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Effect of CCS on the Accumulation of FALS SOD1 Mutant-containing Aggregates and on Mitochondrial Translocation of SOD1 Mutants: Implication of a Free Radical Hypothesis

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Abstract

Missense mutations of SOD1 are linked to familial amyotrophic lateral sclerosis (FALS) through a yet-to-be identified toxic-gain-of-function. One of the proposed mechanisms involves enhanced aggregate formation. However, a recent study showed that dual transgenic mice overexpressing both G93A and CCS copper chaperone (G93A/CCS) exhibit no SOD1-positive aggregates yet show accelerated FALS symptoms with enhanced mitochondrial pathology compared to G93A mice. Using a dicistronic mRNA to simultaneously generate hSOD1 mutants, G93A, A4V and G85R, and hCCS in AAV293 cells, we revealed: (i) CCS is degraded primarily via a macroautophagy pathway. It forms a stable heterodimer with inactive G85R, and via its novel copper chaperone-independent molecular chaperone activity facilitates G85R degradation via a macroautophagy-mediated pathway. For active G93A and A4V, CCS catalyzes their maturation to form active and soluble homodimers. (ii) CCS reduces, under non-oxidative conditions, yet facilitates in the presence of H₂O₂, mitochondrial translocation of inactive SOD1 mutants. These results, together with previous reports showing FALS SOD1 mutants enhanced free radical-generating activity, provide a mechanistic explanation for the observations with G93A/CCS dual transgenic mice and suggest that free radical generation by FALS SOD1, enhanced by CCS, may, in part, be responsible for the FALS SOD1 mutant-linked aggregation, mitochondrial translocation, and degradation.

Keywords

CCS; FALS SOD1; mitochondrial translocation of SOD1 mutants; macroautophagy; molecular chaperone; oxidative stress

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease of motor neurons of the cortex, brainstem, and spinal cord. One form of familial amyotrophic lateral sclerosis (FALS) is linked to more than 100 different missense mutations in the Cu,Zn-superoxide dismutase (SOD1) gene [1-4]. Although the mechanism by which these mutant SOD1s cause fatal neurodegeneration is not clear, experimental results obtained from transgenic

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mice and *in vitro* studies strongly suggest that it is due to a toxic gain of function [4-7]. The proposed mechanisms for this toxic function include enhanced free radicals generation mediated by SOD1-bound copper ion [8-11], and enhanced formation of aggregates due to instability and misfolding of the mutant proteins [4, 12, 13]. The latter suggestion is mainly due to one hallmark of the histological appearance of proteinaceous inclusions in the spinal cord, which contains detergent-insoluble precipitates of mutant SOD1 [14-24]. To this end, overexpression of protein chaperones, such as Hsp70, has been shown to suppress accumulation of SOD1 mutant-containing aggregates and reduce cell death [21, 25-29].

SOD1 acquires its catalytic copper ion by direct interaction with the copper chaperone for SOD1 (CCS) [30, 31]. Initially, SOD1 forms a heterodimer with domain II of CCS, followed by an oxygen-dependent disulfide bond formation between C57 of SOD1 and Cu(I)-binding ligands (C244 or C246) in domain III of CCS [31, 32]. Once the Cu ion is transferred from CCS to the WT SOD1, CCS rapidly dissociates from SOD1 via a disulfide isomerization step to form an intramolecular disulfide bond between C57 and C146 of SOD1. However, with the mutant H48F, which fails to bind Cu ion, it forms a relatively stable heterodimer with CCS, to allow crystallization of the dimer for structural analysis [31-33]. The copper acquisition and formation of the intramolecular disulfide bond in SOD1 are critical for enzyme activity and structural stability, which could prevent the formation of SOD1 aggregates. A recent study using transgenic mice that overexpressed both CCS and G93A mutant showed no detectable SOD1 aggregates in the spinal cord [34]. However, they developed accelerated neurological deficits with a mean survival of 36 days in contrast to 242 days for G93A mice [34]. This finding is inconsistent with the notion that increased aggregate formation due to instability and misfolding of the mutant SOD1 is the primary cause of FALS disease.

To investigate the mechanisms which underlie this observation, we study the effects of CCS on the degradation and mitochondrial translocation of FALS SOD1 mutants using HEK293 or AAV293 cells and a dicistronic mRNA to simultaneously generate hSOD1 mutants and hCCS. Our results reveal that CCS prevents aggregate accumulation by facilitating the maturation of active SOD1 mutants to form active and stable homodimers, and by mediating the removal of inactive SOD1 mutant via a macroautophagy pathway. In addition, we reveal that while CCS overexpression reduces mitochondrial uptake of SOD1 mutants under non-stress conditions, it facilitates mitochondrial translocation of inactive SOD1 mutants under oxidative stress. Together, these results suggest that aggregate formation of SOD1 mutants may not be the primary cause of FALS mediated disease. The potential role of CCS in free radical generation and aggregate accumulation will also be discussed.

