Mutant superoxide dismutase 1 overexpression in NSC-34 cells: Effect of trehalose on aggregation, TDP-43 localization and levels of co-expressed glycoproteins

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Abstract

Protein inclusions rich in mutant Cu,Zn superoxide dismutase (SOD1) have been found in tissues from patients with familial amyotrophic lateral sclerosis (ALS). Here, the mouse motor neuron-like NSC-34 cell line transiently transfected with human SOD1(G93A) fused to enhanced green fluorescent protein exhibited aggregates contrary to cells overexpressing wild-type human SOD1. The aggregates were immunoreactive for ubiquitin but not for the TAR DNA binding protein (TDP-43) that was found in the nucleus. These characteristics mimicked the pathology of mutant SOD1 associated familial ALS. Aggregate formation and mutant SOD1 detergent insolubility were significantly decreased in the presence of millimolar concentrations of trehalose possibly due to its capacity to induce autophagy or to its properties as chemical chaperone. Mutant SOD1, aggregated and non-aggregated, caused decreased levels of coexpressed glycoproteins, which were not due to their intracellular accumulation. These cells may be used to study mechanisms of pathogenesis associated with ALS and to test potential therapeutic compounds.

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Amyotrophic lateral sclerosis is a fatal neurodegenerative disease that affects motor neurons from the spinal cord, brainstem and cortex [7]. Approximately 90–95% of ALS cases are sporadic, whereas 5–10% of cases have familial ALS (FALS), 20% of which presenting dominant mutations in the cytosolic antioxidant protein SOD1.

In vivo, protein inclusions of mutant SOD1 due to misfolding have been found in tissues from FALS patients, animal and cell models [7,15,16], SOD1 misfolding is toxic for the cell via several mechanisms that include dysfunction of organelles, e.g., mitochondria, induction of oxidative stress, chaperone depletion [30], impairment of proteasome function [34], induction of endoplasmic reticulum stress [24] among others. Mutant SOD1 has been shown to be degraded by the proteasome or by autophagy in cell models of FALS [17]. Proteasome degradation involves the ubiquitination of target proteins that are usually short-lived species, whereas autophagy substrates are generally long-lived cytoplasmic proteins, protein complexes or damaged organelles [26].

Trehalose is a disaccharide that has been shown to prevent aggregate formation in a mouse model of Huntington disease [31], to inhibit the formation of fibrillar aggregates of insulin [1], to reduce Aβ aggregation [18], to impair prion protein aggregation [3], and in cell models of oculopharyngeal muscular dystrophy [10]. This effect could be due to its properties as inducer of autophagy or as a chemical chaperone by aiding in the folding of proteins [26].

More recently, aggregation of TDP-43 has been widely described as a pathological hallmark of sporadic ALS and other neurodegenerative diseases, namely frontotemporal lateral dementia [22]. In healthy conditions, TDP-43 is localized in the nucleus, whereas in the pathology phosphorylated protein and C-terminal fragments form aggregates in the cytoplasm. The presence of TDP-43 aggregates in SOD1 associated FALS cases is contradictory, with some groups describing the absence of TDP-43 aggregation in mutant SOD1 FALS pathology [21,19] in contrast with others [25]. Concerning SOD1 animal models the evidence is also contradictory [25,33,28].

The NSC-34 cell line is a mouse neural hybrid cell between neuroblastoma and spinal cord cells that has several characteristics of motor neurons [8]. In previous work, we have found increased fragmentation of the Golgi apparatus in this cell line stably overexpressing mutant SOD1(G93A) similarly to that found in motor neurons of ALS patients [12]. Secretory and membrane glycoproteins cross the endoplasmic reticulum and the Golgi apparatus where they are glycosylated before they are targeted to their final cellular localiza-

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tions. Studies from other groups have shown that overexpression of α-synuclein in yeast, which constitutes a cell model of Parkinson's disease, led to a block in endoplasmic reticulum to Golgi vesicular trafficking [9].

