Cellular redox Systems impact the Aggregation of Cu-Zn Superoxide Dismutase linked to Familial Amyotrophic Lateral Sclerosis
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Running title: Roles of Trx and Grx systems in mutant SOD1 aggregation

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Protein misfolding is implicated in neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), where mutations of superoxide dismutase 1 (SOD1) account for about 20% of the inherited mutations. Human SOD1 (hSOD1) contains four cysteines including C57 and C146 that have been linked to protein stability and folding via forming a disulfide bond, and C6 and C111 as free thiols. But the roles of the cellular redox environment in SOD1 folding and aggregation are not well understood. Here we explore the effects of cellular redox systems on the aggregation of hSOD1 proteins. We found that the known hSOD1 mutations G93A and A4V increased the capability of the thioredoxin (Trx) and glutaredoxin (Grx) systems to reduce hSOD1 compared to wild type hSOD1. Treatment with inhibitors of these redox systems resulted in the increase of hSOD1 aggregates in the cytoplasm of cells transfected with mutants, but not in cells transfected with wild type hSOD1 or those containing a secondary C111G mutation. This aggregation may be coupled to changes in redox state of G93A and A4V mutants upon mild oxidative stress. These results strongly suggest that Trx and Grx systems are the key regulators for hSOD1 aggregation and may play critical roles in the pathogenesis of ALS.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease in which the main feature is a loss of motoneurons and muscle atrophy. About 10% of cases are inherited and described as familial form of ALS (fALS). fALS is associated with mutations in genes C9orf72, SOD1, TDP-43 and FUS, with C9orf72 being the most prevalent and followed by SOD1 (1-3). In fact, the first reported gene related to fALS is the Cu-Zn Superoxide Dismutase 1 (SOD1) (4) and since then, more than a hundred mutations associated with fALS have been identified in human SOD1 (5,6). Among them, point mutation A4V is one of the most invasive and prevalent mutation associated with human ALS (5,7) and G93A is used to produce a widely employed transgenic mice model (8,9). The SOD1 is a cytosolic ubiquitous enzyme that scavenges superoxide radicals in every cell type of the human body including erythrocytes (1,5); however, mutations induce exclusively cell death of motoneurons and to a lesser extent other neurons (10). In a metal coordinated state, SOD1 forms homodimer to accomplish its full enzymatic activity. Cu is necessary for the enzymatic activity, whereas Zn mainly works in maintaining the protein structure (5) (Fig. 1). Conformational change in mutant SOD1 may increase the accessibility of other substrates to Cu in the protein to generate reactive oxygen or nitrogen species. Because most mutations so far associated with fALS do not show reduction in SOD1 enzymatic activity, it has been postulated that the deleterious action of mutated SOD1 is probably due to a gain of toxic function rather than the expected increase of concentration of free radicals (4). Several cellular mechanisms as mitochondrial dysfunction, NMDA mediated excitotoxicity, altered axonal transport, proteasome inhibition, endoplasmic reticulum stress, and so on have been suggested to initiate and promote neuronal death (2,10,11).

A hallmark in neurodegenerative disease is the presence of protein aggregates in the nervous tissue of patients. Indeed, SOD1 makes aggregates in spinal cord of fALS patients and transgenic mice mimicking ALS carrying mutations of SOD1 (8). While it is not fully clear whether the aggregates are toxic or represent a kind of self inactivation system, it is well known that they are the results of protein misfolding (12). Metals are essential for the correct folding of SOD1; the apoenzyme is prone to aggregate (13). Additionally, cysteines have been implicated to be involved in aggregation. SOD1 mutants are susceptible to have more aggregation propensity (14,15) and they are also prone to disulfide reduction, unfolding and misfolding (16,17). SOD1 has four cysteines in positions 6, 57, 111 and 146. According to the crystal structure of SOD1, there is an intramolecular disulfide bond between C57 and C146 (Fig. 1). When cysteines were substituted by site directed mutagenesis to other amino acids,
the intracellular SOD1 aggregates decreased (18-20). C111 is located on the surface of the molecule where it can be oxidized, changing the molecular conformation of the protein (Fig. 1) (21) and it contributes to keep the Cu²⁺ in the correct position (22).

The quaternary structure and stability of SOD1 is controlled by the intramolecular disulfide bond. The equilibrium between oxidized and reduced forms of the disulfide bond of SOD1 should be highly dependent on the redox potential of the cytosolic environment which is mainly controlled by Trx or Grx systems (23). Trx system is composed of NADPH, thioredoxin reductase (TrxR), and Trx, while Grx system is composed of NADPH, glutathione reductase (GR), and GSH, and Grx (23,24). Changes in the cytoplasmic redox potential would influence the reduction or the oxidation of the disulfide bond of WT or mutant SOD1 proteins, rendering reactive free cysteines that could interact with thiol groups of other molecules forming new aggregates. Human Grx1 was reported to be able to reduce apo-hSOD1 (25,26), and overexpression of Grx2 prevents the aggregation of mutant SOD1 in mitochondria, preserves mitochondrial function, and protects neuronal cells against apoptosis induced by mutant SOD1 (27).

