BMB
reports

Inactive extracellular superoxide dismutase disrupts secretion and function of active extracellular superoxide dismutase

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Extracellular superoxide dismutase (EC-SOD) is an antioxidant enzyme that protects cells and tissues from extracellular damage by eliminating superoxide anion radicals produced during metabolism. Two different forms of EC-SOD exist, and their different enzyme activities are a result of different disulfide bond patterns. Although only two folding variants have been discovered so far, five folding variants are theoretically possible. Therefore, we constructed five different mutant EC-SOD expression vectors by substituting cysteine residues with serine residues and evaluated their expression levels and enzyme activities. The mutant EC-SODs were expressed at lower levels than that of wild-type EC-SOD, and all of the mutants exhibited inhibited extracellular secretion, except for C195S EC-SOD. Finally, we demonstrated that co-expression of wild-type EC-SOD and any one of the mutant EC-SODs resulted in reduced secretion of wild-type EC-SOD. We speculate that mutant EC-SOD causes malfunctions in systems such as antioxidant systems and sensitizes tissues to ROS-mediated diseases. [BMB reports 2011; 44(1): 40-45]

INTRODUCTION

Reactive oxygen species (ROS) are produced in the body by several different mechanisms, including cellular respiration, interactions between ionizing radiation and biological molecules, and phagocytosis. ROS produced at lower levels under normal conditions are important mediators of cell signaling events, including differentiation, cell cycle progression, growth arrest, apoptosis, and immunity. However, overproduction of ROS can induce cell and tissue damage as well as various diseases such as UV-induced skin inflammation (1).

The antioxidant enzyme superoxide dismutase (SOD) family comprises three distinct isoforms that are partly responsible for maintaining low levels of ROS by metabolizing superoxide anion into oxygen and hydrogen peroxide (2). Cu/Zn-SOD (SOD1) is localized predominantly in the cytoplasm and the nucleus, and it contains copper and zinc in its active site as cofactors. Mn-SOD (SOD2) is localized in the mitochondrial matrix, and it contains manganese in its active site as a cofactor. The third form of SOD, EC-SOD (SOD3), was discovered recently, and it is present in the extracellular matrix (3). EC-SOD is thought to protect the brain, lungs, and other tissues from oxidative stress (4), and it is highly expressed in the blood vessels, lungs, kidneys, and uterus, whereas lower levels are present in the eyes, skeletal muscle, liver, and brain (5).

Human EC-SOD is a metalloprotein containing copper and zinc ions in its active site, which support enzymatic activities (6). EC-SOD contains several functional domains that include a glycosylation site near its amino terminus (7), a central domain with binding sites for copper and zinc ions (8, 9), and a heparin-binding domain at its carboxyl terminus (10-13). EC-SOD exists as a tetramer composed of two interacting dimers (14, 15), and the tetramer is held together by van der Waals and hydrogen bonding/salt bridge interactions (16).

Disulfide bonds play an important role in the folding of secreted proteins by thiol isomerase enzymes, including protein-disulfide isomerase (PDI) (17, 18). PDI is an enzyme that catalyzes the formation and realignment of disulfide bonds between cysteine residues of synthesized proteins (19, 20). Human EC-SOD is a secreted protein that contains six cysteine residues (9). Specifically, cysteine-219 is involved in the formation of a disulfide-linked home/hetero dimer via intersubunit disulfide bonding, whereas the remaining five cysteine residues are disulfide-bonded via one of two possible intramolecular disulfide bonds, producing enzymatically active (aEC-SOD) and inactive (iEC-SOD) subunits (21, 22). One variant (aEC-SOD) has the enzymatic capacity to scavenge superoxide radicals, whereas the other form (iEC-SOD) lacks enzymatic activity. While the disulfide bonding pattern of aEC-SOD includes a conserved disulfide bond found in all Cu/Zn-containing SODs, this disulfide bond is absent in iEC-SOD (23, 24). Although only two folding variants have been discovered

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DOI 10.5483/BMBRep.2011.44.1.40

Received 9 November 2010, Accepted 22 November 2010

Keywords: Disulfide bond, EC-SOD, Proteasomal degradation, Secretion, Tetramer

so far (9, 25), five folding variants are theoretically possible since each subunit has five cysteine residues that are involved in intramolecular disulfide bonding.