In the present work, we have shown that NSC-34 cells transiently overexpressing mutant SOD1<sup>G93A</sup> exhibited mutant SOD1 but not TDP-43 aggregates. Those aggregates were reduced in the presence of trehalose. Furthermore, a decrease in the levels of concomitantly expressed glycoproteins has been found.

NSC-34 cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM; Gibco), supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen).

NSC-34 cells were transiently transfected with the vector pEGFP-N1 (Clontech) encoding human SOD1<sup>wt</sup> linked at the C-terminus to the enhanced green fluorescent protein tag (pSOD1<sup>wt-EGFP</sup>) and mutant SOD1<sup>G93A</sup> with the same tag (pSOD1<sup>G93A-EGFP</sup>) or vector encoding only the tag (pEGFP) [32], using Lipofectamine<sup>TM</sup> 2000 (Invitrogen). Vectors pMT/β-TP, pCR/EPO and pCDNA3.0/L1 encoding α-tubulin, the cell adhesion molecule L1, respectively, were used in transient or stable transfections. Stably transfected cells by the calmodulin phosphate method were selected with 0.5 mg/ml geneticin and from the pellets (insoluble fraction) after 15 s sonication in RIPA buffer without sodium deoxycholate were analyzed by Western blot. 1% protease inhibitors. Lysates were centrifuged at 15,800 rpm for 10 min. Proteins from the resulting supernatants (soluble fraction), containing 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, 1% protease inhibitor cocktail Complete (Roche), as previously described [11]. For the analysis of detergent insolubility cells were lysed in 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% protease inhibitors. Lysates were centrifuged at 15,800 × g for 10 min. Proteins from the resulting supernatants (soluble fraction), and from the pellets (insoluble fraction) after 15 s sonication in RIPA buffer without sodium deoxycholate were analyzed by Western blot.

Western blot was performed essentially as previously described [11]. The following primary antibodies were used: monoclonal anti-L1 L1-11A (1:2), polyclonal anti-β-TP (1:5000), polyclonal anti-EPO (1:5000, R&D), polyclonal anti-SOD1 (1:15,000, Santa Cruz), monoclonal anti-α-tubulin (1:5000, Sigma). As secondary antibodies anti-rabbit (1:3000) and anti-mouse (1:4000) coupled to horseradish peroxidase (HRP) were used. Proteins were detected by the Immobilon™ Western Chemiluminescent HRP Substrate method (Millipore).

Immunofluorescence was as previously described [12]. Primary antibodies were mouse anti-ubiquitin (1:50, Santa Cruz) and rabbit anti-TDP-43 (1:200, Proteintech Europe Ltd.). The secondary antibodies were 1:500 anti-mouse IgG conjugated to Alexa Fluor 594 and 1:500 anti-rabbit IgG conjugated to Alexa Fluor 594.

For inclusion quantification, aggregate-positive cells were counted as a percentage of total EGFP-positive cell transfectants. If an EGFP-positive cell had one or many aggregates, the aggregate score was one.

In order to mimic the mutant SOD1 aggregates found in ALS [7], NSC-34 cells were transiently transfected with phSOD1<sup>wt-EGFP</sup> or phSOD1<sup>G93A-EGFP</sup>, as reported by Turner et al. [32]. The cells expressing hSOD1<sup>wt</sup> and the corresponding empty vector showed a widespread fluorescence in the cytoplasm (Fig. 1A), whereas in the cells transfected with hSOD1<sup>G93A-EGFP</sup> large and prominent cytoplasmic protein aggregates were observed (Fig. 1B and E). The transfection efficiency was 26 ± 2% and 18 ± 2% (n = 4) for phSOD1<sup>wt-EGFP</sup> and phSOD1<sup>G93A-EGFP</sup> transfected cells, respectively, as determined by FACS analysis. Stably transfected cells did not show aggregates.

It is well described that the aggregates of mutant SOD1 observed in patients and rodent models of ALS stain positively for ubiquitin [2,6]. Accordingly, analysis by immunofluorescence microscopy with an anti-ubiquitin antibody showed that mutant SOD1 aggregates from NSC-34 cells also stained intensively for ubiquitin (Fig. 1B–D).