Materials and Methods

Plasmid and DNA constructs

Plasmids for this study were constructed as shown in Fig. 1A. Plasmids encoding human WT, A4V, G85R, and G93A SOD1 cDNAs in pVL1393 were used as templates as previously described [10]. The plasmid encoding human CCS cDNA in pCCS-HIS was a generous gift from Dr. Valeria C. Culotta (Johns Hopkins University). The pAAV-IRES-hrGFP plasmid (Stratagene, La Jolla, CA) was used to construct a vector system that can co-express SOD1 and CCS in a single mRNA using a viral IRES (internal ribosome entry site) sequence under control of the CMV promoter. *SOD1* gene (wild-type, A4V, G85R, or G93A) was inserted into multiple cloning sites of plasmids and *hrGFP* was replaced with the CCS gene as a second open reading frame (ORF) allowing it to make a dicistronic mRNA for SOD1 and CCS (Fig.1A).

To generate SOD1 from monocistronic mRNA, the 0.5 kb human SOD1 gene, containing BamHI at the 5' end and XhoI at the 3' end was amplified by PCR with pVL1393-SOD1 as template and inserted into pAAV-MCS to generate the pAAV-SOD1 plasmid. The plasmid pAAV-IRES-hrGFP (Stratagene) was used as the vector for the expression of hSOD1 and hCCS from dicistronic mRNA. To replace the hrGFP gene in pAAV-IRES-hrGFP with the hCCS gene, the plasmid was digested with HindIII and BglII, resulting in a 1.1 kb fragment that contains a part of IRES and the hrGFP gene was subcloned into pSP72 (Promega, Madison, WI) to make pSP72-1.1hrGFP. The KpnI site in hrGFP was mutated into the EcoRI site using the Quick change site-directed mutagenesis kit (Stratagene). The hrGFP gene in the pSP72-1.1hrGFP plasmid was replaced with the PCR-amplified 0.8 kb CCS gene that has the NcoI site at the 5' and the EcoRI site at the 3' end. Primers used for hCCS gene amplification were 5'-CAT GCC ATG GCT TCG GAT TCG GGG AAC-3' and 5'-CGG AAT TCC TAT CAA AGG TGG GCA GGG GGC TG-3'. The 1.2 kb HindIII/BglII fragment that contains a part of IRES and the CCS gene was cloned into the same restriction enzyme-treated pAAV-IRES-hrGFP plasmid to generate pAAV-IRES-CCS. Primers used for hSOD1 gene amplification were 5'-CGG GAT CCA TGG CGA CGA AGG CCG TGT GC-3' and 5'-CCG CTC GAG GCT ATT ATT GGG CGA TCC CAA TTA C-3'. The PCR amplified 0.5 kb human wild type (WT) SOD1 gene that has BamHI at the 5' and XhoI at the 3' end was inserted into pAAV-IRES-CCS to generate pAAV-SOD1-IRES-CCS. Mutant SOD1 genes were inserted in the same way and confirmed by nucleotide sequencing (Macrogen USA, Rockville, MD). To generate the FLAG tag at the carboxyl terminus of SOD1, the SOD1 gene was amplified to match the in-frame of the FLAG sequence of pAAV-IRES-hrGFP. Primers used for hSOD1-FLAG gene amplification were 5'-CGG GAT CCA TGG CGA CGA AGG CCG TGT GC-3' and 5'-CCG CTC GAG TTG GGC GAT CCC AAT TAC ACC-3'. The PCR amplified 0.5 kb human SOD1 gene that has BamHI at the 5' and XhoI at the 3' end was inserted into pAAV-IRES-CCS to generate pAAV-SOD1-FLAG-IRES-CCS.

Cell culture and expression of SOD1 and CCS

HEK293 or AAV 293 (Stratagene, La Jolla, CA) cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The recombinant pAAV plasmid, pAAV-SOD1 or pAAV-SOD1-IRES-CCS, was transfected into HEK293 or AAV293 cells in Biocoat 6-well plates (Becton Dickinson Labware, Bedford, MA) using the CaCl₂ transfection method. Cells were harvested 24 hr after transfection for analysis. If required, cells were incubated with epoxomicin (100 nM; Sigma, St. Louis, MO) or 3-methyladenine (5 mM; Sigma) 24 hrs after transfection.

To study the effect of oxidative stress on SOD1 mutant uptake by mitochondria, AAV293 cells were transiently transfected with A4V mutant expression vectors with or without CCS co-expression and incubated for 24 hr. These cells were then treated with 200 μ M of hydrogen peroxide for 15 min prior to cellular fractionation (see below) to determine the mitochondrial levels of A4V.

