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Oxidative Modifications of Cu, Zn-Superoxide Dismutase (SOD1) – The Relevance to Amyotrophic Lateral Sclerosis (ALS)

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease of motor neurons. About 10 % of ALS cases are affected in a familial trait, a subset of which is caused by the mutation of *Cu, Zn-superoxide dismutase (SOD1)* gene (Rosen et al., 1993). Since the identification of the gene for familial ALS, research emphasis for ALS has been placed on uncovering the pathogenic mechanism of motor neuronal death by the disease-causing mutant SOD1. So far over 150 different mutations of *SOD1* gene have been found in familial ALS patients, and they are scattered throughout the entire sequence of the gene regardless of specific functional domains. Transgenic mice that express mutant SOD1, but not wild type SOD1 nor SOD1 knockout mice, develop motor neuron disease, often while retaining normal dismutase activity (Gurney et al., 1994; Reaume et al., 1996). It means that mutant SOD1 gains a new aberrant toxic function apart from the primary enzymatic function of the protein, which has remained uncertain to date. Although the nature of mutant SOD1 toxicity has not been fully determined, conformational abnormalities of mutant SOD1 protein are deeply involved in the pathogenesis of familial ALS (Chattopadhyay & Valentine, 2009). Moreover, recent studies suggest that the phenotype of sporadic ALS also might be regulated by the conformational change of wild type SOD1 (Bosco et al., 2010). I review the recent concept of neuronal toxicity by oxidatively-modified SOD1, which is closely related to its conformational change, in ALS pathogenesis.

2. Cu-mediated oxidative toxicity by mutant SOD1

SOD1 is a major metal-binding enzyme expressed constitutively in tissues, and converts pro-oxidant superoxide radicals to hydrogen peroxide and oxygen (Valentine et al., 2005). In a metal-coordinated state, SOD1 forms homodimer to accomplish its full enzymatic activity. Each subunit coordinates one atom each of Cu and Zn. Cu is necessary for the enzymatic activity, whereas Zn mainly works to maintain the stable structure of the protein. Because Cu is catalytically redox-active and has a potential to oxidize proteins including SOD1 itself, inappropriate reactivity of Cu coordinated in SOD1 can underlie the conformational change of mutant SOD1. Inversely, conformational change in mutant SOD1 may increase the

accessibility of substrates to Cu in the protein to generate reactive oxygen or nitrogen species. There is direct evidence that mutant SOD1 can promote abnormal pro-oxidant reactions cooperated with Cu. Mutant SOD1, unlike wild type SOD1, has a potential to generate hydroxyl radicals (Wiedau-Pazos et al., 1996; Yim et al., 1996) or peroxynitrite (Estevez et al., 1999) by Cu-dependent reaction *in vitro*, which can be inhibited by Cu chelators in cultured cells (Ghadge et al., 1997). Cu-mediated toxicity in mutant SOD1 is also reinforced with the reports that decreasing intracellular Cu, by treatment with Cu chelators or genetic reduction of Cu uptake, alleviates ALS phenotype in mutant SOD1 transgenic mice (Hottinger et al., 1997; Kiaei et al., 2004 ; Nagano et al., 2003; Tokuda et al., 2008). Moreover, metallothioneins, which bind Cu to prevent it from being pro-oxidant, are increased in the spinal cord of mutant SOD1 mice to attenuate the disease expression (Hashimoto et al., 2011; Nagano et al., 2001; Tokuda et al., 2007). These facts suggest that Cu-mediated oxidative chemistry underlies the pathogenesis of familial ALS linked to mutations of *SOD1* gene.

On the other hand, the phenotype of mutant SOD1 mice was not rescued by genetic removal of the Cu chaperone for SOD1 (CCS), which incorporates Cu into the buried active site of SOD1 (Subramaniam et al., 2002). Furthermore, mutant SOD1 still induces the disease in transgenic mice even when the active copper-binding site is totally disrupted by multiple mutations (Wang et al., 2003). These findings had been taken as evidence against the hypothesis of aberrant Cu chemistry in the toxicity of mutant SOD1. However, the theory implicating Cu toxicity cannot be excluded since ectopic binding of Cu away from the active site, for example, could contribute to the pathogenesis. In fact, H46R mutant SOD1, which disrupts Cu binding at the active site, still has the ectopic binding of Cu (Liu et al., 2000).