Using Force-Clamp Atomic Force Spectroscopy, we have previously shown that a nucleophilic attack of C111 towards either C57 or C146 in a SOD1 single molecule can occur. The reactivity of C111 towards the conserved disulfide bond in WT and G93A or A4V mutant SOD1 is different (28). Our aim here is to investigate the effects of Trx and Grx systems on the redox state of wild type, G93A, and A4V mutant human SOD1 (hSOD1) to evaluate the contribution of cellular redox environment change on the SOD1 aggregation and ALS progression.

RESULTS

Reduction of hSOD1 by thioredoxin and GSH-Grx systems in vitro-To detect whether the mutations in ALS-linked SOD1 affect the susceptibility of the SOD1 to reductants, we investigated the reduction of WT, mutants G93A and A4V of hSOD1 by GSH-Grx and Trx systems (Fig. 2A). When native oxidized hSOD1 (Fig. 2A, lane 6) was incubated with strong reductants including a high concentration of GSH (10 mM) (Fig. 2A, lane 1) or DTT (10 mM) (Fig. 2A, lane 11) the proteins were fully reduced, which is consistent with previous report (25). A lower concentration of GSH (1 mM) alone could reduce disulfides in mutant G93A hSOD1 partially, but not in WT hSOD1 (Fig. 2A, lane 2). GR plus NADPH did not affect the redox state of SOD1 proteins and could not increase the reduction efficiency of 1 mM GSH for the oxidized WT and mutant SOD1 proteins (Fig. 2A, lanes 3 and 4). Presence of Grx1 in the GSH-Grx system enhanced the reduction of disulfide in G93A SOD1 protein. In contrast whole GSH-Grx system has a little reduction of disulfide in WT and A4V SOD1 proteins (Fig. 2A, lane 5).

All the oxidized SOD1 proteins were not substrates of TrxR1 (Fig. 2A, lane 7), only the whole Trx system could reduce them. Trx system reduced the oxidized A4V and G93A mutant SOD1 proteins efficiently, but only gave a slight reduction for oxidized WT hSOD1 (Fig. 2A, lanes 8-10). Increasing the concentration of Trx1 did not enhance the reduction capacity (Fig. 2A, lanes 8-10). Mutation of SOD1 did not result in significant SOD1 activity change (Fig. 2B). The activities of recombinant proteins hSOD1 were 5.6 ± 0.6 U/μg for WT, 4.3 ± 1.8 U/μg for G93A, and 8.3 ± 1.4 U/μg for A4V, respectively. The presence of thiol dependent redox systems did not change the enzymatic activity of hSOD1. Neither GSH nor the individual reductants of the Trx system affected the SOD1 activity of G93A or A4V mutants (data not shown).

Regulation of SOD aggregation in the cells by thioredoxin and Grx systems-To explore whether Trx and GSH-Grx systems are key regulators for the aggregation of hSOD1 in the cells, we generated the schwannoma RT4-D6P2T SCs cells stably expressing hSOD1-GFP fusion proteins in order to visualize their cellular location. We used aurothioglucose (ATG) to inhibit the cytosolic TrxR1 and buthionine sulfoximine (BSO) that blocks the synthesis of GSH in cells to see the effect of Trx and Grx systems on SOD1 aggregation. Three different fluorescent cells, WT hSOD1-GFP, G93A
hSOD1-GFP and A4V hSOD1-GFP were used to observe insoluble protein accumulations (Fig. 3A). Under resting conditions fluorescence is spread over the cytoplasm (Fig. 3B, upper panels). In all the untreated cells very few fluorescent bright spots in the cytoplasm were observed (Fig. 3B, upper panels).

ATG inhibits TrxR1 by targeting the essential selenocysteine residue and BSO blocks GSH synthesis by inhibiting glutathione synthase and thus treatment with 100 µM ATG or BSO for 24 hours caused the alteration of reducing capacity of Trx system and GSH systems, respectively. These treatments did not affect the cell viability (data not shown). Using confocal microscope, no remarkable morphologic changes were observed in WT hSOD1-GFP cell line. In contrast, in cells overexpressing G93A hSOD1-GFP and A4V hSOD1-GFP, we detected condense GFP fluorescent spots, mostly spherical, with sizes ranging from 0.2 µm² to 20 µm² and apparently located in the cytoplasm with no special distribution (Fig. 3B, middle and bottom panels). The cells were analyzed with confocal florescence microscopy at higher magnification (400x) to see the contents of aggregates in cells (Fig. 3C). In WT cells, treatment with ATG did not show any increase in the aggregates amount, but the treatments with BSO resulted in a slight increase. The aggregates in the cells expressing G93A hSOD1-GFP and A4V hSOD1-GFP showed significant increase upon the treatments with BSO and ATG. These results indicated that the cellular redox environment changes which regulated by Trx and GSH systems are important for the mutant hSOD1 aggregation in the cytoplasm.