Cell fractionation

Phosphate-buffered saline (PBS)-washed HEK293 or AAV293 cells transfected with plasmids for 24 hr before they were sonicated in PBS containing 1% Triton X-100 in the presence of protease inhibitor cocktail (Sigma). For fractionation, cell lysates were centrifuged at 100,000 $\times$ g for 20 min using an Optima Ultracentrifuge (Beckman Coulter, Fullerton, CA), and the supernatants were used for analysis as the "nonionic detergent-soluble fraction", and the "nonionic detergent-insoluble fraction" was obtained by dissolving the pellets in PBS containing 5% SDS with brief sonication. For preparation of total cell

lysates containing both detergent-soluble and -insoluble fractions, cells were lysed in SDS buffer and sonicated. Protein concentrations were determined with the RC-DC protein assay kit (Bio-Rad, Hercules, CA) or the BCA protein assay kit (Pierce, Rockford, IL).

Mitochondrial and cytosolic fractions were prepared as described previously [35]. Briefly, transfected cells were homogenized with a tight-fitting glass-Teflon motorized homogenizer (500 rpm, 30 strokes) in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA. After 30 min incubation on ice, the lysates were centrifuged at 600×g for 8 min at 4 °C. The resultant supernatant was then centrifuged at 5,500×g for 15 min to obtain mitochondrial fractions.

Western blotting and antibodies

Proteins from extracts were loaded onto 10%-20% gradient pre-made gels and subjected to electrophoresis using either SDS-PAGE or non-reduced PAGE. Separated proteins were then transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA), probed with an appropriate antibody, and quantitated with the Odyssey infrared system (Lincoln, NE, USA). The results presented in the figures represent a typical observation from indicated number of independent experiments. The primary antibodies used were as follows: sheep polyclonal anti-human Cu,Zn SOD1 (574597) (1:2000; Calbiochem, La Jolla, CA, USA), rabbit polyclonal anti-human Cu,Zn SOD1 (RDI-SODabRx) (1:5000; Fitzgerald, Concord, MA, USA), mouse monoclonal anti-mitochondrial Hsp70 (MN3-028) (1:5000; Affinity Bioreagents, Golden, Co.), mouse monoclonal anti-human CCS (2A1) (1:5000; Abcam, Cambridge, MA, USA), mouse monoclonal anti-actin (1:10000; Sigma), and mouse monoclonal anti-Hsp70 (W27) (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For ECL detection (Amersham, Piscataway, NJ, USA), horseradish peroxidase conjugated secondary antibody was used and incubation was performed at 1:10,000 dilution. For the blot and quantitative analysis of protein bands the LI-COR Odyssey (Lincoln, NE, USA) was used, Alex Fluor goat anti-rabbit 800 or goat anti-mouse 680 secondary antibodies (Invitrogen) was used at 1:10,000 dilution.

SOD activity staining

Cell extracts were electrophoresed on native PAGE gel (18%) (Invitrogen, Carlsbad, CA) for 12 hr. The gel was soaked in 20 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM riboflavin and 2 mM *o*-dianisidine for 1 hr. The gel was then placed in a dry tray and illuminated with fluorescent light for 10-20 minutes. The dark-yellowish color indicates the presence of SOD activity in the bands.

Statistical Analysis

An unpaired two-tailed distribution Student's *t* test (Microsoft Excel) was used to analyze the data and results were expressed as means ± SEM.

Results and Discussion

CCS co-expression reduces formation of high molecular-weight species (HMWS) and detergent-insoluble aggregates of FALS SOD1 in cells

To investigate the effects of CCS on the formation of high molecular-weight species (HMWS) in cells overexpressing SOD1, we constructed vectors for the concomitant expression of hSOD1 and hCCS from dicistronic mRNA. As shown in Fig. 1A, the plasmid pAAV-IRES-hrGFP was used. hSOD1 cDNA (wild-type, A4V, G85R, or G93A) was inserted into multiple cloning sites of the vector and the hrGFP gene was replaced by the hCCS gene as a second open reading frame to produce a dicistronic mRNA for SOD1 and CCS.

AAV293 cells were transiently transfected with either pAAV-SOD1-GFP (Fig. 1B, lanes 2, 4, 6, and 8) or pAAV-SOD1-CCS (Fig. 1B, lanes 3, 5, 7, and 9). When mutants A4V (lane 4), G85R (lane 6), and G93A (lane 8) were expressed without CCS overexpression, HMWS bands (marked with asterisks) were clearly detected by Western blot. However, overexpression of WT SOD1 (lane 2) showed only small amounts of HMWS. In contrast, when CCS was co-expressed with SOD1 (lanes 3, 5, 7, and 9), no HMWS were detected for the four SOD1s. Over expression of CCS also eliminated detectable SOD1 aggregates in the spinal cords of G93A/CCS mice [34], and reduced the detergent-insoluble SOD1 in cultured cells [36]. Surprisingly, we found that the levels of SOD1 monomers were generally lower in the CCS co-expressed cells, most notably in G85R/CCS co-expressed cells (Fig. 1B, lane 7). This CCS-induced reduction is not caused by down-regulation of SOD1 expression by CCS or the formation of HMWS, based on the observation that the detection of HMWS is only dependent on CCS co-expression and independent of the total SOD1 levels (Fig.S1). These results indicate that CCS exerted its capacity to suppress the accumulation of HMWS post-translationally.