Therefore, we constructed five variant expression vectors via substitution of each cysteine residue with serine residues and evaluated the expression levels and enzyme activities of the mutants. Except for the C195S mutant, all of the mutant EC-SODs had lower expression levels due to proteasomal degradation and exhibited reduced extracellular secretion. Furthermore, the mutant EC-SODs did not show enzymatic activity, unlike the C195S mutant.

RESULTS

Expression of wild-type and mutant EC-SOD

It has been reported that EC-SOD has six cysteine residues, and disulfide bond formation among these cysteine residues dsefines whether it is active or inactive (21). Active EC-SOD is secreted while inactive EC-SOD is not secreted. We constructed EC-SOD expression vectors that produce mutant EC-SODs in which the cysteine residues were substituted with serine residues at positions 45 (C45S), 107 (C107S), 189 (C189S), 190 (C190S), and 195 (C195S). We next determined whether or not the EC-SOD expression vectors would produce and secrete EC-SODs. The expression vectors were transfected into HEK293 cells, and 48 hours after transfection, the cells and media were harvested for Western blot analysis. Although wild-type EC-SOD as well as the C45S, C107S, C189S, C190S, and C195S mutants were expressed, the C45S, C107S, C189S, and C190S mutants had lower expression levels than wild-type EC-SOD and the C195S mutant. In addition, only wild-type EC-SOD and the C195S mutant were secreted (Fig. 1A). To define whether or not expression of the EC-SODs was regulated at the mRNA level, RT-PCR was performed. The results show that there were no transcriptional differences among the expression vectors, indicating that the expression of EC-SOD was regulated post-transcriptionally (Fig. 1B).

C45S, C107S, C189S, and C190S mutants are degraded

To determine whether or not the lower production of the C45S, C107, C189S, and C190S mutants resulted from degradation of the proteins, we treated HEK293 cells transfected with pEC-SOD, pC45S, pC107S, pC189S, pC190S, or pC195S mutant with the proteasome inhibitor MG-132 (10 μM) 18 h after transfection. Twenty-four hours after MG-132 treatment, the cells and culture media were collected, and Western blot analysis was performed. MG-132-treated cells had similar protein levels, whereas non-treated cells transfected with pC45S, pC107S, pC189S, or pC190S mutant had lower protein production (Fig. 2A, B). These results indicate that the decreased

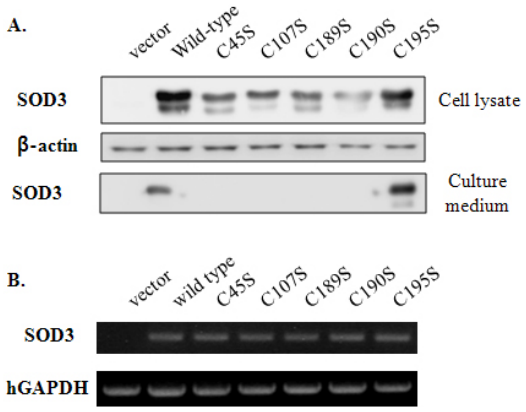

Fig. 1. EC-SOD expression. (A) Cell lysates and culture media of wild-type and mutant EC-SOD-transfected HEK293E cells were analyzed by Western blot analysis. (B) mRNA levels of the EC-SODs were determined by RT-PCR. EC-SOD expression in HEK293E cells transfected with wild-type and mutant EC-SOD was observed in the cell lysates; however, secreted EC-SOD in cultured media was found in the wild-type and C195S mutant EC-SOD-transfected cells. The mRNA levels of EC-SOD did not change in all groups, even though the protein levels were different. β-actin protein and hGAPDH mRNA served as loading controls.