To further understand how the two cellular thiol-dependent redox systems changes upon the treatment with ATG and BSO, we detected protein levels of TrxR1, Trx1 and GR in the cells using Western Blotting (Fig. 4). In all cells, the expression level of TrxR1 was elevated upon the treatment with BSO, but not in cells treated with ATG (Fig 4, line 1). No significant differences were detected in term of GR level in all the cells (Fig. 4, line 5). Interestingly, treatment with BSO did not cause dramatic changes for Trx1 in cells expressing WT SOD1, but resulted in the enhancement of Trx1 in the cells expressing mutant SOD1; treatment with ATG induced an increase of Trx1 in cells overexpressing A4V SOD1, not the cells overexpressing WT SOD1 (Fig. 4, line 3). These results indicate that the inhibition of electron transfer in one pathway may activate the expression of the other system. The cells overexpressing WT SOD1 also exhibited some difference in term of the response to the oxidative stress compared to the cells expressing mutant SOD1.

C111 is critical for the hSOD1 aggregation upon alteration of Trx and GSH systems-Since C111 has been shown to be a critical amino acid residue in the redox regulation of SOD1 and may affect aggregation and formation of inclusion body-like structure of mutant SOD1 (29,30), we prepared stably transfected cell lines with the expression of SOD1 harboring C111 mutation to detect the effects of alteration of Trx and GSH systems on SOD1 aggregations (Fig. 5). Compared to the results of Fig. 3, similar with the cells expressing WT hSOD1, cells expressing C111G hSOD1 had little aggregates upon the treatment with BSO and ATG (Fig 5A). For the cells expressing G93A and A4V mutant hSOD1, treatment with ATG or BSO showed an increase in aggregation (Fig. 3), while mutations in C111 prevented the aggregate formation upon the treatment with ATG and BSO in all the cells (Fig. 5A, middle and right panels). The amount of aggregates per cell in G93A/C111G and A4V/C111G expressing cells kept at the same level upon the treatment with BSO and ATG (Fig. 5B). This cumulative plot of probability of the size of hSOD1 aggregate in cells showed that treatment with BSO and ATG (Fig. 5B). This cumulative plot of probability of the size of hSOD1 aggregate in cells showed that treatment with BSO and ATG enlarged the probability of aggregates with bigger area in mutation G93A and A4V (Fig. 5C, upper panels), but once the C111 was mutated the size of aggregates decreased in both cases, and distributed like untreated conditions (Fig. 5C, lower panels). These results indicate that C111 in hSOD1 is a critical amino acid residue for the formation of SOD1 aggregate during thiol dependent redox environment change.
**ROS level detection under GSH depletion**- Treatment with BSO and ATG can cause a redox environment change, which may result in an alteration of ROS production. We used a luminol chemiluminescence to detect ROS level upon the treatment with BSO. Superoxide reacts with luminol, which is oxidized and light is emitted and detected (31). After the treatment with BSO, ROS level significantly increased in the cells expressing WT hSOD1, C111G, A4V, and A4V/C111G cells (Fig. 6).

**SOD1 redox state in the cells**- In addition to studying the effects of treatment with ATG and BSO on cell morphology, we have analyzed redox state of hSOD1-GFP molecules in the cells harboring single and double mutant hSOD1 by redox western blotting (Fig. 7). In the cells expressing WT-SOD1-GFP, there were no changes for the ratio of reduced and oxidized form of SOD1 upon the treatment with BSO or ATG. In cells expressing G93A SOD1-GFP, treatment with either BSO or ATG increased the ratio of oxidized forms (Fig. 7). In cells expressing A4V SOD1-GFP, the oxidized forms were increased significantly in both treatment conditions compared to untreated cells. We also found that mutation at C111 made the redox state of G93A/C111G and A4V/C111G SOD1 independent on cellular redox environment change caused by treatment with ATG or BSO (Fig. 7).