The proteinacious inclusions in the spinal cords of FALS patients and FALS-transgenic mice contain non-ionic detergent-insoluble aggregates of SOD1 [16-22]. We examined whether CCS overexpression also affects the formation of detergent-insoluble aggregates of FALS SOD1 as those HMWS observed in Figs. 1B. Figure 2 shows Triton-soluble (fractions 1 and 2) and -insoluble fraction (fraction 3) obtained from AAV293 cell lysates transiently transfected with SOD1 mutants, after ultracentrifugation at 100,000×g. In the absence of CCS co-expression, large amounts of A4V (lane 9) and G85R (lane 15) mutants, and a much smaller quantity of WT (lane 3), were detected in the Triton-insoluble fraction (fraction 3). Endogenous SOD1 was also weakly detected from various fractions (lanes 13, 16). However, co-expression of CCS with A4V (lane 12) or G85R (lane 18) mutants markedly reduced the amount of detergent-insoluble mutant detected. The HMWS of SOD1 mutants that were suppressed by CCS co-expression shown in Fig. 1B are likely similar to those detected in the Triton-insoluble fraction.

Copper insertion at the active site and intramolecular disulfide-bond formation of SOD1 are essential for structural stability as well as enzyme activity [32, 37]. These post-translational modifications involve an intermolecular disulfide formation between C57 of SOD1 and C244 or C246 of CCS domain III [31]. Mutation of these residues is known to disrupt the copper transfer and disulfide oxidation activities of CCS [38-41]. To examine the role of disulfide-bond formation and copper delivery on the CCS-dependent reduction of mutant SOD1 aggregate, we mutated CCS with C244S and C246S. The activity staining shown in Fig. 3A reveals that double mutations impaired CCS ability to deliver copper ion into the active site of WT SOD1 and A4V mutants. However, this mutated CCS is capable of suppressing the accumulation of detergent-insoluble SOD1 fractions of A4V (Fig. 3B, lane 6 and 8). Thus, CCS can function as a molecular chaperone, in addition to its known copper chaperone activity. Our results are in line with the report showing that protein-protein interaction between SOD1 and domain II of CCS is sufficient to suppress the formation of SOD1 mutant aggregates [36]. These results demonstrate that co-expression of SOD1 with CCS, independent of its copper chaperone activity, will lead to elimination of SOD1-containing HMWS and aggregates, independent of whether SOD1 is an active mutant, such as A4V and G93A, or an inactive mutant, such as G85R.

Effect of CCS on FALS SOD1 degradation pathways

To investigate the mechanism by which CCS eliminates the accumulation of FALS SOD1 aggregates shown above, the potential of CCS mediating the turnover of FALS SOD1 was studied. Several reports indicated that mutant SOD1 proteins are more rapidly degraded than WT SOD1 via both the proteasome [15, 21, 42-45] and macroautophagy [46] pathways. In

general, most cytosolic and misfolded proteins are degraded by the ubiquitin-mediated proteasome pathways and by autophagy-lysosome pathways [47, 48]. We examined whether CCS could induce the degradation of FALS SOD1 mutants, using well established specific inhibitors to identify the degradation pathways.

After HEK293 cells were transiently transfected with plasmids for mutant SOD1, with or without CCS, they were then treated with known degradation pathway specific inhibitors, epoxomicin for proteasome [49, 50] and 3-methyladenine (3-MA) for macroautophagy [47, 51-53] for 12 or 24 hrs. It should be pointed out, that HEK293 cells were used for the inhibitor studies because we found 3-MA induced cell death in AAV293. Following treatment with each inhibitor, Triton-soluble and -insoluble fractions from the lysates were fractionated and proteins (5 µg) were loaded to a 10-20% Tris-Glycine gel and subjected to SDS-PAGE in the presence of a reducing agent. The immunoblot data are depicted in Figs. 4. Fig 4A-4C shows typical Western blots revealing the effect of epoxomicin (100 nM) and 3-MA (5 mM) in protecting A4V, G85R and CCS from degradation in cells expressing either A4V or G85R mutant with or without CCS co-transfection. Fig. 4D shows the levels of SOD1 or CCS affected by the indicated inhibitor expressed as fold increase relative to that of control cells. Without CCS co-expression, treatment of epoxomicin (100 nM) resulted in a 1.5- to 2-fold increase in A4V levels in both soluble and insoluble fractions relative to that observed in the absence of this inhibitor. Treatment with 3-MA (5 mM) also exhibited a 1.5- to 2-fold increase in A4V protein. When A4V was co-expressed with CCS, the data show a less than 2-fold increase in soluble A4V levels due to epoxomicin or 3-MA treatment. However, the CCS protein in the soluble fraction was elevated nearly 5-fold due to 3-MA treatment. This result indicates that CCS is degraded mainly via the macroautophagy pathway.

