).

Preparation of cell lysates and fractionation

At the end of the treatments, cells were detached from the monolayer by a plastic scraper, extensively washed in PBS (Phosphate Buffered Saline: phosphate buffer 10 mM, KCl 2.7 mM, NaCl 137 mM, pH 7.4), resuspended in hypotonic PBS (1:2, v:v), and sonicated for 10 s. Whole cell lysates were used for copper content assessment, whilst SOD1 activity was assayed in a cleared supernatant, obtained by centrifugation for 30 min at 23,000×*g*.

Copper levels were measured by atomic absorption spectrophotometry after digestion with nitric acid as described (Arciello et al. 2010). SOD1 activity was determined by in-gel inhibition of the staining with nitro blue tetrazolium, as described (Lombardo et al. 2003).

For Western blotting analyses, cells were detached, washed and treated with a lysis buffer (Tris–HCl 10 mM, 150 mM NaCl, 1% Triton X-100, 10% proteases inhibitor cocktail (Sigma, St Louis, MO, USA)) for 30 min on ice, followed by centrifugation (23,000×*g*, 30 min). Supernatants were stored at −80°C until use. Protein content was assayed as described (Arciello et al. 2010).

The isolation of purified mitochondria was carried out by a sucrose gradient as described previously (Arciello et al. 2010).

Western blotting analyses

Immunoelectrophoresis was performed following standard 12 or 7.5% SDS PAGE. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and CCS were identified by monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). VDAC (Voltage-Dependent Anion Channel), a protein marker for the mitochondrial membrane, was identified by a monoclonal antibody obtained from Molecular Probes (Eugene, OR, USA). Protein-antibody complexes were identified with the Super Signal Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The densitometric analyses of blots were performed by a computerized image processing system (Gel-Pro Analyzer, Media Cybernetics Inc., Bethesda, MA, USA).

Statistical analysis

The results are presented as mean ± standard deviation. Data were analysed by Student's *t* test. The images of Western blots shown are representative of at least three analyses performed on samples from at least three separate experiments.

Results

Effects of copper depletion in human neuroblastoma cells SH-SY5Y

The treatment of SH-SY5Y cultured cells with the copper-chelator Trien produces approximately 60% decrease of the intracellular copper content (Fig. 1a). CCS, the protein which delivers copper to SOD1 and it is known to quickly sense copper paucity (Bertinato and L'Abbé 2003) increases in SH-SY5Y cells treated with Trien (Fig. 1b), confirming the condition of copper depletion.

Copper depletion deeply affects the activity of the copper-dependent enzyme SOD1, as evidenced by an

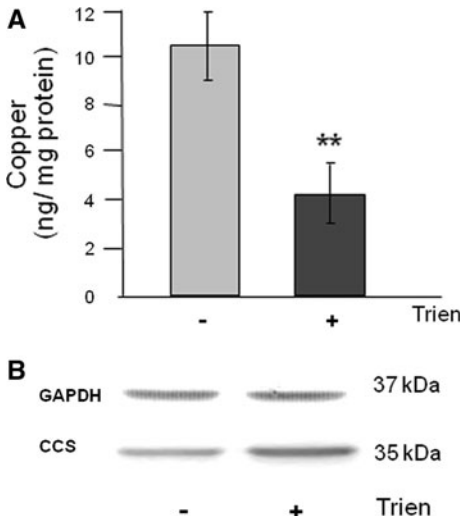

Fig. 1 Effects of Trien treatment on the intracellular copper content and Copper Chaperone for Superoxide dismutase (CCS) level in human neuroblastoma cells SH-SY5Y. Trien (125 μM, final concentration) was added to the cell medium 24 h after plating, and cells analysed 3 days later. **a** Copper content measured by atomic absorption spectrophotometry. n = 4, ** $P < 0.005$. **b** Western blotting analysis of the copper chaperone CCS. 20 μg protein were applied to each lane and a monoclonal antibody was used. GAPDH was used as the loading control

in-gel colorimetric assay (Fig. 2a), that shows about 80% decrease. Immunoreactive SOD1, instead, was affected by only 50%, as demonstrated by Western blotting analysis (Fig. 2b). This difference suggests that in copper-depleted human neuroblastoma cells SH-SY5Y about 30% of SOD1 protein is present as the inactive copper-free enzyme. This assumption is demonstrated by the recovery of SOD1 activity in extracts from copper-depleted cells upon incubation with 1 mM $CuSO_4$ for 3 h at room temperature (Fig. 2c). The addition of exogenous copper to homogenates of control cells, not treated with Trien, did not show any change of activity (not shown).