**MS-MS analysis for the disulfide formation in recombinant proteins**- To investigate whether the role of C111 in the hSOD1 aggregation involves the formation of disulfides, we used MS-MS to detect the disulfide formation in recombinant A4V SOD1 proteins. The protein was analyzed as purified. Besides the structural disulfide between C57 and C146, the peptide V(4)VCVLK(9)-H(80)VGDLGNVTADKDGVADVSEDSVISLSGDHCII(114) was identified with high confidence both with 5+ precursor with the p value of 1.68e-6 and with 6+ precursor with the p value of 8.9e-8 (Fig. 8A and B), indicating that the disulfide of C6 - C111 was formed. Figure 8C and D showed the extracted ion chromatography of peptide L(144)ACGVIGIAQ (153) –H(80)VGDLGNVTA DKDGVADVSEDSVISLSGDHCII GR(114) and MS/MS fragmentation annotation of the HCD spectrum of the 5+ peptide, indicating that disulfide between C111 and C146 (p value of 5.25e-4) was present. In contrast, disulfide between C57 and C146 was detected, but disulfides of C6-C111 and C111-C146 were not detected in purified recombinant WT hSOD1 protein.

**DISCUSSION**

ALS is a complex disease, in which the main targets are the cortical, brain stem or spinal cord motoneurons. The intracellular protein aggregate of SOD1 is a hallmark character in nervous tissue of fALS patients (32). It has been described that cysteines play an important role in ALS-linked SOD1 misfolding (14,33-40). Trx and Grx systems are the two major cellular disulfide reductase systems. Trx family proteins control protein folding (41), regulating the protein disulfide formation. We show here that both Trx and Grx systems can reduce the oxidized forms of ALS-linked A4V and G93A hSOD1 mutants (Fig. 2A), which is consistent with previous reports (25-27). The WT hSOD1 is much more resistant to the reduction by both systems. Disulfide reduction and metal ion removal may trigger SOD1 aggregation (42), but in fact disulfide bonds cause the initiation of its fibrillation (43). In our study the reduced mutant hSOD1 was detected to be catalytically active.

Oxidative stress has been indicated to be a key factor for the neuron cell injury during ALS progression (11). ROS level is reported to be increased in ALS patients because antioxidant enzymes including glutathione S-transferase, peroxiredoxins and redox sensitive transcription factor Nrf2 are down-regulated and NADPH oxidase activity is elevated in motor neuronal cells (32). Here we have found that the interruption of Trx system by the treatment with ATG or depletion of GSH by BSO treatment caused the increase of ROS level and aggregation of mutant SOD1, but not WT SOD1 (Fig. 3). This is consistent with the result that redox state of WT SOD1 was hardly affected by the treatment of ATG and BSO (Fig. 7), though ROS level was increased in all tested cells including those expressing WT SOD1 following the treatment with BSO (Fig. 6). Trx dependent peroxidase 2 (Prx2), and
glutathione peroxidase 1 (GPx1) are colocalized with SOD1 in neuronal Lewy body like hyaline inclusions in the spinal cords of fALS patients (44).

An important observation is that C111 is critical for the formation of hSOD1 aggregates (Fig. 5) (45). Although C111 is not the only residue to mediate misfolding/aggregation, it is the only Cys residue present in SOD1 from primates and its role in ALS pathophysiology has been considered (28,45). Due to its location in the surface of the protein, C111 may interact with the C57 or C146 and reorganizes intramolecular disulfide bond (20) and would reorient the Cu2+. As it has been described, glutathionylation of C111 increases the proportion of highly fibrillogenic hSOD1 monomers (46,47), interrupting the dimer contact at the interface stereochemically and causing the dissociation. Indeed the point mutation of C111 in A4V and G93A mutant SOD1 proteins reduces the amount of aggregates (Fig. 5) (30,48,49).

A potential mechanism involving the role of C111 in the hSOD1 mutant protein aggregation is proposed, as shown in Fig. 9, which also showed how Trx and GSH-Grx systems participate in this process. Mutation in hSOD1 dimers exposes its conserved C57-C146 disulfide on the protein surface in either one or both subunits (28,29), where it is easy to be reduced by Trx and Grx system into dithiol form followed by the dimer dissociation. We can also find non conserved disulfide bond forms, as C6-C111 and C146-C111 in SOD1 dimers, which are in a balance due to the Trx-GSH/Grx redox systems. High accessibility of the exposure to Trx system and Grx system may lead to intermolecular thiol/disulfide exchange reactions, the dissociation of the dimers and misfolding when they are not in an equilibrium (29,37,50). The monomers, which are released by the protein reduction, could promote the non-native and less stable disulfides. Then under the oxidation state including the treatment with BSO or ATG, mainly C111 can then attack intermolecularly a cysteine from another SOD1 molecule, particularly C146 which is exposed to form intermolecular disulfide. C146 was previously indicated to be involved in the formation of SOD1 insoluble multimers and aggregates (37,51), and it is in agreement with our results as disulfide bonds between C111 and C146 were identified in MS experiment. These intermolecular disulfide bonds will induce the formation of insoluble high molecular weight aggregates and it is not necessary for the proteins to be fully reduced to start polymerization (Fig. 9) (52,53). Therefore, the formation of the insoluble SOD1 oligomeric inclusions, which is commonly described in fALS cases, is promoted by the modification or destabilization of the redox cytosolic environment.