Similar experiments were carried out with cells transfected with G85R or G85R co-expressed with CCS. When the cells were overexpressed with G85R alone, treatment with epoxomicin or 3-MA led to a 2.5- to 3-fold elevation of the observed G85R levels in the detergent-soluble fractions relative to that observed in the absence of either inhibitor. This inhibitor effect is more pronounced compared to the results obtained with A4V experiments (Fig. 4D). Furthermore a larger inhibitor effect was observed with the detergent-insoluble fractions, a 3-fold increase in G85R level with epoxomicin and a 3.5-fold increase with 3-MA treatments. These results suggest that in the absence of CCS, misfolded or aggregated G85R is degraded efficiently by both proteasome and macroautophagy pathways. However, when CCS was co-expressed with G85R, 3-MA treatment yielded a much more pronounced effect, an ~7-fold increase, while only an ~3-fold increase due to epoxomicin treatment. The effects of inhibitors on the level of CCS protein are similar to the results obtained from A4V experiments, indicating that clearance of CCS is mainly mediated by macroautophagy pathways. It should be pointed out that the levels of endogenous wild-type SOD1 observed in cells overexpressed with G85R were not affected by 3-MA and only slightly elevated by epoxomicin (data not shown).

CCS is known to form a transient heterodimer with SOD1 to deliver a Cu ion to the active site of SOD1 and to induce the formation of an intramolecular disulfide between C57 and C146 of SOD1 [31-33]. However, when the SOD1 mutant fails to bind a Cu ion at its active site, such as H48F, the CCS-H48F heterodimer has been shown to be relatively stable for crystallization for structural analysis [31-33]. Similarly G85R has been shown to exist as a monomer with reduced disulfide and lacking metal ions in G85R overexpressing mice (54), and as shown in Fig. 5A, it forms a relatively stable heterodimer with CCS base on the fact that it co-precipitated with CCS. In addition, the C244/246S mutant of CCS fails to bind Cu(I) at its domain III and transfer copper to SOD1 [54]. The absence of bound copper at domain III has been shown to enhance interaction between CCS and SOD1 [41]. Thus,

quasi-stable G85R-CCS and A4V-CCS(C244/246S) complexes are likely responsible for the robust effect of CCS and CCS mutant on the degradation of G85R mutant (Fig. 4D) and A4V mutant (Fig. 3B), respectively. Furthermore, the data in Fig. 4D also show that co-expression of CCS causes G85R to be degraded, like CCS itself, primarily via a macroautophagy pathway.

Overexpression of Hsp70 has also been reported to reduce the accumulation of mutant SOD1-containing aggregates in cells [21, 25-29]. To determine whether the reduction of aggregates accumulation mediated by CCS and Hsp70 is mechanistically related, we examined the interaction between SOD1, CCS, and Hsp70. Results from immunoprecipitation experiments show that when cell extracts were immunoprecipitated with anti-Hsp70 antibody, the protein band of CCS was not detected in extracts obtained from CCS overexpressed cells (Fig. 5B, lane 2 and 4, and Fig. 5C lane 2). This indicates that CCS could not form a stable complex with Hsp70 under our conditions. However, a strong protein band of SOD1 was detected only in extracts obtained from cells transfected with A4V-FLAG (Fig. 5B, lane 3) or with G85R (Fig. 5C, lane 1). Together, these results indicate that the mechanisms by which CCS and Hsp70 reduce the cellular accumulation of SOD1 aggregates are distinctly different. Hsp70 binds only to a misfolded SOD1 mutant and leads to its degradation, while CCS interacts with SOD1 mutants to serve as a copper chaperone to yield an active and stable homodimer in the case of G93A and A4V, and as a molecular chaperone to facilitate removal of inactive G85R mainly via a macroautophagy pathway. This notion is consistent with the facts that Hsp70 fails to co-precipitate with WT SOD1 in cells overexpressing WT-SOD1, or with SOD1 mutant when it is co-overexpressed with CCS (Figs. 5B and 5C) under our experimental conditions.

CCS overexpression reduces mitochondrial accumulation of SOD1 mutants

Both SOD1 and CCS have been reported to be found in the intermembrane space of mitochondria [55, 56]. In addition, Son et al [34] showed that G93A/CCS dual mice exhibit severe mitochondrial vacuolar pathology. Subcellular fractionation studies revealed an enriched G93A mutated SOD1 in their mitochondria. These results lead Son et al to suggest that enrichment of SOD1 mutants in mitochondria is induced by CCS overexpression and is, in part, responsible for the accelerated neurological deficits [34]. Here, we examined the effect of CCS co-expression on the accumulation of SOD1 mutants in mitochondria of AAV293 cells.