Effects of copper depletion in NSC-34-derived cells

The same copper-depletion procedure was applied to NSC-34-derived cells. The cells treated with Trien were induced to express Wt or G93A mutant hSOD1 by the addition of 2 μg/ml doxycycline to the culture medium in the last 24 h of the Trien treatment. Both the cells expressing the Wt hSOD1 and those with G93A hSOD1 showed a similar decrease of copper content (about 60%) (Fig. 3a), also comparable to that observed in Trien-treated SH-SY5Y cells. NSC-34-derived cells not expressing hSOD1s responded to the Trien treatment, in terms of copper reduction, similarly to the NSC-34 cells expressing the hSOD1s.

In Fig. 3b Western blotting analysis of NSC-34-derived cell lines, induced by doxycycline and treated with Trien, is reported. The level of CCS, as detected by immunoreactivity, increased, as a confirm of the achievement of copper depletion. Both the endogenous mouse SOD1 and the human isoform are detected, showing a different apparent molecular weight (16 vs. 18 kDa). The protocol of doxycycline treatment was selected in order to obtain the induction of a low level of hSOD1s, comparable between Wt cells and G93A cells, conditions under which the G93A mutant is only mildly toxic to the cells (Arciello et al. 2010). Since the level of hSOD1s is much lower than that of mouse SOD1, the protein loading had to be high enough to allow the detection of the hSOD1s, but this did not allow the detection of the decrease of mouse SOD1 upon copper deficiency, because of a saturation problem. When the analysis was repeated with a lower protein loading, decrease of immunoreactive mouse SOD1 was detected (results not shown). Immunoreactivity for both Wt and G93A hSOD1 shows a comparable decrease following copper depletion, which accounted for about 70 and 80% respectively (Fig. 3b).

Under native gel electrophoresis, used to evaluate SOD1s activity, human isoforms show a slower electrophoretic mobility than the mouse enzyme. Upon Trien treatment, the decrease of the activity of all SOD1s isoforms occurs (Fig. 3c). A band with an electrophoretic mobility intermediate between the mouse and the human enzyme can be detected in NSC-34-derived cell extracts, probably representing human/mouse heterodimers (Mattiazzi et al. 2002). Densitometric analysis of the bands demonstrates that hSOD1 activity decreases by about 90%, for both Wt and G93A (Fig. 3c). In NSC-34-derived cells, as observed in SH-SY5Y cells, the decrease in hSOD1 protein level upon Trien treatment is lower than the decrease in activity under the same conditions, indicating that also in this case a copper-free SOD1 exists in the cells following copper-depletion.