The treatment of BSO and ATG interrupts the GSH-Grx and Trx system, causing mild oxidative stress and a subsequent cellular response including the Nrf-2 regulated antioxidant expression for compensation. For example, the treatment with ATG can specifically inhibit TrxR1, because Trx1 may get the electrons from GSH-Grx system, a less efficient electron donor for Trx1, and it is kept in the reduced form (54). However, the change in Trx system causes the mutant SOD1 aggregation, indicating that the mutant SOD1 is susceptible to the cytoplasmic oxidative stress. Many environmental factors including some toxic heavy metal ions, some pharmaceutical compounds can cause the inhibition of TrxR1, inducing oxidative stress (55). Aging is also a factor which can cause the alteration of Trx and GSH-Grx systems. All these factors may thus contribute to the pathophysiology via the change of Trx and GSH-Grx systems. In fact, TrxR1 haplotypes affect the onset of fALS (56). In this regard, a role of Trx system in ALS was also shown to be involved in the cell apoptosis process (57). The G93A mutant SOD1 can induce dissociation of MST1 (mammalian sterile 20-like kinase 1) from Trx1. This resulted in the activation, in a ROS dependent manner, of p38 mitogen protein kinase in SOD1 (G93A) mice spinal cord motoneurons (57). In summary, we showed here that mutations of SOD1 affects the susceptibility toward reduction by Trx and GSH-Grx systems. The inhibitors for TrxR and GSH can trigger mutant SOD aggregation formation in cells. These results strongly suggest that Trx and Grx systems are key players for SOD1 progression and ALS pathogenesis.
EXPERIMENTAL PROCEDURES

Chemicals and Proteins—Human Trx1, recombinant rat TrxR1, Grx1, Rabbit anti-human Trx1, and anti-rat TrxR1 antibodies were from IMCO Ltd. (Stockholm, Sweden; www.imcocorp.se). GR and GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals were from Sigma.

Human SOD1 expression and purification—The plasmids to express human SOD1 were constructed by the insertion of human SOD1 gene in PGEX 4T-1 plasmid. The human SOD1 mutant proteins were obtained by site-directed mutagenesis kit (QuickChange kit, Stratagene). The expression of either wild type (WT) or mutations G93A and A4V was induced in bacteria for overnight by 0.4 mM isopropyl beta-d-thiogalactopyranoside at room temperature, with 250 ml LB medium cultures. Cells were harvested by centrifugation and resuspended in ice cold 250 mM NaCl, 20 mM sodium phosphate, pH 7.5, sonicated and centrifuged at 15,000 × g for 20 min. The resultant supernatant was incubated with 0.7 ml of Glutathione Sepharose™ 4B beads (Amersham Pharmacia Biotech, Freiburg, Germany) for 1 h at 4°C to isolate the recombinant proteins. Finally, the recombinant proteins were eluted by thrombin cleavage in 250 mM NaCl, 2.5 mM CaCl₂, 20 mM sodium phosphate, pH 7. The concentration of proteins was determined by Lowry or Bradford methods using BSA as the standard.

In vitro hSOD1 reduction assays—The oxidized human SOD1 proteins (0.5 µM) were incubated with GSH-Grx system including 1 mM GSH, 100 nM GR, 2 µM Grx1, 200 µM NADPH or Trx system including 100 nM TrxR1, 200 µM NADPH, 10-40 µM Trx1 in 1.0 M PBS buffer for 1h at room temperature. In order to prepare fully reduced proteins, 10 mM DTT was used in a separate experiment. Afterwards, the resulting free cysteines of proteins were alkylated with 30 mM iodoacetamide (IAM), at 37°C for 30 minutes. After these treatments, proteins were separated on a 12% non-reducing SDS-PAGE. The proteins were then transferred into a nitrocellulose membrane to perform Western Blot analysis and detected using a sheep polyclonal antibody against human Cu-Zn SOD1 (1:2500 dilution) (Calbiochem, Darmstadt, Germany). Western Blot bands were quantified using ImageJ software.