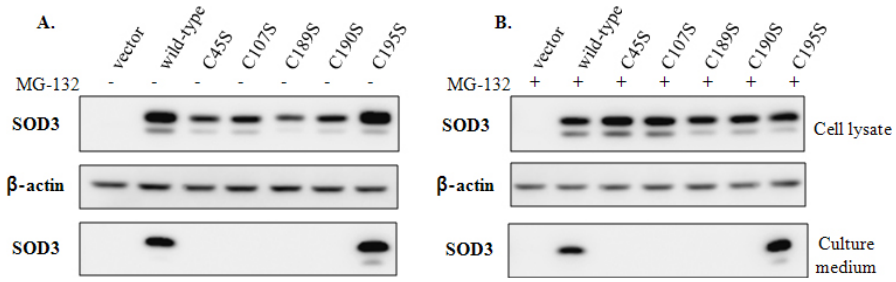

Fig. 2. Proteasomal degradation of mutant EC-SODs. Wild-type and mutant EC-SODs were transfected into HEK293E cells in the absence (A) or presence (B) of 10 μM MG-132, a proteasome inhibitor. The cell lysates and culture media were analyzed by Western blotting. Higher expression levels of intracellular EC-SOD were observed in both wild-type and C195S mutant EC-SOD-transfected cells compared to those in cells transfected with other mutant EC-SODs in the absence of MG132, and the decreased expression levels of other transfected mutant EC-SODs were recovered at similar levels as those in wild-type and C195S mutant EC-SOD-transfected cells in the presence of MG132. However, secreted EC-SOD in the extracellular space was not detected in mutant EC-SOD-transfected cells in the absence or presence of MG-132, except in the C195S mutant EC-SOD-transfected cells.

protein levels of the C45S, C107S, C189S, and C190S mutants were caused by proteasomal degradation.

Wild-type EC-SOD subunit binds to the C45S, C107S, C189S, C190S, and C195S mutant subunits

EC-SOD is a tetramer composed of two dimers that are linked by disulfide bonds (25). Therefore, we determined whether or not the EC-SOD tetramer could be composed of secretable and nonsecretable subunits. To distinguish wild-type EC-SOD from the C45S, C107S, C189S, C190S, and C195S mutants, Myc and His tags were attached to the C-termini of wild-type EC-SOD and of the C45S, C107S, C189S, C190S, and C195S mutants, respectively. We next determined whether or not the wild-type EC-SOD subunit could bind to the nonsecretable subunits. HEK293 cells were co-transfected with pEC-SOD-Myc along with pC45S-His, pC107S-His, pC189S-His, pC190S-His, or pC195S-His and then harvested 48 h after transfection. The cell lysates were immunoprecipitated with anti-Myc antibody, and then Western blotting was performed with anti-His antibody. As shown in Fig. 3, wild-type subunits could bind to the C45S, C107S, C189S, C190S, and C195S subunits. The results imply that the EC-SOD tetramer was composed of wild-type subunits along with one of the C45S, C107S, C189S, or C190S mutant subunits.

Co-expression of pC45S, pC107S, pC189S, or pC190S mutants with wild-type EC-SOD inhibits the secretion of wild-type EC-SOD

To determine whether or not the EC-SOD tetramer composed of secretable and nonsecretable subunits might be secreted, HEK293 cells were co-transfected with pEC-SOD-Myc along with pC45S-His, pC107S-His, pC189S-His, pC190S-His, or pC 195S-His. Forty-eight hours after the transfection, cells and media were collected. Fig. 4A shows that the production of wild-type EC-SOD was not affected by co-expression with the C45S, C107S, C189S, C190S, and C195S mutants. On the other hand, co-expression of the C45S, C107, C189S, and C190S mutants inhibited the secretion of wild-type EC-SOD (Fig. 4B). Therefore, EC-SOD composed of nonsecretable (C45S, C107S, C189S, and C190S) and secretable (wild-type and C195S) subunits may not be secreted.

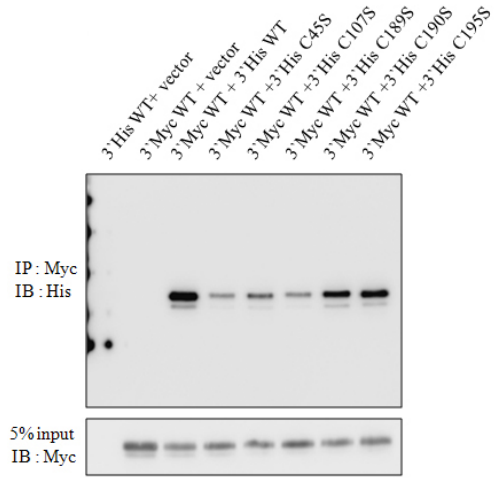

Fig. 3. Mutant EC-SODs bind to wild-type EC-SOD. Myc-tagged wild-type EC-SOD was co-transfected with the His-tagged form of each mutant EC-SOD in HEK293E cells. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by Western blot analysis using anti-His antibody to detect mutant EC-SOD expression. The results show that the mutant EC-SODs bound to wild-type EC-SOD. Myc protein served as a loading control.

