Superoxide dismutases enhance H_2O_2-induced DNA damage and alter its site specificity

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Abstract Superoxide dismutases (SODs) are involved in the protection of cells from oxygen toxicity. However, several papers have reported that the overexpression of CuZn-SOD causes oxidative damage to cells. We investigated a mechanism by which an excess of SODs accelerates oxidative stress. The presence of CuZn-SOD, Mn-SOD or Mn(II) enhanced the frequency of DNA damage induced by hydrogen peroxide (H_2O_2) and Cu(II), and altered the site specificity of the latter: H_2O_2 induced Cu(II)-dependent DNA damage with high frequency at the 5′-guanine of poly G sequences; when SODs were added, the frequency of cleavages at thymine and cytosine residues increased. SODs also enhanced the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine by H_2O_2 and Cu(II). We conclude that SODs may increase carcinogenic risks, e.g. of tumors in Down syndrome.

Key words: Superoxide dismutase; DNA damage; Hydrogen peroxide

1. Introduction

Superoxide dismutases (SODs) are an essential defense against superoxide anion radical ($O_2^{\bullet-}$), and involved in protecting the cell from oxygen toxicity [1,2]. However, at high concentrations, SODs may function as peroxidase. Transfectants of mouse epidermal cells that overproduce CuZn-SOD were sensitized to the toxic effects of an extracellular burst of $O_2^{\bullet-}$ plus hydrogen peroxide (H_2O_2), resulting in DNA strand breakage and cell death [3,4]. Human HeLa and mouse L-cell clones that overproduce CuZn-SOD showed an increase in lipid peroxidation [5]. Incubation of CuZn-SOD with H_2O_2 increased the lipid peroxidation [6]. The level of lipid peroxide was significantly higher in the brain of transgenic mice carrying human CuZn-SOD [7]. In addition, overexpression of CuZn-SOD may participate in the high occurrence of cancers in Down syndrome patients [7–9]. The mechanism by which excess SODs can induce oxidative stress in vivo remains to be clarified.

In this study, we investigated the effects of SODs, on H_2O_2-induced DNA damage in the presence of Cu(II), and on the site specificity of the latter, using 5′-^{32}P-labeled DNA fragments obtained from the c-Ha-*ras*-1 and the *p53* genes. We also analyzed the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA, using a high-performance liquid chromatograph equipped with an electrochemical detector (HPLC–ECD).

2. Materials and methods

2.1. Materials

Restriction enzymes (*Apa*I, *Ava*I, *Eco*RI, *Hin*dIII, *Pst*I, *Sty*I and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. Calf intestine phosphatase was from Boehringer Mannheim. [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) was from Dojin Chemicals Co., Kumamoto, Japan. Acrylamide, bisacrylamide and piperidine were from Wako Chemicals Co., Osaka, Japan. $CuCl_2$ and $MnCl_2$ were from Nacalai Tesque, Kyoto, Japan. Calf thymus DNA, CuZn-SOD (3000 U/mg from bovine erythrocytes) and Mn-SOD (from *Escherichia coli*) were from Sigma. Nuclease P1 was from Yamasa Shoyu Co., Chiba, Japan.

2.2. Preparation of 5′-^{32}P-labeled DNA fragments

DNA fragments were obtained from the human *p53* tumor suppressor gene [10] and the c-Ha-*ras*-1 proto-oncogene [11]. The DNA fragment of the *p53* tumor suppressor gene was prepared from pUC18 plasmid. The singly 5′-^{32}P-labeled 443 bp fragment (*Apa*I 14 179–*Eco*RI* 14 621), 348 bp fragment (*Sty*I 13 155–*Eco*RI* 13 507) and 211 bp fragment *Hin*dIII* 13 972–*Apa*I 14 182) were obtained according to a method described previously [12]. DNA fragments were also prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 proto-oncogene [13,14]. The singly labeled 341 bp fragment (*Xba*I 1906–*Ava*I* 2246) and 261 bp fragment (*Ava*I* 1645–*Xba*I 1905) were obtained according to a method described previously [13,14]. The asterisk indicates ^{32}P-labeling.