3. Increased affinity for Cu in mutant SOD1

To clarify a possible aberrant interaction of mutant SOD1 with Cu outside the active site in the context of familial ALS, we characterized the affinity for Cu of the mutants by immobilized metal affinity chromatography (IMAC), a method that separates proteins based on their affinities with an immobilized metal such as Cu (Watanabe et al., 2007). Mutant SOD1 commonly exhibited an aberrant fraction with **h**igh **a**ffinity for **C**u (SOD1HAC), in addition to that with **l**ow **a**ffinity for **C**u (SOD1LAC) seen in wild type SOD1 as well. SOD1HAC was detected whether the mutants were expressed in yeasts, mammalian cells or spinal cords of transgenic mice, while an unknown cellular factor(s) other than SOD1 was needed for its generation (Nagano, unpublished data). We observed SOD1HAC even in H46R or G85R mutant SOD1, the mutants that do not efficiently incorporate Cu into the active site, and therefore the immobilized Cu is likely to interact with SOD1 outside the active site, on a solvent-facing surface of the protein. Considering that mutant SOD1 is separated into two distinct fractions (SOD1LAC and HAC) and the interaction of proteins on IMAC is determined by topology of metal-coordinating residues on solvent-facing surfaces (Porath et al., 1975), conformational transition from the native to non-native state is implied to be critical for the increased affinity for Cu in SOD1HAC.

4. Monomerization of SOD1 by cysteine oxidation

Then what is the determinant of conformational transition for SOD1HAC in mutant SOD1? Human SOD1 has four cysteine residues—Cys6, Cys57, Cys111 and Cys146—in a subunit.

Two of them (Cys57 and Cys146) form an intramolecular disulfide bond that maintains the rigid structure and enzymatic activity of SOD1 protein, whereas the remaining two (Cys6 and Cys111) are present as cysteines having free sulfhydryl groups. Of the latter, Cys6 is deeply buried in the protein molecule and less accessible by substrates, while Cys111 is located on the surface of the protein near the dimer interface. Substitution of serine for Cys111 (C111S) is known to increase the structural stability and resistance to heat inactivation of wild type SOD1 (Lepock et al., 1990), implying that the mode of Cys111 may regulate the conformational state of mutant SOD1. H46R mutant SOD1, which has an ectopic binding to Cu as mentioned above, has been reported to bind the metal at Cys111 (Liu et al., 2000). We hypothesized that Cys111 might be a candidate site in human SOD1 that could enhance the coordination of the protein with immobilized Cu. Indeed, C111S substitution eliminated the increase of Cu binding in mutant SOD1. Moreover, the protein degradation assay in cell culture indicated that the decrease of SOD1HAC by C111S substitution well correlated with the stability of each mutant protein. That is, the stability was lower, the affinity for Cu was higher in mutant SOD1. In agreement with our findings, a previous report indicated that the decreased stability of mutant SOD1 correlated with its toxicity and the disease progression rate in familial ALS patients (Sato et al., 2005).

Next, to examine whether other cysteine residues play the same role as Cys111 in mutant SOD1, we introduced C57S substitution into the protein. In contrast to the effect of C111S, C57S substitution rather increased Cu binding, and did not rescue the instability of the mutant. C57S substitution prevents the disulfide bond between Cys57 and Cys146, which is supposed to make SOD1 protein difficult to keep its structure and stabilize. Although the function of the disulfide bond in SOD1 is not fully elucidated, it could be related to either the dimerization of SOD1 or the metal binding process at the active site or both. Thus, mutant SOD1 with C57S may become conformationally further destabilized, exposing Cu-interaction sites to enhance Cu affinity of the protein.

We performed further biochemical characterization of SOD1HAC and determined what properties could cause its formation and toxicity. Since mutant SOD1 is known to susceptible to intramolecular disulfide reduction (Tiwari & Hayward, 2003), we employed a cysteine-modifying reagent to estimate the redox status of cysteine residues in SOD1HAC. We found that sulfhydryl groups of free cysteine residues, especially of Cys111, were oxidized in SOD1HAC while the residues remained reduced in SOD1LAC (Kishigami et al., 2010). The intramolecular disulfide bond between Cys57 and Cys146 was unchanged in both components.