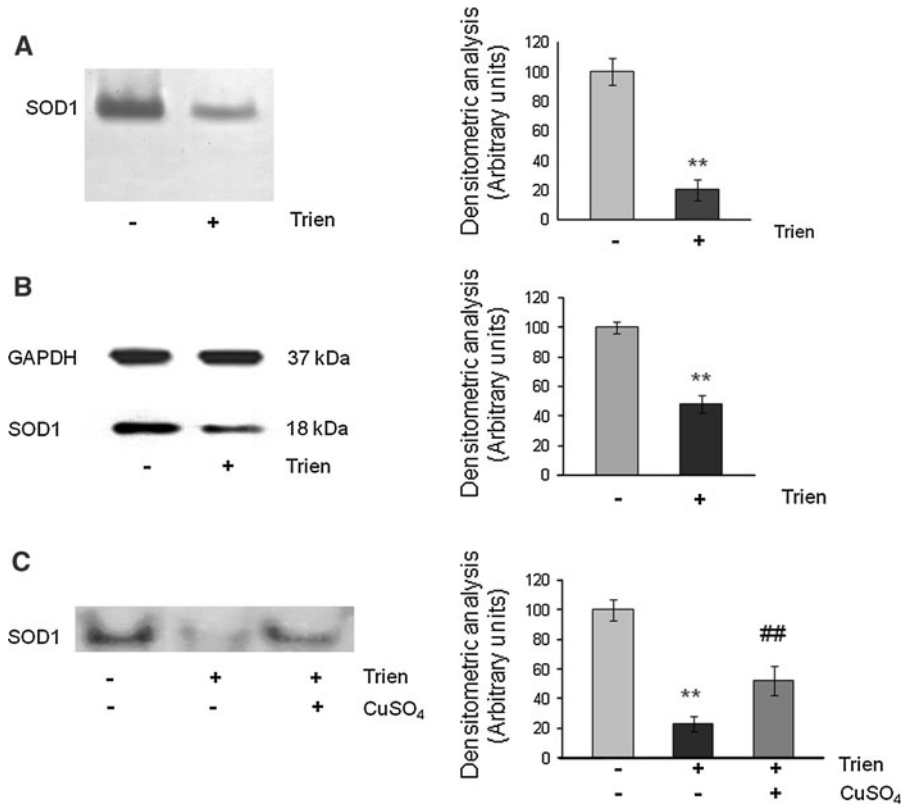

Fig. 2 Effects of copper depletion on SOD1 activity and protein level in human neuroblastoma cells SH-SY5Y. **a** SOD1 activity was detected by specific staining after non-denaturing PAGE. 50 μg protein was applied to each lane. The gel shown is representative of three different experiments. Histograms represent the mean ± SD of the densitometric analyses of the bands (n = 3, ** $P < 0.005$). **b** Western blotting analysis of immunoreactive SOD1. 20 μg of total protein was loaded on each lane. The blot shown is representative of five different experiments. Densitometric analysis of the bands obtained SOD1 is expressed as mean ± SD (n = 5, ** $P < 0.005$). **c** In a set of experiments, 1 mM $CuSO_4$ was added to cell homogenates for 3 h at room temperature and in-gel activity assay of SOD1 performed. The gel shown is representative of three different experiments. Histograms represent the mean ± SD of the densitometric analyses of the bands (n = 3, ** $P < 0.005$ vs. untreated; $^{\#\#}$ $P < 0.005$ vs. Trien-treated)

Content of SOD1 in mitochondria of copper-depleted neuronal cells

Lack of copper in the active site of SOD1 does not allow the correct folding of the protein and seems to favour the association of SOD1 to the mitochondria, where it is normally located as a minor fraction (between 1 and 5% of total cell SOD1 in yeast (Field et al. 2003), 1% or less in mammalian cells (Ferri et al. 2006)). In order to analyse the effect of copper-depletion on the intracellular localization of SOD1, we have isolated a highly purified mitochondrial fraction from Trien-treated SH-SY5Y cells or NSC-34-derived cells by ultracentrifugation on a discontinuous sucrose gradient. Western blotting analysis shows that the mitochondrial fraction isolated from copper-depleted SH-SY5Y cells contains a higher amount of SOD1 than that isolated from copper-adequate cells (Fig. 4a). Mitochondria isolated from Trien-treated NSC-34-derived cells also show a higher amount of hSOD1s in Western blotting analysis, both in the case of the Wt protein and for G93A (Fig. 4b). This analysis was repeated loading a lower amount of protein to the gel, and demonstrated that also mouse SOD1 is present in mitochondria and that its amount is affected by the Trien treatment (not shown).

In order to test whether the higher amount of hSOD1 associated with mitochondria under copper depletion affects the mitochondrial phenotype in NSC-34-derived cells, the morphology of mitochondria was observed by fluorescence microscopy after