AAV293 cells were transiently transfected with A4V or G85R vectors with and without CCS co-expression. Mitochondrial and cytosolic fractions were prepared as described in *Materials and Methods* for immunoblot as well as activity analysis. Figure 6A reveals that overexpression of CCS diminishes the mitochondrial accumulation of A4V (lane 2) or G85R (lane 4) relative to those obtained in the absence of CCS (lane 1 for A4V, lane 3 for G85R). The observation that CCS overexpression reduces mitochondrial content of SOD1 appears to be in contrast with the results obtained from G93A/CCS dual transgenic mice [34]. The activity staining of the native gel depicted in Fig. 6B shows that the cytosolic fraction of SOD1 is enzymatically much more active when co-expressed with CCS (comparing lanes 3 and 4), as expected. However, the A4V mutant found in mitochondria was inactive, despite the fact that it was co-expressed with CCS (lane 2). These results are in agreement with the report that demetallated SOD1 (inactive), but not holoenzyme, is translocated into mitochondria [26]. In addition, these authors also showed that heat shock protein in cytosol binds to mutant SOD1s and prevents its uptake onto mitochondria. Our results are consistent with the notion that CCS inserts a Cu ion into the active site of A4V to form a stable holoenzyme and prevents its uptake into mitochondria. In the case of the inactive mutant G85R, CCS forms a relatively stable G85R-CCS complex to induce its degradation and to prevent its uptake by mitochondria. In this regard, the accumulated G93A mutant detected in

the mitochondria of 12- and 36-day old CCS/G93A dual mice [34] is likely demetallated (inactive) due to its oxidative inactivation in the cytosol.

Cellular stress induces mitochondrial accumulation of SOD1 mutants

To address the apparent contradiction between the effect of CCS co-expression on mitochondrial accumulation of SOD1 mutant reported for G93A/CCS mice [34] and that in our observations with AAV293 cells, we investigated the potential role of oxidative stress. To this end, AAV293 cells were transfected with A4V mutant expressing vectors with or without CCS co-expression for 24 hr, followed by treating these cells with 200 μ M hydrogen peroxide for 15 min. The cell lysates were then fractionated and the quantity of A4V mutant in the mitochondrial and cytosolic fraction was determined by western blot analysis as described in the *Materials and Methods* section. As shown in Fig. 7, the quantity of A4V detected in mitochondria further confirms that in the absence of hydrogen peroxide co-expression of CCS suppresses the accumulation of SOD1 mutant in mitochondria (Fig. 7A, lane 1 and 2, Fig. 7B). However, hydrogen peroxide treatment of these cells leads to an increase in mitochondrial content of the A4V mutant (Fig. 7A lane 5 and 6, Fig. 7B). It should be pointed out that, as predicted, the enzymic activity of A4V in mitochondria was found to be inactive (data not shown). These observations suggest that oxidative stress can induce mitochondrial accumulation of inactive SOD1 mutants. Thus, the enhanced accumulation of G93A mutants in the mitochondria of 12- and 36-day old G93A/CCS dual mice could well be derived from the translocation of stress-induced inactivated SOD1 mutants.

Concluding Remarks and Proposed Mechanism

Our data are consistent with and they also provide a potential mechanistic explanation for the observation that overexpression of CCS in G93A SOD1 mutant transgenic mice exhibited no detectable SOD1 aggregates in their spinal cords, yet it led to accelerated neurological deficits with enriched G93A mutant detected in their mitochondria [34]. With the SOD1 mutant-CCS co-expressing system, we demonstrated that in addition to its known copper chaperone activity which leads to the formation of an active and stable SOD1, CCS, with or without its copper chaperone activity, can function as a molecular chaperone to facilitate the turnover of inactive G85R mutant, mainly via a macroautophagy pathway (Fig. 8). As shown, CCS forms a soluble heterodimer with nascent SOD1 to deliver copper ions to the active site of SOD1 (WT, A4V, G93A, etc.) by an oxygen-dependent mechanism to yield a fully active SOD1 homodimer (31-33) (Fig. 8, species, *a*). The dissociated CCS is degraded mainly via a macroautophagy pathway. The relatively stable heterodimer, formed between the inactive G85R and WT CCS, as well as that between A4V and inactive CCS (C244/246S) mutant, is degraded in a similar fashion as CCS. This observation indicates that the heterodimer (e.g. species, *b*) is recruited to the CCS-mediated degradation pathways. The misfolded and detergent-insoluble SOD1s produced in the absence of CCS are degraded by both proteasome and macroautophagy pathways. Hsp70 may participate in removing detergent-insoluble SOD1 via a CCS independent pathway, because Hsp70 fails to co-precipitate with the WT SOD1 or SOD1 mutant in cells that overexpress WT SOD1 or SOD1 mutants co-express with CCS (Figs. 5 B and C). This indicates that chaperon-mediated autophagy may also be involved in the degradation of detergent-insoluble SOD1s.