The dimeric structure of SOD1 is destabilized in pathogenic SOD1 mutants (Furukawa & O'Halloran, 2005). We therefore investigated the dimer/monomer status of SOD1LAC and SOD1HAC using gel filtration chromatography. SOD1HAC eluted predominantly as a monomer, whereas SOD1LAC consisted of a dimer structure. It means that SOD1HAC formation is concordant with the loss of dimeric stability to form a monomer.

We further employed nitrosoglutathione or hydrogen peroxide, cysteine-oxidizing reagents, in wild type SOD1 to mimic the sulfhydryl oxidation of Cys111 in SOD1HAC. We observed that the reaction actually modified Cys111, and engendered SOD1HAC. Moreover, we saw that Cys111-modified wild type SOD1 lost its dimeric conformation and mainly consisted of a monomer in SOD1HAC. Conversely, intersubunit crosslinking between Cys111 of each subunit prevented mutant SOD1 from monomerizing and developing SOD1HAC. These results mean that Cys111 is labile to be oxidized by endogenous agents such as

nitrosoglutathione or hydrogen peroxide in familial ALS-linked mutant SOD1, which is the first step for the substantial monomerization of the protein and increase of the Cu affinity probably by exposing a Cu-accessible interface of the dimer.

5. Oxidative stress by cysteine-oxidized SOD1

In case that SOD1 is monomerized through conformational destabilization mediated by Cys111 oxidation, Cu coordinated at the ectopic binding site can be redox-active. To see whether SOD1HAC causes an aberrant redox reaction, we measured thiol oxidase activity, a Cu-dependent activity that is reported in human SOD1 (Winterbourn et al., 2002). We found that mutant or Cys111-oxidized wild type SOD1 developed the thiol oxidase activity when it was loaded with Cu, and that the activity was decreased by C111S substitution or intermolecular crosslinking of Cys111. Because SOD1 modified at Cys111 possesses the thiol oxidase activity, it is unlikely that Cys111 itself is the direct binding site for Cu. These results indicate that cysteine-oxidized SOD1 may exert the potentially toxic pro-oxidant activity through ectopic binding of Cu to SOD1HAC at a site within the dimer interface, which becomes exposed upon the dissociation of SOD1. The thiol oxidase activity of mutant SOD1 can promote oxidative stress because of the exhaustion of glutathione, the major free thiol and antioxidant. The activity may also oxidize cysteine residues of other proteins, deteriorating various cell functions (Fig. 1).
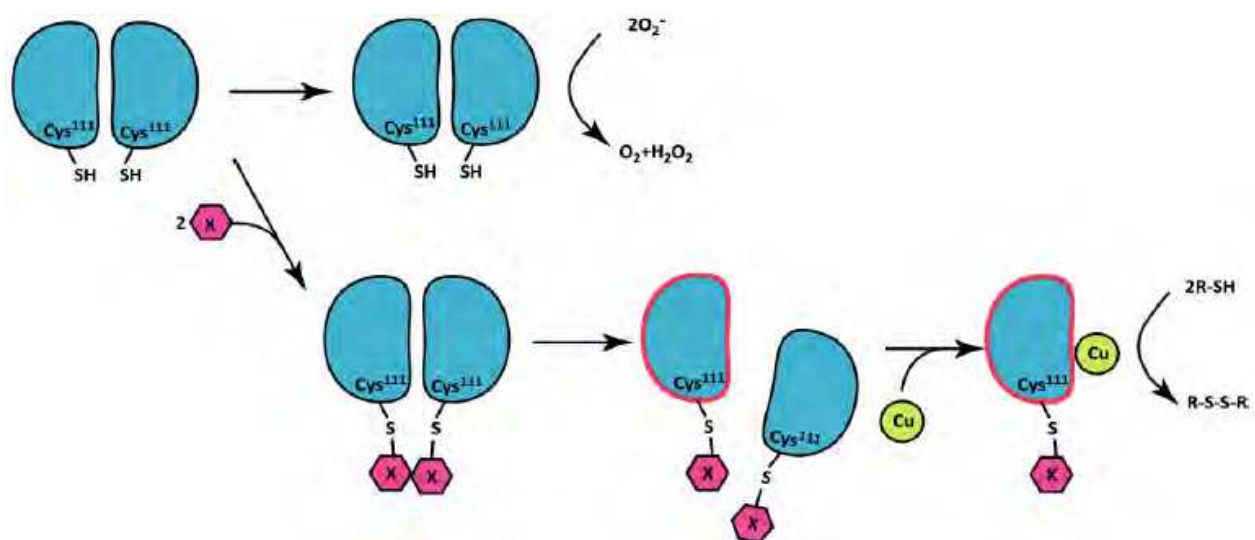

Fig. 1. Proposed model of mutant SOD1 toxicity. Modification of Cys111 leads to dissociation of SOD1 dimers into monomers. Cu, either resulting from rearrangement of the active site or from an external source, becomes ectopically bound to the former dimer interface surface, where it can now catalyze thiol oxidase activity (Kishigami et al., 2010).