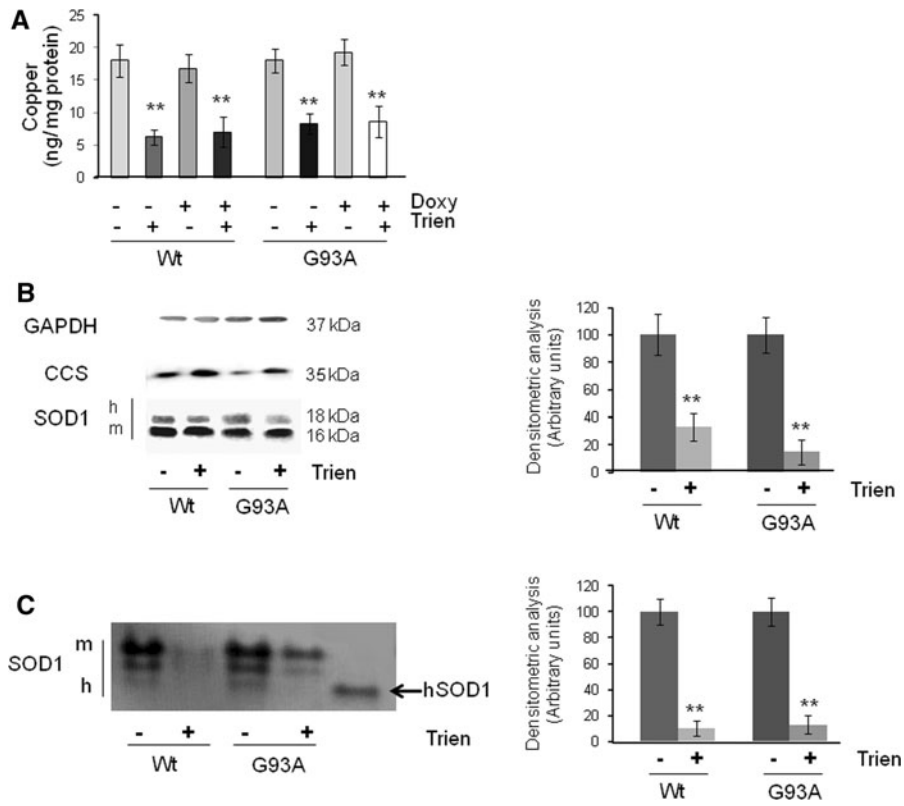

Fig. 3 Effects of Trien treatment on the intracellular copper content, CCS level and SOD1 in mouse motoneuronal NSC-34-derived cells. Trien (125 μM, final concentration) was added to the medium of Wt or G93A-transfected NSC-34 cells 24 h after plating and maintained for up to 3 days. Doxycycline (2 μg/ml medium) was added the last 24 h, to induce the expression of the human SOD1s. **a** Copper content was measured by atomic absorption spectrophotometry. n = 4, ** $P < 0.005$. **b** Contents of SOD1 and the copper chaperone CCS proteins were detected by Western blotting analysis. 20 μg of total protein were loaded on each lane. A monoclonal (for CCS) or a polyclonal (SOD1) antibody was used. GAPDH was used as the loading control. The blot shown is representative of five different experiments. Histograms represent the mean ± SD of the densitometric analyses of the bands of hSOD1 (n = 5, ** $P < 0.005$). **c** SOD1 activity was detected by specific staining after non-denaturing PAGE. 50 μg protein was applied to each lane. The *arrow* indicates a sample of SOD1 purified from human erythrocytes, used as a mobility standard. The gel shown is representative of three different experiments. Histograms represent the mean ± SD of the densitometric analyses of the bands (n = 3, ** $P < 0.005$)

immunolabelling of SOD2 (a mitochondrial matrix enzyme) (Fig. 5). The mitochondrial network of NSC-34 cells expressing Wt hSOD1 is well defined and well distributed. Conversely, upon G93A-hSOD1 expression, swollen mitochondria are detectable and the organelle network is barely visible. Trien treatment does not produce a significant change of the mitochondrial network in both lines.

Discussion

In the present report we demonstrate that in two different neuronal cell models (namely, human neuroblastoma cells SH-SY5Y and mouse motoneuronal NSC-34-derived cells) copper depletion induces an increase of the amount of SOD1 in mitochondria. This may be associated with the occurrence of a fraction of SOD1 stable in the copper-free form. Indeed, under copper deficiency the decrease in SOD1 activity is always higher than the decrease of SODI protein level and in homogenates of copper-depleted SH-SY5Y cells recovery of SOD1 activity can be achieved upon addition of exogenous copper. This finding is in line with previous work from this and other groups (Rossi et al. 1994; Lombardo et al. 2003; DiSilvestro 1989; Steinkühler et al. 1991). In the present report, this matter was reinvestigated in