Our findings also show that CCS co-expression consistently leads to a reduction of mitochondrial accumulation of SOD1 in AAV 293 cells, except when it is coupled with oxidative stress conditions. In addition, the SOD1s found in mitochondria are inactive, consistent with the report showing that demetallated SOD1 and not the holoenzyme is taken into mitochondria [26]. However oxidative stress induces mitochondrial accumulation of SOD1. Together these findings suggest that the observed accumulation of G93A mutant in

the mitochondria of 12- and 36-day old CCS/G93A dual transgenic mice [34] could be the consequence of oxidative or other cellular stress induced by overexpression of G93A and CCS. To this end, we and others have shown that the copper in SOD1 mutants and in WT SOD1 can catalyze free radicals generation using H₂O₂ and small anions as substrates [8-10, 57-59]. Overexpression of CCS should favor the formation of reactive SOD1 mutants (e.g. Fig. 8, species **a**) and copper bound heterodimers (e.g. Fig. 8, species **b**). Both of these copper-containing species are capable of catalyzing free radicals generation which in turn trigger various free radical-mediated cascading reactions and lead to oxidative inactivation of SOD1s and their subsequent copper releases. While the released copper ions could catalyze additional free radicals formation to further induce cellular stress, the demetallated SOD1 can be translocated into mitochondria by a yet to be elucidated mechanism.

These findings, together with those reported by Son *et al.* [34] showing that G93A/CCS dual mice with severe mitochondrial pathology and accelerated neurological deficits without detectable HMWS in their spinal cords, suggest that the formation of mutant SOD1-containing aggregates may not be the primary mechanism by which FALS mutant SOD1 induces ALS. The copper ion mediated free radical-generating reactions of FALS SOD1 [8-10], enhanced by CCS overexpression, may be the initial event which leads to FALS SOD1-linked aggregation, translocation and degradation. The toxic-gain-of-function by the enhanced free radical generation mediated by FALS SOD1 is also in accord with a recent finding showing that mRNA oxidation occurs early in motor neuron deterioration in ALS [60], in view of the fact that mRNA oxidation is mediated by free radical reactions [61].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SOD1	Cu,Zn superoxide dismutase
CCS	copper chaperone for SOD1
HMWS	high molecular-weight species
3-MA	3-methyladenine
E	epoxomicin

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Fig. 1. Effect of simultaneous expression of FALS hSOD1 and hCCS on SOD1 containing HMWS in AAV 293 cells

(A) Schematic representation of plasmid vectors used to produce dicistronic mRNA for hSOD1 and hCCS co-expression. B, BamHI; X, XhoI; N, NcoI; K, KpnI; RI, EcoRI. (B) Detection of mutant SOD1 with or without CCS revealed with reducing gel (see *Materials and Methods*). Top panel shows a hSOD1 blot visualized by ECL chemiluminescence. Asterisks depict the high molecular-weight species. The CCS proteins were also detected by the sheep polyclonal hSOD1 (574597) antibody due to the high homology of the two proteins. Middle panel shows the identical SOD1 blot as the top panel, except with shorter exposure time. Lower panel represents a CCS blot. This figure shows typical results obtained with three independent experiments.

Fig. 2. Co-expression of CCS reduced the nonionic detergent insoluble fraction of FALS A4V and G85R SOD1 mutants

As described in *Materials and Methods*, “PBS-soluble fraction” (lane 1), “nonionic detergent-soluble fraction” (lane 2), and “nonionic detergent-insoluble fractions” (lane 3) were obtained and analyzed. Proteins (5 ug) were loaded onto 10-20% gradient gels. Blots were obtained with rabbit polyclonal antibody (RDI-SODabRx) to Cu,Zn SOD1, or with monoclonal antibody to CCS, and visualized by ECL chemiluminescence.

Fig. 3. Effects of co-expressing WT and CCS mutant on the activity and aggregate of SOD1
(A) WT or A4V SOD1 was expressed in AAV293 cells with WT CCS, mutant CCS (C243/245S), or without CCS by transient transfection. The activity of SOD1 was detected in *In-gel* assay as described in *Materials and Methods*, and shows a dark yellow color. **(B)** Fractionated proteins obtained from AAV293 cells co-expressed with WT or mutant CCS or without CCS co-expression were analyzed on SDS-PAGE. S, nonionic detergent-soluble; I, nonionic detergent-insoluble; - These data represent a typical set of three independent experiments.