DISCUSSION

Human EC-SOD is a tetrameric enzyme that eliminates superoxide anion by converting it to oxygen and hydrogen peroxide. The subunit contains six cysteine residues (9). Cysteine at position 219 participates in intersubunit disulfide bonding, whereas the other residues are involved in the formation of intrachain disulfide bonds (20-23). Therefore, five different folding variants are theoretically possible, but only two variants have been discovered to date (9, 24). The two folding variants were identified by analyzing EC-SOD purified from human aorta (14). Therefore, to investigate the role of each cysteine residue, we constructed five variant mutant expression vectors and evaluated their expression levels and enzyme activities. Mutant EC-SODs exhibited blocked extracellular secretion and lost their enzymatic activities (data not shown). At the same time, mutant EC-SODs had lower expression levels due to proteasomal degradation (Fig. 1-3).

The two folding variants of EC-SOD, based on disulfide bonding pattern, are classified as aEC-SOD (enzymatically active) and iEC-SOD (enzymatically inactive). They can be distinguished based on their combination of free and bound cysteine residues, which determines whether or not secretion takes place. aEC-SOD contains an unbound cysteine residue at 195 and can be secreted, but iEC-SOD has an unbound cysteine residue at 45 and is not secreted (23). We thus constructed artificial aEC-SOD and iEC-SOD by substituting each of the five cysteine residues with serine residues and then evaluating their effects on disulfide bond formation and secretion. In our results, C195S mutant showed similar patterns as wild-type regarding protein expression, secretion, and enzymatic activity. However, the other mutants showed lower expression levels due to proteasomal degradation, in addition to blocked secretion and a lack of enzymatic activity (Fig. 1-3). According to our results and those reported by Petersen *et al*., the free cysteine residue at 195 is essential for the activity of the enzyme (24).

It was interesting that the constructed iEC-SODs could not be secreted and were degraded in proteasomes, which was confirmed by MG-132 treatment. Actually, the C195S mutant of aEC-SOD and C45S mutant of iEC-SOD were confirmed to

have undergone proper folding and possess enzymatic activity (23). Both aEC-SOD and iEC-SOD exhibited the same properties as observed in our results, such as enzyme activity and proteasomal degradation. Generally, nascent or misfolded glycoproteins are targeted for degradation (26, 27). Furthermore, proteasomal degradation via endoplasmic reticulum (ER)-associated degradation (ERAD) functions to remove misfolded proteins based on the strict protein quality control system of the ER (26, 27). Therefore, proteasomal degradation and inhibited secretion of the iEC-SODs may have been due to misfolding, resulting in structurally incomplete enzymes, and glycosylation. EC-SOD could form both tetramers and octamers in the presence of heparin-Sepharose when purified from human aorta, and these constructs consist of aEC-SOD and iEC-SOD folding variants (28). We showed that aEC-SODs, including wild-type and the C195S mutant, bind to iEC-SODs such as the C45S, C107S, C189S, and C190S mutants. The binding of aEC-SOD with iEC-SOD resulted in degradation and inhibited secretion (Fig. 3, 4).

In summary, iEC-SODs with substituted cysteine residues, except for the C195S mutant, were characterized by degradation, blocked secretion, and loss of enzymatic activity. In addition, we found that both the mutant EC-SODs and wild-type EC-SOD, which had activity, were not secreted upon tetramer formation between wild-type EC-SOD and the mutant EC-SODs. It remains to be clarified, but this finding indicates that iEC-SODs can aggregate with EC-SODs having enzymatic activity, resulting in decreased levels of EC-SOD in the extracellular matrix. Therefore, we wish to elucidate the mechanism of formation and further research the causes of aEC-SOD and iEC-SOD generation.

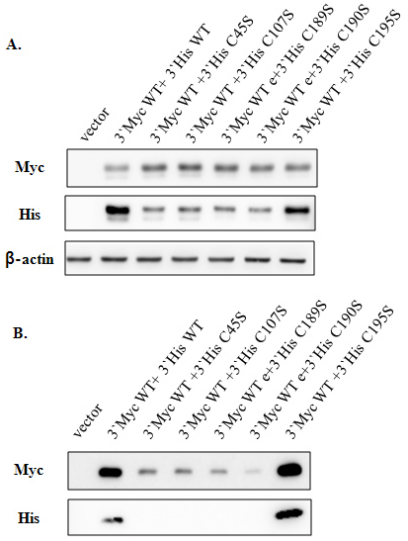

Fig. 4. Mutant EC-SODs are inhibited in extracellular secretion. HEK293E cells were co-transfected with Myc-tagged wild-type EC-SOD and His-tagged mutant EC-SOD, and the expression levels of EC-SOD in the lysates (A) and culture media (B) were analyzed by Western blot analysis. Both the wild-type EC-SOD and mutant EC-SODs were expressed in intracellular fractions, and mutant EC-SOD expression levels were decreased in both intracellular and extracellular fractions compared to expression levels in wild-type and C195S mutant EC-SOD-transfected cells, respectively. The co-transfection of wild-type and mutant EC-SODs blocked extracellular secretion of the EC-SODs, excluding that of the C195S mutant. β-actin protein served as a loading control.