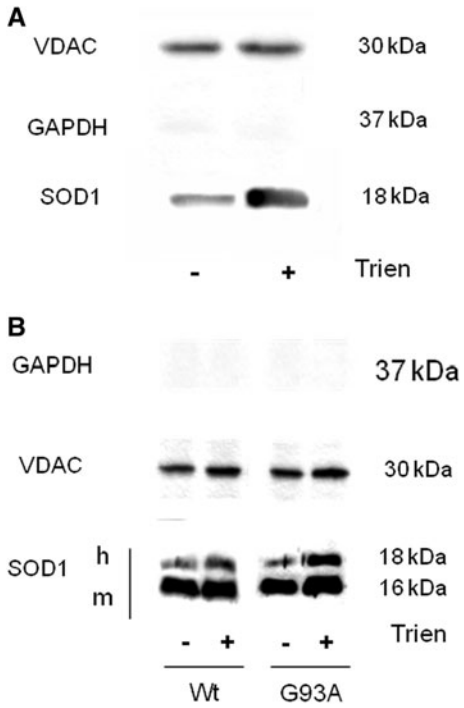

Fig. 4 Copper depletion increases the content of SOD1 in mitochondria of SH-SY5Y cells (**a**) and of NSC-34-derived cells (**b**). Trien (125 μM, final concentration) was added to the medium of cells 24 h after plating and maintained for up to 3 days. Doxycycline (2 μg/ml medium) was added to Wt hSOD1- or to G93A hSOD1-transfected NSC-34 cells the last 24 h of Trien treatment, to induce the expression of the human SOD1s. Pure mitochondrial fraction was obtained by sucrose gradient. Western blotting analyses shown are representative of at least three independent experiments. 50 μg of mitochondrial protein was applied to each lane. SOD1 was detected by a polyclonal antibody. The cytosolic enzyme GAPDH was used to probe mitochondria purity, whilst VDAC, a mitochondrial membrane protein, was used as the loading control

the light of (i) the knowledge of the existence of a fraction of SOD1 in mitochondria (Okado-Matsumoto and Fridovich 2001; Sturtz et al. 2001); (ii) the suggested mechanism of SOD1 entrance in the mitochondrial IMS; and (iii) the possible relevance of both copper imbalance and mutant SOD1s noxious effects on mitochondria in fALS.

Although it is now well accepted that a small percent of the total cell SOD1 associates with mitochondria (Okado-Matsumoto and Fridovich 2001; Sturtz et al. 2001; Field et al. 2003; Ferri et al. 2006; Vande Velde et al. 2008), because of the lack of a targeting sequence, the import mechanism has never been clearly explained. SOD1 does not show the amino-terminal pre-sequence directing proteins of cytosolic origin to mitochondria, though it may contain internal targeting sequences involving cysteine residues, which, on the other hand, characterize the majority of the IMS proteins. These are supposed to enter the mitochondria via the TOM portal and the MIA pathway, involving the formation of a mixed disulfide bond between a cysteine residue of the imported protein with the IMS receptor Mia40 (Terziyska et al. 2005; Chacinska et al. 2009). It has been postulated that a fraction of SOD1 translated in the proximity of mitochondria may be recruited by the import machinery, and transferred inside mitochondria before folding of the protein occurs. In yeast, it was shown that SOD1 enters the mitochondria in the non-metallated and unfolded form, gains copper inside the organelle by intervention of CCS, then the formation of the disulfide bond occurs and the protein is retained in the mitochondria (Sturtz et al. 2001). The results of the present work support this hypothesis; indeed, we demonstrated, in two neuronal cell models, that the shortness of copper favours the entrance of SOD1 in the mitochondria.

Under copper depletion, the activity of cytochrome *c* oxidase (the major mitochondrial copper enzyme) is strongly decreased both in SH-SY5Y cells (Lombardo et al. 2003) and in NSC-34-derived cells (Arciello et al. 2010). Thus, under such conditions, the mitochondria-associated SOD1 is also likely to remain inactive because copper-devoid; however, it may have an intra-chain disulfide bond (Furukawa et al. 2004), which may allow its partial folding and retention in the mitochondrion.

The role of copper on the toxic action of mutant SOD1 linked to fALS is debated. Copper depletion only slightly improves viability of cultured cells and survival of animal models, without neutralizing the toxicity of mutant SOD1 efficiently (Hottinger et al. 1997; Kiaei et al. 2004; Tokuda et al. 2008). Furthermore, genetic ablation of CCS, the chaperone delivering copper to SOD1, does not improve onset and progression of motor neuron disease in transgenic G93A mice (Subramanian et al. 2002); we can speculate that in that experiment the absence of CCS leads to a significant reduction in the amount of copper-loaded mutant SOD1, and hence in an increased load of mutant SOD1 into mitochondria. Conversely, CCS over-expression accelerates SOD1-linked disease without the hallmarks of cytosolic