6. Role of cysteine-oxidized mutant SOD1 in familial ALS

What is the role of Cys111 modification on the neuronal toxicity by mutant SOD1? In the spinal cords of familial ALS patients and mutant SOD1 transgenic mice, degenerating motor neurons contain SOD1-positive inclusion bodies, suggesting that mutant SOD1 is conformationally misfolded and subject to aggregate (Chattopadhyay & Valentine, 2009). As seen in other neurodegenerative diseases, abnormal protein accumulation in neurons can impair their cellular functions such as axonal transport (Tateno et al., 2009), oxidative phosphorylation in mitochondria and protein degradation machinery.

Various factors can cause conformational rearrangement or misfolding of mutant SOD1, including decreased metallation (Hayward et al., 2002), hydrophobicity (Tiwari et al., 2005) and reduction of repulsive charge (Sandelin et al., 2007). Modification of amino acid residues, especially by oxidative stress, can be a critical factor to enhance the misfolding of mutant SOD1 (Rakhit & Chakrabartty, 2006). Cysteine is in particular susceptible to oxidative modification, since its sulfhydryl moiety is readily attacked by redox active substrates such as glutathione or peroxides to form S-S or S-O covalent modification. Sulfhydryl groups also crosslink each other to form intra- or inter-molecular disulfide bond, which have important roles to maintain or disrupt physiological conformation of proteins. Oxidative reactivity and modification of Cys111, such as glutathionylation (Kajihara et al., 1988; Schinina et al., 1996) and peroxidation (Fujiwara et al., 2007), was documented with human or chick wild type SOD1, although the effect of which on the enzymatic activity or dimer stability had not been determined. Because Cys111 is located on the edge of the dimer interface of each subunit, the modification of Cys111, especially when a large molecule such as glutathione is adducted to the residue, can interrupt the dimer contact at the interface stereochemically and cause the dissociation of SOD1. Molecular dynamics simulations of SOD1 imply that the region including Cys111 is important for the residue interaction network in the protein, and likely to affect the dimer interface through the network and may disrupt their coupled motions (Khare & Dokholyan, 2006). In fact, glutathionylation of Cys111 has been confirmed with native human SOD1 in erythrocytes (Nakanishi et al., 1998; Wilcox et al., 2009), and it was noted that the modification caused SOD1 liable to monomerize and decrease its enzymatic activity (Wilcox et al., 2009). The SOD1 monomer is prone to form aggregates that might be the origin of intracellular inclusions found in motor neurons with SOD1-linked familial ALS. Supporting that, Cys111-peroxidized SOD1 was detected in the neuronal inclusions of mutant SOD1 mice (Fujiwara et al., 2007).

Oxidative modification of cysteine residues, including Cys111, is also possible to be involved in the aggregation process of mutant SOD1. High molecular weight dimers and multimers of mutant SOD1 can be detected in the spinal cords of transgenic mice in parallel to the disease onset (Deng et al., 2006; Furukawa et al., 2006). They are detergent-insoluble and reversed by reductants, supposing that disulfide-mediated crosslinking at cysteine residues is a major factor for mutant SOD1 to form aggregates and ALS phenotype. Cysteines forming the intramolecular disulfide bond (Cys57 and Cys146) are possibly involved in the crosslinking, since the disulfide bond between the residues is labile to be reduced (Tiwari & Hayward, 2003) and cause aberrant oxidation in mutant SOD1. The disulfide-reduced mutant SOD1 is actually enriched in the spinal cord of transgenic mice (Jonsson et al., 2006). The reduced form of mutant SOD1 can also translocate into the intermembrane space of mitochondria cooperated by CCS (Field et

al., 2003), which may be components of aggregates in mitochondria (Deng et al., 2006; Ferri et al., 2006) and harmful to the mitochondrial function. However, intermolecular disulfide bonds mediated at free cysteines (Cys6 and Cys111) can also be components of the detergent-insoluble SOD1 aggregates (Banci et al., 2007; Niwa et al., 2007). In either case, apo SOD1 is more prone to the disulfide-linked oxidative aggregation than holo SOD1 (Banci et al., 2007; Furukawa & O'Halloran, 2005). That is in concert with the notion that immature SOD1 is the pathogenic species in familial ALS (Seetharaman et al., 2009).

