PRO-OXIDANT ACTIVITY OF CU,ZN-SUPEROXIDE DISMUTASE

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Whereas Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is an essential antioxidant enzyme for superoxide dismutation *in vivo* (1), many reports have shown that an elevated level of Cu,Zn-SOD induces cell killing (2, 3), increases lipid peroxidation (4, 5), and interferes with the transport of neurotransmitters (4, 6). All of these observations are archetypical results inducible by oxygen radicals. Cu,Zn-SOD (7) and FALS mutants (8) are known to develop neurodegeneration. These findings suggest that under certain conditions Cu,Zn-SOD may behave directly or indirectly as a pro-oxidant.

In addition to the usual superoxide dismutation activity, Cu,Zn-SOD is known to exhibit anion binding capacity (9, 10), inactivation by its own reaction product H_2O_2 (11-13), and the purported peroxidase activity (14). Here we summarize our findings that Cu,Zn-SOD has free radical ($\cdot OH$ and scavenger-derived radicals)-generating activity (15, 16). The active Cu,Zn-SOD can catalyze the generation of free $\cdot OH$ radicals from H_2O_2 . In the presence of H_2O_2 and radical scavengers, especially with anionic character, both free $\cdot OH$ radicals and scavenger-derived radicals are produced (15, 16). We also found that the free radical-generating function of several FALS-associated Cu,Zn-SOD mutants is enhanced relative to that of the wild-type enzyme, while the dismutation activities remain unchanged (17, 18).

To monitor the production of free radicals, two spin traps, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and *N*-tert-butyl- α -phenylnitrone (PBN), were used in our studies (15) to convert transient free radicals to stable free radicals adducts according to the following reactions:

The nature of the trapped free radical was identified by EPR spectroscopy. When $O_2^{\cdot -}$ (dissolved KO_2 in dried dimethylsulfoxide) was added to the $NaHCO_3/CO_2$ buffer at pH 7.6 containing 100 mM DMPO, EPR resonance lines of DMPO-superoxide adduct (DMPO-OOH) were observed. When similar experiments were carried out in the presence of Cu,Zn-SOD, DMPO-hydroxyl radical adduct (DMPO-OH) signals were detected in place of the DMPO-OOH signals. Addition of both Cu,Zn-SOD and catalase inhibited the formation of DMPO-OH adducts, indicating that H_2O_2 is needed for the formation of DMPO-

OH adducts. DMPO-OH formation also required active Cu,Zn-SOD, because heat-inactivated Cu,Zn-SOD or Mn-SOD failed to produce these radical adducts. The direct addition of H_2O_2 in place of $O_2^{\cdot -}$ produced similar results. In the presence of $\cdot OH$ radical scavengers with anionic character such as HCO_2^- or N_3^- , DMPO adducts of the scavenger-derived radicals, DMPO- $CO_2^{\cdot -}$ or DMPO- $N_3^{\cdot -}$, were observed in addition to DMPO-OH. The concentration ratio between DMPO-OH and DMPO-scavenger-derived radical adducts observed in these experiments reflects competition reactions between DMPO and scavengers for free $\cdot OH$ radicals. Surprisingly, with neutral $\cdot OH$ radical scavengers, such as ethanol, the formation of DMPO-hydroxyethyl radical adducts was insignificant and the concentration of DMPO-OH formed was unaffected. In contrast, with a different spin trap, PBN, in place of DMPO, ethanol yields PBN-hydroxyethyl radical adducts as expected in the competition reaction for free $\cdot OH$ radicals. These seemingly contradicting results may be caused by the different affinities of these spin traps for the positively charged active channel leading to the active site of Cu,Zn-SOD, where the $\cdot OH$ radicals are generated. To prove this hypothesis, we carried out experiments to measure binding constants of the spin traps to the active site by using a negatively charged chromogen, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (16).

The ABTS is known to react with an $\cdot OH$ radical with a diffusion-controlled rate (Reaction 3) to produce a stable oxidized free radical, $ABTS^{\cdot +}$, which has strong absorption at 415 nm ($\epsilon_{415} = 3.6 \times 10^4 M^{-1} \lambda$) and 660 nm ($\epsilon_{660} = 1.2 \times 10^4 M^{-1}$).