Since the presence of TDP-43 aggregates in mutant SOD1 associated FALS is contradictory in humans and animal models [21,19,25,33,28], here we also investigated in this experimental cell model if mutant SOD1 aggregation would be associated with endogenous TDP-43 aggregation by immunofluorescence microscopy. TDP-43 was predominantly detected in the nucleus of the cells as in healthy conditions and no aggregates were detected in the cytoplasm (Fig. 1F). The results showed the lack of TDP-43 pathology in a cell model of SOD1 FALS according to that previously described for patients by some authors [21,19]. This corroborates the notion that misfolded SOD1 and TDP-43 cause disease by different mechanisms.
Since it is known that trehalose reduces protein aggregation in models from several neurodegenerative diseases [26], the effect of this disaccharide on mutant SOD1 aggregates has been studied here. Mutant transfected cells were incubated with 1 or 10 mM trehalose for 72 h. The percentage of cells containing aggregated SOD1 was statistically significantly decreased in the presence of 10 mM trehalose (Fig. 2A). The effect of trehalose was also monitored using a different assay for protein aggregation: detergent insolubility that is usually found for aggregated proteins including SOD1G93A [32]. Using the buffer 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100 mutant SOD1 was consistently found to be enriched in the pellet fraction whereas wild-type SOD1 was found in the soluble fraction (Fig. 2B). When the cells were incubated with 10 mM trehalose there was a statistically significant decrease in the amount of insoluble mutant SOD1 (Fig. 2C), using the one-way ANOVA method for analysis of variance followed by Dunnett’s post hoc test (n = 4). However, when instead, cellular extracts were incubated with 1 or 50 mM trehalose for 48 h at 4 °C, 48 h after transfection, there was no decrease in the amount of insoluble mutant protein (Fig. 2D), using the same statistical analysis as above (n = 4).

These results together indicated that trehalose prevented the de novo formation of aggregates or induced their degradation in the cell but it did not promote the resolubilization of the aggregated protein. In other studies, trehalose also prevented aggregate formation of PrPSc in prion-infected cells [3] as well as polyglutamine induced protein aggregation [31]. In those cases the authors suggested that the effect of the disaccharide might be due to its stabilizing effect through interaction with the protein, thus stabilizing its folding and, therefore, acting as a chemical chaperone. Such mechanism could also explain the decreased mutant SOD1 aggregation in the presence of trehalose observed in this work.

Alternatively, trehalose has been reported to induce mTOR independent autophagy [26], which could be responsible for the degradation of mutant SOD1. Supporting this hypothesis were the observations that mutant SOD1 was degraded by autophagy in a cell model of FALS [17], and that this degradative pathway is induced in the SOD1G93A mouse model [20]. Also in other neurodegenerative diseases, autophagy has been shown to be responsible for the degradation of misfolded and aggregated proteins, for example, huntingtin and α-synuclein, the hallmarks of Huntington and Parkinson’s diseases [26,27].

Previously, we have reported that a higher percentage of NSC-34 cells stably overexpressing SOD1G93A had fragmented Golgi apparatus. To investigate if this would have consequences for the secretion or glycosylation of proteins, NSC-34 cells were doubly and stably transfected with vectors encoding wild-type or SOD1G93A without or with the EGFP tag, together with a secretory model glycoprotein, β-trace protein (β-TP). β-TP is a major glycoprotein of the human cerebrospinal fluid [14]. Clones NSC-34/SOD1wt, NSC-34/SOD1G93A, NSC-34/SOD1wt-EGFP, and NSC-34/SOD1G93A-EGFP were selected that expressed similar amounts of human SOD1. The percentage of cells with fragmented Golgi was 33 ± 8%, 44 ± 7%, 37 ± 2% and 50 ± 5% (n = 2), respectively, as evaluated by immunofluorescence microscopy using the GM130 Golgi marker. These values are higher than those previously reported for cells stably transfected with only wild-type or mutant SOD1 [12], which could be due to the stress of the double transfection. Cells stably transfected with mutant SOD1 with the EGFP tag (Fig. 3A) or without the tag (data not shown) showed a lower amount of β-TP in the