SOD1 Activity assay. SOD1 activities were measured according to the SOD1 activity assay Kit from Enzo life sciences (Farmingdale, USA). Briefly, in this colorimetric based assay, superoxide ions were generated from the conversion of xanthine and oxygen to uric acid by xanthine oxidase. The superoxide anion then converted WST-1 to WST-1 formazan, a colored product with absorbance at 450 nm. SOD1 reacted and reduced the superoxide ion concentration and thereby decreased the rate of WST-1-formazan production. Reduction in the production of WST-1-formazan was used to represent SOD1 activity in experimental samples. In this experiment, 0.1, 1 and 10 units of SOD1 from human erythrocytes were used as standards.

Construct of the stably hSOD1 transfected cell lines—Fluorescent hSOD1-GFP protein was generated in plasmid pEGFP-N1 by inserting human SOD1 DNA sequences. Mutations were performed as indicated in section Human SOD1 expression and purification. The schwannoma cell line RT4-D6P2T SCs (ATCC, Spain) were transfected with these plasmid using Lipofectamine 2000 (Invitrogen) according to instructions of the manufacturer. The cells were grown in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C, and 5% CO₂.

To screen hSOD1-GFP positive transfected cell lines, cells were washed in PBS, then dissociated by trypsin treatment and diluted tenfold in cell Sorter buffer and filtrated to prevent cell aggregation. GFP positive cells were selected in a FACSCalibur and BD CellQuest Pro software (Becton Dickinson, Mountain View, CA), in which the excitation wavelength was set at 488 nm and the fluorescence emission was detected at 585 nm. Isolated fluorescent cells were grown and subsequently submitted to a process of
selection. Even though, cells were routinely checked for the purity to get cell lines containing the following constructs: WT hSOD1-GFP, C111G-GFP, G93A hSOD1-GFP, G93A/C111G, A4V hSOD1-GFP, and A4V/C111G hSOD1-GFP.

**Detection of protein aggregation in cells**—The SOD1-GFP stably transfected cell lines were grown at a confluence of 80% and then treated with 100 µM aurothioglucose (ATG), or 100 µM buthionine sulfoximine (BSO) for 24 hours. The cells were fixed with paraformaldehyde (4%) and prepared for the detection of GFP aggregation with a confocal microscope (Zeiss Axioplan 2 microscope). Nuclei were stained with ToPro (1:1000). Digital images were taken by an AxioCamHRm Camera and AxioVision 4.2 software. Size of aggregates and distribution of aggregates per cell were determined using ImageJ software.

**Detection of redox state of SOD1 in the cells**—Cells treated with 100 µM ATG or BSO were washed with 1 ml of PBS, scratched and centrifuged at 1600 rpm for 5 min. Cells were resuspended in 100 µl of lysis buffer (100 mM NaCl, 1 mM EDTA, and 20 mM IAM, 10 mM Tris, pH 7.4) containing 1% Nonidet P40, and Protease Inhibitor Cocktail (1: 100 dilution) (PIC, Roche Diagnostics, Mannheim, Germany,) and sonicated at 50 Watts for 10-15 s. The resulting lysates were centrifuged at 50,000 g for 10 minutes in a Beckman Airfuge to remove insoluble pellet. The supernatants were saved for analysis and mixed with the loading buffer with or without 20 mM DTT. The experiments of SDS-PAGE and Western Blots were performed as indicated above.

**Intracellular ROS production**—To detect ROS production, a luminal-dependent chemiluminescence (CL) method was utilized (31). Briefly, cells (1 x 10⁶) were treated with BSO for 24 h, and then harvested by centrifugation. The cells were washed twice in 4 ml 1x PBS and resuspended in 100 µl 1x PBS (pH 7.2). Subsequently the suspension was homogenized by sonication. The resulting homogenates were used for the measurement of ROS. A stock solution of 2 mM of luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione) was prepared by dissolving it in a drop of 10 M NaOH, diluted in 10 mM Tris-HCl, pH 8.0 and stored in the dark. An aliquot of cell homogenate (20 µl) was added with 40 µl of luminol stock solution to a hemolysis tube containing 500 µl of 10 mM Tris-HCl, pH 8.0. When ROS reacted with luminol, emitted light was captured by a photomultiplier (R374, Hamamatsu) fed at high voltage (700-800 V, High Power Supply, C-9525-01, Hamamatsu). The resulting signal was amplified in a low noise amplifier (P16, Grass), filtered at 0.5 Hz in a Bessel filter (Frequency Devices) and digitized at 25 Hz using WinWCP (v3.3.3) software (Professor John Dempster, Strathclyde University). Results are expressed as arbitrary units, obtained by quantification of areas under the curves of emission, as a function of time and amount of protein.