Addition of active Cu,Zn-SOD also produced $ABTS^{\cdot +}$ in the presence of H_2O_2 . However, it showed a binding isotherm with ABTS that yielded a dissociation constant for $ABTS^{\cdot +}$ -Cu,Zn-SOD of $K_d = 7.1 \pm 0.5 \mu M$. The formation of $ABTS^{\cdot +}$ was inhibited by the presence of DMPO or PBN in the reaction mixture. By performing a competition reaction study, K_d values for DMPO and PBN were obtained as 0.63 and 11 mM, respectively. A radical scavenger, formate anion, also inhibits the formation of $ABTS^{\cdot +}$, whereas ethanol does not. The results together indicate that DMPO and anionic scavengers have easy access inside the positively charged active channel of Cu,Zn-SOD, whereas PBN and neutral ethanol stay outside of the channel. Consequently, DMPO or anionic scavengers are in a position to intercept the newly formed $\cdot OH$ radicals and

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also drastically reduce the probability for the reaction of $\cdot\text{OH}$ radicals with ethanol. Therefore, DMPO-hydroxyethyl radical adducts could not be detected in the Cu,Zn-SO-catalyzed reaction. PBN and ethanol, however, are able to compete in the bulk solution for free $\cdot\text{OH}$ radicals released from the active site of Cu,Zn-SOD and thus both PBN-hydroxyethyl radical and PBN-OH can be detected. Conversely, these results indicate that free $\cdot\text{OH}$ radicals and scavenger-derived radicals can reach the bulk solution and may cause oxidative damage to the biological environments. Therefore, the enhanced free radical-generating activity induced by a burst of H_2O_2 formation, elevated level of Cu,Zn-SOD, or mutation of the enzyme may exert the deleterious effects *in vivo*. We demonstrated such an effect in FALS (17, 18).

Most of the FALS mutants have point-mutation sites in conserved interaction regions critical to the subunit fold and dimer contact, rather than residues in the active-site or in the electrostatic active channel (19-20). Initial studies of Cu,Zn-SOD activity in erythrocytes and brain tissues of FALS patients carrying mutations at the *SOD1* locus demonstrated reduced Cu,Zn-SOD dismutation activity compared to that of normal individuals (20). This reduction in SOD dismutation activity may facilitate the pathway of oxidative damage to cause FALS symptoms. However, several studies with transgenic mice (8, 21), transfected cells (22, 23), and lymphoblasts of patients (24) revealed that levels of total Cu,Zn-SOD dismutation activities remain high or higher than normal, which suggests that the FALS mutations in *SOD1* may act through a dominant cytotoxic gain-of-function (8, 21-24). We investigated whether there are any differences in the free radical-generating function between the wild-type and FALS mutant Cu,Zn-SOD. For this purpose, we cloned the wild-type, G93A, and A4V cDNA of human Cu,Zn-SOD, overexpressed them in insect cells, purified the proteins, and measured their enzymic activities. Our results showed that the mutant and wild-type enzymes contain one copper ion per subunit and have identical dismutation activities. However, the free radical-generating activities of the mutants, as measured by the spin trapping method at low H_2O_2 concentration, are enhanced relative to that of the wild-type enzyme in the following order: wild-type < G93A < A4V. This is due to the decrease in the K_m value of H_2O_2 , wild-type (44 mM) > G93A (25 mM) > A4V (13 mM), while the k_{cat} is identical for these enzymes (4.0/min). Thus, the FALS symptoms are not associated with the reduction in the dismutation activity of the mutant enzyme. In view of the fact that the intracellular concentration of the H_2O_2 is in the low or submillimolar range, the differential K_m values observed with the mutant enzymes should play a dominant role in the severity of the various FALS. In this regard, one expects the much reduced K_m value of the A4V mutant obtained in this study to correlate with the severity of A4V FALS patients. Rosen *et al.* (25) and Juneja *et al.* (26) have shown in their clinical study that the A4V mutation is both the most commonly detected and the most clinically aggressive form associated with FALS patients. They found that about 40% of FALS families

subjected for their study have the A4V mutation and these patients survive only an average of 1.2 years after the onset of the disease, as compared to 2.4 years for the average survival of G93A FALS patients. Therefore, our results together with those reported by Rosen *et al.* (25) indicate that the K_m values for H_2O_2 of different FALS mutants are associated with the aggressiveness of the disease progression. Wiedau-Pazos *et al.* also observed that several FALS mutants have enhanced DMPO-OH generating reactivity (27).

In conclusion, we have shown that Cu,Zn-SOD has the free radical-generating function, which involves free $\cdot\text{OH}$ radicals and other scavenger-derived radicals. This function may be different from a peroxidase activity in the classical sense that does not involve free $\cdot\text{OH}$ radicals as an intermediate. Thus, Cu,Zn-SOD may behave as a pro-oxidant in certain situations including a genetic disorder FALS.

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