It is still controversial whether the cysteine-mediated misfolding or aggregation of mutant SOD1 is the origin of the protein's toxicity. Removal of free cysteines, especially of Cys111, strongly reduced the ability of mutant SOD1 to form disulfide crosslinking and aggregates, and improved cell viability in cultured cells (Cozzolino et al., 2008; Niwa et al., 2007). Moreover, glutaredoxins, which specifically catalyze the reduction of protein-SSG-mixed disulfides, significantly increased the solubility of mutant SOD1 and protected neuronal cells (Cozzolino et al., 2008; Ferri et al., 2010). On the other hand, the intermolecular disulfide binding at cysteines is shown to have a limited effect on the aggregation of mutant SOD1 (Karch & Borchelt, 2008). Even in this case, Cys111-modified mutant SOD1 may cause neuronal toxicity independently of the aggregation, by oxidative stress such as thiol oxidase activity we have shown (Kishigami et al., 2010).

7. Role of cysteine-oxidized wild type SOD1 in sporadic ALS

In sporadic ALS, there had been no direct evidence that SOD1 was involved in the pathogenesis of the disease, except that some mutations of *SOD1* gene expressed familial ALS in a low penetration rate with seemingly 'sporadic' cases. The link between SOD1 and sporadic ALS was first introduced by the detection of SOD1 specifically modifiable with a chemical compound commonly in familial and sporadic ALS, although the molecular basis for it has not been determined in detail (Gruzman et al., 2007). It indicates that a similar conformational change in mutant and wild type SOD1 can trigger the phenotype of familial and sporadic ALS in common. In *in vitro* study, wild type SOD1 acquires toxic properties of mutant SOD1 through oxidation by hydrogen peroxide (Ezzi et al., 2007), implying that cysteine-oxidized wild type SOD1 may be a contributor to motor neuronal death in sporadic ALS.

Recently, a conformation-specific antibody generated against misfolded mutant SOD1 has been shown to recognize wild type SOD1 only when the protein was sulfonylated (-SO$_3$H) at Cys111, and the antibody immunostained motor neurons in the spinal cords of sporadic ALS patients, but not of SOD1-unrelated familial ALS patients or controls (Bosco et al., 2010). Chemically oxidized or purified wild type SOD1 from sporadic ALS spinal cords inhibited kinesin-based fast axonal transport as did mutant SOD1, supposing that Cys111-mediated conformational change or misfolding of SOD1 is a shared pathological denominator of familial and sporadic ALS. Interestingly, most of the sporadic ALS-derived toxic SOD1 was soluble and non-aggregated, meaning that misfolding or monomerization is sufficient for SOD1 to gain the toxic property such as oxidative insult we have shown in mutant and wild type SOD1 (Kishigami et al., 2010). Further studies *in vivo* will be required to clarify the detailed mechanism of SOD1 toxicity mediated by oxidation of cysteine residues including Cys111.

8. Conclusion

The findings mentioned above indicate that oxidative modification of SOD1 at cysteine residues is a critical factor to contribute to the oxidative stress, inclusion pathology and degeneration of motor neurons commonly to familial and sporadic ALS. Based on them, steric inhibition of cysteine oxidation, monomerization or exposure of the dimer interface can be the first-line treatment strategy of this incurable disease.

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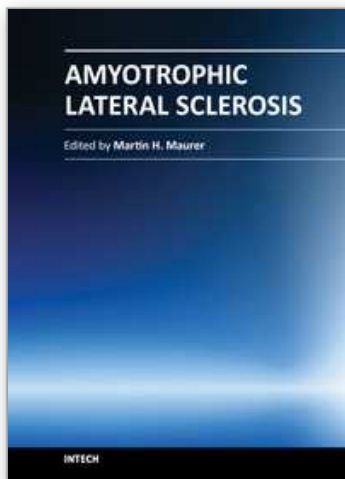
Amyotrophic Lateral Sclerosis

Edited by Prof. Martin Maurer

Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

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