**Mass spectrometry analysis**—Recombinant SOD1 A4V mutant protein was dissolved in 2 M Urea, 50 mM ammonia bicarbonate buffer, and digested overnight at 37°C with sequencing grade modified trypsin (Promega, 5% weight of SOD1 A4V). The digests were then desalted by stage tips (Thermo Fisher Scientific), dried by speedvac, and stored at -20°C till further analysis.

The digests were dissolved in 0.1% formic acid and injected into a C18 EASY-Spray column (Thermo Fisher Scientific), 50 cm x 75 µm ID with 2 hour gradient coupled with Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific). The mass spectra were obtained with full scan at 120,000 resolution in the Orbitrap, and MS/MS with top speed method at 3 second with 15,000 resolution in the Orbitrap using HCD energy at 35%. The resulting raw files were analyzed by Pepfinder 2.0 (Thermo Fisher Scientific) with mass accuracy of 5 ppm.

**Statistic analysis**—Mean, Standard Error of the Mean (SEM) and t-test significances were calculated in IgorPro (Wavemetrics) and GrapPad Software.
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CONFLICT OF INTEREST
The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS
CA, JL, CS, and AH conceived the study. CA, JL, CS, AH designed experiments. CA, JL, YZ, HY, JB performed experiments, CA, JL, CS, AH wrote the paper. All authors analyzed the results, and approved the final version of the manuscript.

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The abbreviations used are: ALS, Amyotrophic Lateral Sclerosis; ATG, aurothioglucose; BSO, buthionine sulfoximine; GR, glutathione reductase; Grx, glutaredoxin; GPx, glutathione peroxidase; GSH, glutathione; IAM, iodoacetamide; Prx, Trx dependent peroxidase, peroxiredoxin; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; Trx, thioredoxin; TrxR, thioredoxin reductase; DTT, dithiothreitol; WT, wild type.
FIGURE LEGENDS

Figure 1. Representation of Cu-Zn human Superoxide Dismutase 1 molecule (SOD1). A. Lineal representation of SOD1 indicating the position of cysteines. Each subunit coordinates one atom of each of Cu and Zn. The disulfide bond between C57 and C146 determines a flexible loop, which contains C111. B. Left panel, the dimeric form of human SOD1, PDB accession number 2C9V, is represented using VMD software. Each monomer shows the eight beta-sheets. One monomer is labeled in magenta and the other in cyan. Cu$^{2+}$ is depicted in red and Zn$^{2+}$ in blue. Cysteines are labelled in yellow. Notice that cysteines are located face to face in the interface between dimers. Right panel, corresponds to the same representation in which the molecule have been turned 180º over its X axis. C. A surface representation of the molecule. C111 is the unique cysteine that has access to the surface of the molecule. The right panel, as in panel B, shows the molecule after a rotation of 180º.

Figure 2. Effects of the mutation in SOD1 on its reduction by GSH-Grx or Trx systems and catalytic activity. A. After WT, G93A, and A4V hSOD1 proteins were incubated with components of GSH-Grx system and Trx system for 1h, the proteins were separated on a non-reducing SDS-PAGE and SOD1 were detected by Western Blotting. Lane 1, 10 mM GSH; lane 2, 1 mM GSH plus 100 nM GR and 200 µM NADPH; lane 4, 100 nM GR and 200 µM NADPH; lane 5, 1 mM GSH, 100 nM GR, 200 µM NADPH, plus 2 µM Grx1; lane 6, control; lane 7, 100 mM TrxR1, 200 µM NADPH; lane 8, 100 mM TrxR1, 200 µM NADPH plus 10 µM Trx1; lane 9, 100 mM TrxR1, 200 µM NADPH plus 20 µM Trx1; lane 10, 100 mM TrxR1, 200 µM NADPH plus 40 µM Trx1; lane 11, 10 mM DTT. B. Effects of mutation in SOD1 on its activity. SOD1 activity was assayed by detecting scavenging effects on superoxide generated by xanthine and xanthine oxidase. Open circles correspond to xanthine oxidase activity to produce superoxide. Open squares, open triangles and inverted open triangles correspond to the activity of 0.1, 1 and 10 units of human erythrocytes SOD1, respectively. The activity of recombinant hSOD1 is represented as red circles (WT), blue circles (G93A) and green circles (A4V). The figure is a representative of three experiments.