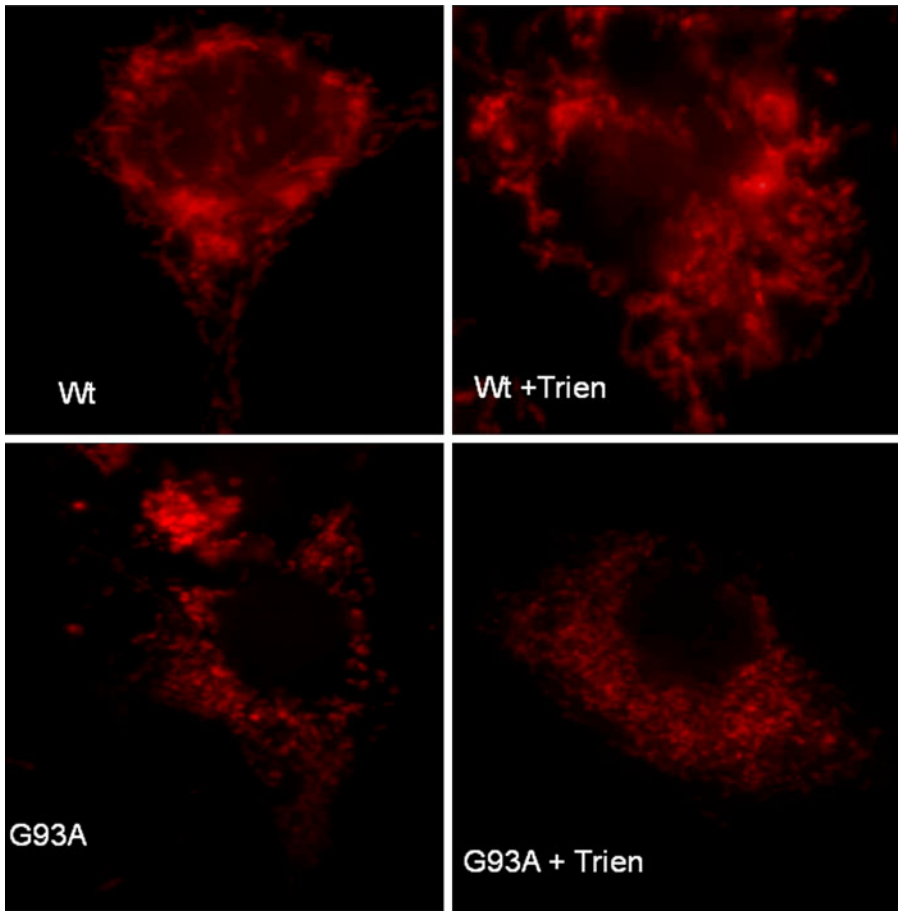

Fig. 5 Mitochondrial morphology and network of copper-adequate or copper-deficient mouse motoneuronal NSC-34 cells expressing Wt or G93A human SOD1. Cells were incubated ± Trien for 3 days and with doxycycline to induce hSOD1 expression. Cells were labeled with a polyclonal antibody against the mitochondrial enzyme SOD2, followed by TRITC-conjugated secondary antibody and observed under a fluorescence microscope (60× objective)

misfolding and aggregation seen in other mutant SOD1 models (Son et al. 2007). In our experiments, we confirm that under copper depletion the level of CCS is increased upon copper deficiency in both neuronal cell types studied. This finding extends previous studies performed in other experimental models demonstrating that copper regulates CCS protein level (Prohaska et al. 2003; Bertinato and L'Abbé 2003) and indicates that also neuronal cells are able to sense copper deficiency and increase CCS in an attempt to prioritize the utilization of copper when it is scarce.

A small fraction of both wild-type and mutant SOD1 is associated with mitochondria, but only those cells expressing the mutant protein display a damaged mitochondrial phenotype. Under copper depletion, the level of both proteins increases in the mitochondrial fraction, but the mitochondrial morphology is not further affected by G93A SOD1. This may be explained by the fact that a relatively small amount of mutant protein is sufficient to elicit mitochondrial damage, and the further increase of its amount in this localization does not correspond to a proportional organelle derangement. Alternatively, if copper is necessary for the toxic function of mutant SOD1, it is possible that the additional intra-mitochondrial mutant protein does not find proper copper levels, remains in a copper-free state and thus is unable to provoke further damage. The latter hypothesis is in line with previous reports of a beneficial, albeit small, effect of copper chelators in the mouse model for ALS (Hottinger et al. 1997; Kiaei et al. 2004; Tokuda et al. 2008).

In conclusion, copper deficiency is not a factor which may worsen mitochondrial damage, which is one of the earliest events in ALS associated with mutant SOD1.

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