2.3. Detection of damage on isolated DNAs

The standard reaction mixture prepared in a microtube (1.5 ml Eppendorf) consisted of H_2O_2, $CuCl_2$, 5′-^{32}P-labeled DNA fragments and calf thymus DNA in 200 μl of 10 mM bicarbonate buffer (pH 7) containing 5 μM DTPA. After incubation at 37°C for 20–60 min, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min, where indicated, and treated as described previously [13]. The experiments were performed in air-saturated solution. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the Maxam–Gilbert procedure [15] using a DNA sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated

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Abbreviations: DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; HPLC–ECD, HPLC equipped with an electrochemical detector; $O_2^{\bullet-}$, superoxide anion radical; $^{\bullet}OH$, free hydroxyl radical; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; SOD, superoxide dismutase

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DNA fragments. Inactivated CuZn-SOD was prepared by heating at 121°C for 20 min.

2.4. Measurement of 8-oxodG formation in calf thymus DNA

The amount of 8-oxodG was measured by a modified method of Kasai et al. [16]. Calf thymus DNA (100 μM/base) was incubated with indicated concentrations of H_2O_2 in the presence of 20 μM $CuCl_2$ in 10 mM bicarbonate buffer (pH 7) containing 5 μM DTPA at 37°C for 60 min. After ethanol precipitation, the DNA was digested to the nucleosides by incubation with nuclease P1 and alkaline phosphatase and analyzed with an HPLC-ECD as described previously [17].

2.5. Measurement of SOD activity

SOD activity was measured by using the ferricytochrome *c* reduction assay [18,19].

3. Results

3.1. Effects of SODs on the damage of DNA induced by H_2O_2 in the presence of metal ions

CuZn-SOD and Mn-SOD enhanced DNA damage induced by H_2O_2 and Cu(II) (Fig. 1). The enhancement due to Mn-SOD was slightly weaker than CuZn-SOD (Fig. 1A). The intensity of DNA damage increased depending on the concentrations of H_2O_2 (Fig. 1A) or SOD (Fig. 1B). CuZn-SOD and Mn-SOD did not cause DNA damage in the presence of H_2O_2 alone (data not shown). The DNA damage was enhanced by piperidine treatment, suggesting that CuZn-SOD enhanced not only backbone breakage but also base damage. Although active CuZn-SOD enhanced the DNA damage induced by H_2O_2 and Cu(II), inactivated CuZn-SOD did not (data not shown). These results suggest that active CuZn-SOD participated in the enhancement of the DNA damage. Similar results were obtained, when recombinant human CuZn-SOD was added instead of bovine erythrocyte CuZn-SOD (data not shown). Frederick et al. reported that Mn(II) catalytically scavenges $O_2^{\bullet-}$ [20]. In our study, Mn(II) enhanced the DNA damage to the same extent as SODs. CuZn-SOD treated with Chelex 100 resin to remove metals also enhanced the DNA damage (data not shown). Therefore, the enhancement of DNA damage was not due to contaminant trace metal ions in SOD preparations. Therefore, SODs appear to serve the same function as Mn(II). In addition, SODs were not significantly degraded by the levels of H_2O_2 used.

3.2. Comparison of the formation of 8-oxodG induced by H_2O_2 and Cu(II) in the absence and in the presence of CuZn-SOD or Mn-SOD

CuZn-SOD enhanced the formation of 8-oxodG induced by H_2O_2 and Cu(II) (Fig. 2). 8-oxodG formation was monitored in calf thymus DNA treated with various concentrations of H_2O_2 in the presence of Cu(II). When CuZn-SOD was added, the formation of 8-oxodG induced by H_2O_2 and Cu(II) increased significantly. Similar results were obtained, when Mn-SOD was added instead of CuZn-SOD.

3.3. Comparison of site specificity of Cu(II)-mediated DNA damage induced by H_2O_2 and Cu(II) in the absence and in the presence of CuZn-SOD or Mn-SOD

H_2O_2 induced Cu(II)-dependent DNA damage with a high frequency at the 5′-guanine of GG and GGG sequences in the human c-Ha-*ras*-1 proto-oncogene (Fig. 3A). When CuZn-SOD or Mn-SOD was added, the cleavages at thymine and cytosine residues increased (Fig. 3B,C).