Figure 3. Effect of inhibition of Trx1 by ATG or depletion of GSH by BSO on hSOD1 aggregation in cultured cells. A. SOD1 amount in transfected cell lines. B. The SOD1-GFP stably transfected cells including expressing WT, G93A, A4V SOD1 were treated with 100 µM ATG, or 100 µM BSO for 24 hours. GFP aggregation in the cells were detected with a confocal microscope. C. The amounts of aggregates in each cell were analyzed by florescence microscopy at higher magnification (400x). Mean value found at WT (n=69), WT with BSO (n=91), WT with ATG (n=103), A4V (n=70), A4V with BSO (n=168), A4V with ATG (n=95), G93A (n=85), G93A with BSO (n=91), and G93A with ATG (n=85). (*P<0.05; **P<0.01, treated cells versus untreated cells)

Figure 4. Alteration of protein level of the thiol dependent redox system caused by treatment with ATG or BSO. Stably transfected cells expressing WT, G93A, A4V SOD1 were treated with 100 µM ATG, or 100 µM BSO for 24 hours. Then the cells were harvested and the proteins were detected by Western blotting.

Figure 5. Effect of C111 in hSOD on hSOD1 aggregation upon the treatment with ATG or BSO. A. The SOD1-GFP stably transfected cells expressing C111G, G93A/C111G, A4V/C111G SOD1 were treated with 100 µM ATG, or 100 µM BSO for 120 hours. GFP aggregation in the cells was detected using confocal microscope. B. The amounts of aggregates in each cell were analyzed by florescence microscopy at higher magnification (400x). Mean value counted at C111G (n=49), C111G with BSO (n=78), C111G with ATG (n=82), A4V/C111G (n=70), A4V/C111G with BSO (n=68), A4V/C111G with ATG (n=105), G93A/C111G (n=115), G93A with BSO (n=104) and G93A with ATG (n=89). C. Cumulative plot of probability of the size of hSOD1aggregates. The probability of finding a specific interval size of aggregate was
plotted accumulatively. Black lines correspond to untreated conditions; red lines to ATG treatment, and blue lines to BSO treatments.

Figure 6. ROS level detection in the cell lysates. Cells (1 x 10^6) were treated with BSO for 24 h and then lysed in PBS buffer. ROS level in the lysates were measured by a luminol-dependent chemiluminescence method. (Data were obtained from n=6, *P<0.05, **P<0.01, ***P<0.001)

Figure 7. Analysis of the redox state of SOD1 in the cell lysates. After RT4-D6P2T cells expressing WT and mutant hSOD1 were treated with BSO and ATG, the cells were lysed in the buffer containing 20 mM IAM to block the redox state of the proteins. The protein were separated in non-reducing SDS PAGE (left lanes) or in reducing condition (right lanes – DTT) and detected by Western blotting.

Figure 8. MS/MS Analysis of purified recombinant A4V hSOD1 protein. A. Extracted ion chromatography of peptide V(4)VCVLK(9)-H(80)VGDLGNVTADKDGVADVISEDVISLSGDHCIIGR(114), and the isotopic pattern of the 6+ ions. B. MS/MS fragmentation annotation of the HCD spectrum of the 6+ peptide, indicating the disulfide bond between C6 and C111. C. Extracted ion chromatography of peptide L(144)ACGVIGIAQ(153)-H(80)VGDLGNVTADKDGVADVISEDVISLSGDHCIIGR(114), and the isotopic pattern of the 5+ ions. D. MS/MS fragmentation annotation of the HCD spectrum of the 5+ peptide, indicating disulfide bond between C111 and C146, upper fragments are counted from peptide (144)LACGVIGIAQ(153), while the bottom fragments are counted from the other peptide.

Figure 9. A potential mechanism for mutant hSOD1 aggregation. Trx and GSH-Grx systems may play dual roles in the hSOD1 mutant protein aggregation. A. Wild type SOD1 (WT) is highly resistant to GSH-Grx reducing conditions because the disulfide bond is located intramolecularly. B. On the contrary, mutant hSOD1 protein is easier to be reduced by Trx system and GSH-Grx system, reflecting that disulfide bonds in the molecule are mostly exposed to the external environment compared to hSOD1 WT protein. A balance stabilized by redox systems will induce the formation of three disulfide bond patterns, which can be reduced by the destabilization of the systems. C. The hSOD1 monomer is prone to form aggregates when the redox balance is disturbed by ATG or BSO and Trx and GSH-Grx systems are not in equilibrium. Some monomers could return to their original form and some others could enhance an intermolecular disulfide bond formation. This panel shows an example of how the aggregates can be organized, but other combinations would be possible. The Trx system and GSH-Grx system are quite susceptible to be interrupt by environmental factors and others like aging, which can result in mild oxidative stress. Under such conditions, balance between thiol dependent antioxidant system and reactive oxygen species will be altered, which may cause the formation of intermolecular disulfide and further insoluble high molecular weight aggregates.
Figure 1

A

SOD1

C6
C111
C57
C146

B

C

111
6
111
57
146
57
6
146
111
6
111
57

Figure 1
A

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Cellular redox Systems impact the Aggregation of Cu-Zn Superoxide Dismutase linked to Familial Amyotrophic Lateral Sclerosis

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