MATERIALS AND METHODS

Construction of mutant EC-SOD plasmids

Human EC-SOD cDNA was used to construct the mutant EC-SOD plasmids using a QuikChange Multi Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, CA). The mutant EC-SOD plasmids were amplified by PCR using pcDNA3.1 Zeo-EC-SOD as a template. The PCR reaction consisted of 30 cycles of annealing at 60°C for 1 min and extension at 60°C for 10 min. The forward and reverse primers used for the mutant EC-SODs were as follows: C45S, 5'-CTCCACGCCGCCAGCCAGGTGCAGCCG-3' and 5'-CGG CGCACCTGGCTGGCGGCGTGGAG-3'; C107S, 5'-CTGAGC CAGGGCAGCGAGTCCACCGGG-3' and 5'-CCCGGTGGAC TCGCTGCCCTGGCTCAG-3'; C189S, 5'-CGGCGGCTGGCCA GCTGCGTGGTGGGC-3' and 5'-GC-CCACCACGCAGCTGG CCAGCCGCCG-3'; C190S, 5'-CGGCTG-GCCTGCAGCGTGG TGGGCGTG-3' and 5'-CACGCCCACCACGCTGCAGGC-CAG CCG-3'; and C195S, 5'-GTGGTGGGCGTGAGCGGGCCCGG GCTC-3' and 5'-GAGCCCGGGCCCGCTCACGCCCACCAC-3' (the mutant sites are underlined). The amplified sequences of the mutant EC-SODs in the expression vectors were verified by sequence analysis.

Cell culture and *in vitro* transfection

HEK293E (HEK293-EBNA) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and cultured at 37°C in a 5% CO_2 incubator. The cells were transiently transfected with EC-SOD using Attractene reagent according to the manufacturer's instructions (Qiagen, CA).

Reverse transcription PCR analysis

Total RNA of 293E cells transfected with each EC-SOD was prepared using TRIzol reagent (Invitrogen, CA), and cDNA was synthesized using a Reverse Transcription Kit (Qiagen). The PCR reaction consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The forward and reverse primers for EC-SOD were 5'-GCTGGCGAGGACGACCTGGG-3' and 5'-GGCGGCCTTG CACTCG CTCTC-3', and those for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-CATCTTCCAGGAGC CAGACC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The amplified PCR products were resolved by agarose gel electrophoresis and visualized under UV light after staining with ethi-

dium bromide.

Western blot analysis

Wild-type and mutant EC-SOD-transfected cell lysates were prepared with RIPA buffer [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1% NP-40, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitors cocktail] on ice for 30 min. Protein concentrations from whole-cell lysates and cultured supernatants were determined using the Bradford method (Bio-Rad, CA). The proteins were resolved on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, MI). The membranes were blocked in 5% skimmed milk in TBS-T [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature and subsequently probed with primary antibodies at 4°C overnight. Membranes were then washed with TBS-T, probed with horseradish peroxidase-conjugated secondary antibodies (Invitrogen), and visualized using WEST-one reagent (iNtRON Biotechnology, Korea) using a LAS-3000 (Fuji Photo Film, Japan). Antibody specific for EC-SOD was obtained from Abcam (Cambridge, MI), and anti-Myc and anti-His antibodies were purchased from Applied Biological Materials (Belgium).

Immunoprecipitation

His- or Myc-tagged wild-type and mutant EC-SOD-transfected cell pellets were lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and protease inhibitors cocktail] on ice. The lysates were precleared with protein G agarose (Thermo Scientific, IL) at 4°C for 2 h and then incubated with anti-Myc antibody at 4°C overnight. The immune complexes were subsequently precipitated by the addition of protein G Sepharose beads at 4°C for 2 h with gentle centrifugation and washed with TBS-T, after which SDS-PAGE was performed.

Acknowledgements

The work was supported by a grant (2010-0002058) from Future-based Technology Development Program through National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology.

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