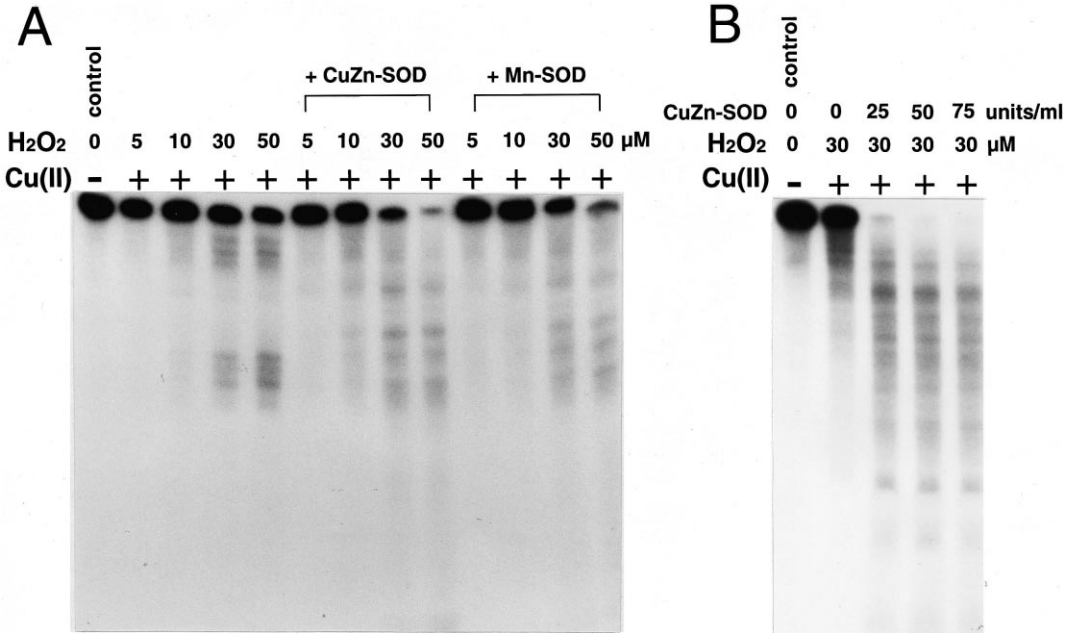

Fig. 1. Autoradiograms of ^{32}P-labeled DNA fragments incubated with SODs and H_2O_2 in the presence of Cu(II). A: The reaction mixture contained the 5′-^{32}P-labeled 261 bp fragment, 10 μM per base of sonicated calf thymus DNA, 20 μM $CuCl_2$, indicated concentrations of H_2O_2, and 150 U/ml (1 μM) of CuZn-SOD or Mn-SOD in 200 μl of 10 mM bicarbonate buffer (pH 7) containing 5 μM DTPA. B: The reaction mixture contained the 5′-^{32}P-labeled 443 bp fragment, 10 μM per base of sonicated calf thymus DNA, 20 μM $CuCl_2$, 30 μM H_2O_2, and indicated concentrations of CuZn-SOD in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 20 min (A) or 60 min (B), the DNA fragments were treated with 1 M piperidine for 20 min at 90°C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing the gel to an X-ray film.