![Fig. 2. Effect of trehalose on SOD1G93A-EGFP aggregation and insolubility in NSC-34 cells.](image-url)
supernatant in comparison with the wild-type SOD1 transfected cells. The decrease was not due to its intracellular accumulation since it was not detected in the cellular extracts. Furthermore, there were no detectable alterations in the N-glycosylation of β-TP produced in mutant transfected cells as evaluated from their hydrolysis with peptide-N-glycosidase F (which removes high-mannose-, hybrid- and complex-type glycans) and endoglycosidase H (which removes only high-mannose- and hybrid-type glycans). β-TP from both cell lines contained complex-type N-glycans (data not shown) as expected for this protein [14]. These results suggested that the fragmentation of the Golgi apparatus did not lead to detectable intracellular accumulation of secreted glycoproteins or to major changes in their glycosylation.

To study this effect in mutant SOD1 aggregate-containing cells, NSC-34 cells were transiently co-transfected with vectors encoding hSOD1G93A-EGFP and β-TP using Lipofectamine™ 2000. These cells exhibited extensive Golgi apparatus fragmentation (data not shown). A lower level of β-TP was also detected in the supernatant of the mutant transfected cells that was not due to its intracellular accumulation (Fig. 3B). To investigate if this effect would be observed with other model glycoproteins, NSC-34 cells were transiently co-transfected with vectors encoding hSOD1G93A-EGFP and the secretory glycoprotein erythropoietin (EPO) or the plasma membrane glycoprotein L1. EPO is a type I cytokine that regulates human erythropoiesis, and that, in the central nervous system, may have a neuroprotective role [5]. L1 is involved in the development of the nervous system and is a survival factor for motor neurons [23]. Similarly to β-TP, the level of EPO from the supernatant of transfected cells was lower in comparison with wild-type SOD1 transfected cells, and the decrease was not due to intracellular accumulation (Fig. 3C). L1 was also detected at a lower level in mutant SOD1 transfected cells (Fig. 3D).

The decreases observed could have several explanations. First, the possibility that the proteins would be ubiquitinated and degraded via the proteasome has been explored. However, cellular L1 was not ubiquitinated, as observed from the Western blot analysis with an anti-ubiquitin antibody of immunoprecipitated L1 (data not shown). This result suggested that the decreased level of L1 was not due to degradation by the proteasome.

Second, it is known that mutant SOD1 triggers endoplasmic reticulum stress in NSC-34 cells [24], and that endoplasmic reticulum stress leads to a general decrease of protein synthesis [13]. Therefore, the cell lines stably co-expressing wild-type hSOD1-EGFP or hSOD1 G93A-EGFP, and β-TP from supernatants and cellular extracts of NSC-34 cells overexpressing mutant SOD1. (A) β-TP from supernatants and corresponding cell extracts (stable overexpression). C: positive control, 10 ng human hemofiltrate β-TP. (B) β-TP from supernatants and corresponding cell extracts (transient overexpression). C: positive control, 10 ng recombinant human EPO from CHO cells. (D) L1 from cell extracts. Supernatants and cell extracts were obtained from 2 × 10⁵ cells. Open arrowheads are proteolytic products. (1) Empty vector pEGFP; (2) hSOD1wt -EGFP; (3) hSOD1G93A -EGFP. These results are representative immunoblots from at least three different experiments.
and membrane glycoproteins, independently of the presence of aggregates.

In conclusion, the effect of trehalose in reducing mutant SOD1 aggregation suggests a possible beneficial role on the disease that requires further studies. Furthermore, mutant SOD1 overexpression causes decreased levels of several glycoproteins due to an unknown mechanism. This work opens novel avenues in the study of ALS.

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