Fig. 4. Effects of proteasome inhibitor, epoxomicin, and macroautophagy inhibitor, 3-methyladenine, on the accumulation of FALS mutant SOD1 and CCS levels

FALS SOD1 mutants were expressed in HEK293 cells with or without CCS by transient transfection. Twenty-four hours after transfection, media were replaced with fresh ones that contained the indicated inhibitors and incubated for another 12 hrs or 24 hrs. Fractionated proteins (5 ug) were loaded onto each lane and subjected to SDS-PAGE in the presence of reducing agent in 10-20% Tris-Glycine gel. **(A)** Level of A4V and CCS monitored by Western blot after treatment with 100 nM epoxomicin for 12 hrs or 24 hrs as indicated. **(B)** A4V and CCS monitored by Western blot after treatment with 5 mM 3-methyladenine (3-MA) for 24 hrs. **(C)** G85R and CCS monitored by Western blot after treatment with 100 nM epoxomicin or 5 mM 3-MA for 24 hrs. **(D)** The relative change in SOD1 and CCS levels due to epoxomicin or 3-MA treatments are expressed in fold increase relative to control in each group. The histogram represents means $\pm$ SEM ($n \geq 5$) of fold increase relative to that observed in the absence of indicated inhibitor. S, nonionic detergent-soluble; I, nonionic detergent-insoluble; -, control; E, epoxomicin; M, 3-MA; Endo, endogenous SOD1.

* $p < 0.05$; ** $p < 0.01$ vs. control.

Fig. 5. Complexes formed between G85R and CCS, and between SOD1 and Hsp-70 detected by co-immunoprecipitation

AAV 293 cells were transiently transfected with (A, C) G85R-GFP or G85R-CCS vectors and with (B) FLAG-tagged WT-SOD1 or A4V mutant expression vectors with or without CCS co-expression. At 48 hr post-transfection, cells were lysed in lysis buffer (20 mM HEPES, 1% Triton X-100, 10% glycerol, pH 7.4) that contained protease inhibitor cocktail (Sigma). After brief sonication, cell lysates were centrifuged ($10,000\times g$ for 5 min) and the supernatant was collected. Rabbit polyclonal antibody that does not cross-react with CCS, against SOD1 (RDI-SODabRx) (A) or mouse monoclonal antibody against Hsp70 (MN3-028) (B,C) were added to a supernatant aliquot containing 500 μg of cellular proteins and incubated for 1 hr at $4^{\circ}C$. To isolate the SOD1 antibody conjugated proteins (A) or the Hsp70 antibody conjugated proteins (B,C), Protein A-conjugated agarose beads (Pierce) were added and the mixture incubated for another 1 hr at $4^{\circ}C$. After washing with wash buffer, the immunoprecipitates were recovered with $1\times$ SDS sample buffer, separated by SDS-PAGE, and probed with rabbit anti-SOD1 (RDI-SODabRx), mouse monoclonal anti-CCS (2AI) and anti-Hsp70 antibodies (MN3-028) as indicated. IB, immunoblotting; IP, immunoprecipitation.

Fig. 6. Overexpressed CCS prevented the mitochondrial accumulation of A4V and G85R
 AAV 293 cells were transiently transfected with A4V or G85R mutant expression vectors with or without CCS co-expression. Mitochondrial and cytosolic fractions were prepared as described in *Materials and Methods* and the proteins were separated by SDS-PAGE (**A**) and native gel (**B**). Mitochondrial Hsp70 (mtHsp70), α -tubulin, and mitochondrial SOD2 (for native gel) were used as marker proteins for mitochondrial and cytosolic fractions. An immunoblot membrane was probed with anti-SOD1, anti-CCS, anti-mtHsp70 and anti- α -tubulin antibodies. The activity of SOD1 was detected in *In-gel* assay (**B**) as described in *Materials and Methods*, and shows a dark yellow color. These data represent results obtained from three independent experiments.

Fig. 7. H₂O₂ induces A4V accumulation in mitochondria of AAV 293 cells co-express with A4V and CCS

AAV 293 cells were transiently transfected with A4V mutant expression vectors with or without CCS co-expression. One day after transfection, the cells were treated with 200 μ M H₂O₂ for 15 min. **(A)** Mitochondrial and cytosolic fractions were prepared and analyzed by Western blotting as described previously. **(B)** Relative mitochondrial levels of A4V in CCS co-expressed cells in the presence or absence of H₂O₂ were plotted relative to those observed without CCS co-expression. Values are means $\pm$ SEM (*n* = 5). **p* < 0.05 vs. control.

Fig. 8. A proposed mechanistic scheme showing the effect of CCS on protein aggregation and degradation pathways

The scheme illustrates the CCS-dependent and independent degradation pathways inhibited by 3-MA, a known specific inhibitor for macroautophagy pathway, or epoxo, an established proteasome inhibitor, respectively.