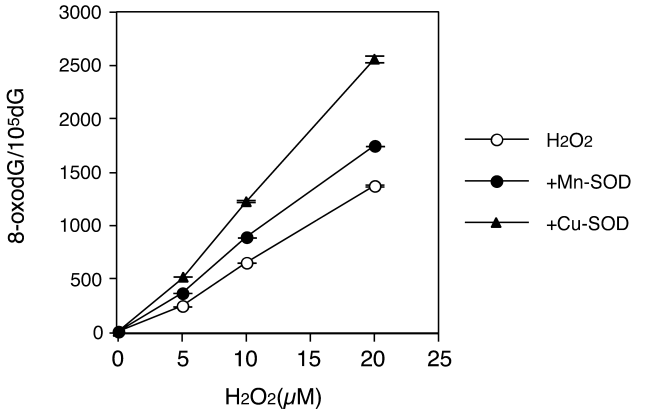

Fig. 2. Formation of 8-oxodG in calf thymus DNA induced by H_2O_2 and Cu(II) in the presence of CuZn-SOD or Mn-SOD. Calf thymus DNA fragments (100 μM per base) were incubated with 20 μM $CuCl_2$ and indicated concentrations of H_2O_2 (○) in the presence of 50 U/ml (0.3 μM) Mn-SOD (●) or CuZn-SOD (▲) at 37°C for 60 min. After ethanol precipitation, the DNA was digested into nucleosides with nuclease P1 and calf intestine phosphatase, and analyzed with an HPLC–ECD. Results are expressed as mean ± S.D. of values obtained from three independent experiments.

4. Discussion

This study revealed that CuZn-SOD efficiently enhances oxidative DNA damage and alters the site specificity of the latter. The formation of 8-oxodG induced by H_2O_2 and Cu(II) increased in the presence of CuZn-SOD. Similar effects were observed, when Mn-SOD or Mn(II) was used instead of CuZn-SOD. Several papers have reported that CuZn-SOD enhances DNA damage. CuZn-SOD, but not Mn-SOD, is damaged by free radicals induced by its own product H_2O_2, resulting in the inactivation and the fragmentation of the enzyme [21,22]. Copper ions released from damaged CuZn-SOD react with H_2O_2 to produce free hydroxyl radical ($^{\bullet}OH$), which may damage DNA [23–25]. In addition, CuZn-SOD exhibits peroxidase activity in the presence of H_2O_2, leading to self-inactivation and the formation of a potent enzyme-bound oxidant such as the SOD-Cu(II)-$^{\bullet}OH$ species, which is similar to free $^{\bullet}OH$ [19]. These mechanisms, where only CuZn-SOD causes oxidative DNA damage, are different from the mechanism clarified in our study, since our results show that not only CuZn-SOD but Mn-SOD and Mn(II) also enhances oxidative DNA damage. The mechanism we propose for the enhancement by SODs of DNA damage induced by H_2O_2 and Cu(II) is as follows:

$$2Cu(II) + H_2O_2 \rightarrow 2Cu(I) + O_2 + 2H^+ \quad (1)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(I)OOH + H^+ \rightarrow {}^{\bullet}OH + OH^- + Cu(II) \quad (2)$$

We previously reported that the formation of dioxygen occurs by the reaction between Cu(II) and H_2O_2 as shown in Eq. 1 [26]. The reaction between Cu(I) and H_2O_2 yields Cu(I)OOH, which may damage DNA (Eq. 2). On the other hand, it has been proposed that H_2O_2 reduces Cu(II) into Cu(I) (Eq. 3) which reacts with H_2O_2, leading to the formation of $^{\bullet}OH$ (Eq. 2) [27–29]:

$$Cu(II) + H_2O_2 \rightleftarrows Cu(I) + O_2^{\bullet-} + 2H^+ \quad (3)$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2 \quad (4)$$

A two-electron transfer as described in Eq. 1 is considered to be thermodynamically more favorable than a one-electron transfer as shown in Eq. 3. As shown in Eq. 4, CuZn-SOD

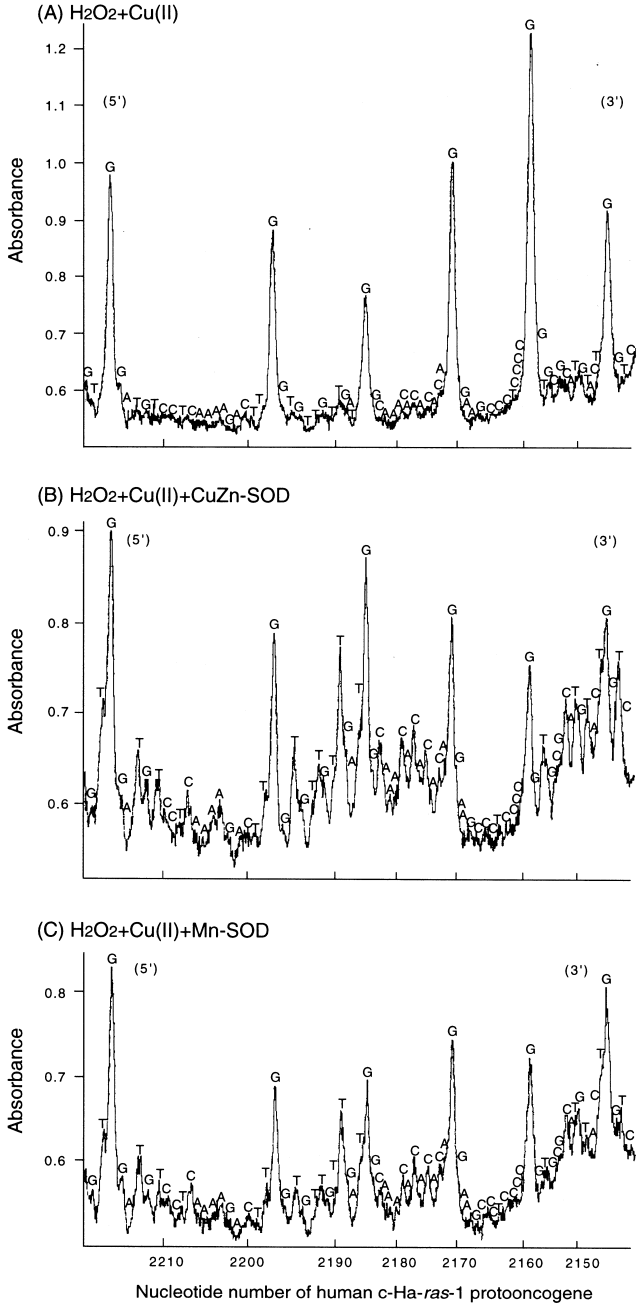

Fig. 3. Site specificity of DNA cleavage induced by H_2O_2 and Cu(II) in the presence of CuZn-SOD or Mn-SOD. The reaction mixture contained the 5′-^{32}P-labeled 341 bp fragment, 10 μM per base of sonicated calf thymus DNA, 20 μM $CuCl_2$, 60 μM (A) or 30 μM H_2O_2 (B, C) and 50 U/ml (0.3μM) CuZn-SOD (B) or Mn-SOD (C) in 200 μl of 10 mM bicarbonate buffer (pH 7) containing 5 μM DTPA. After incubation at 37°C for 40 min, the DNA fragments were treated by the method described in the legend of Fig. 1. The relative amounts of oligonucleotides produced were measured with a laser densitometer. The horizontal axis shows the nucleotide number of the human c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site (11).

provokes the dismutation of $O_2^{\bullet-}$ formed in the reaction of Eq. 3. Therefore, SOD can displace the reaction of Eq. 3 in the forward direction, provoking the accumulation of Cu(I). The latter reacts with H_2O_2 to yield excess Cu(I)OOH, resulting in the enhancement of DNA damage [26]. This function is distinct from the inactivation and fragmentation of the enzyme, resulting in the release of Cu(II). It is noteworthy that not only CuZn-SOD but also Mn-SOD displayed an enhancing effect on the DNA damage induced by H_2O_2 and Cu(II).

In this study, H_2O_2 could induce Cu(II)-mediated DNA damage at the 5′-guanine of GG and GGG sequences of the human c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. The sequence specificity of DNA damage at the 5′-side of consecutive guanines may be explained by the distribution of electrons of HOMO. A large portion of the electrons of HOMO is located on the 5′-guanine of GG and GGG sequences [30]. Therefore, metal ions bind more preferentially to poly G sequences than to single Gs [31]. A peroxide bridge may be formed between two Cu(II) complexes at consecutive guanines. Finally, by Fenton-like reaction, H_2O_2 produces $^{\bullet}OH$, which mediates DNA strand breakage at the 5′-guanine of GG and GGG sequences [28,32]. When SODs were added, cleavages at the level of thymine and cytosine residues increased. Excess Cu(I) formed by reactions involving SODs may induce the proton transfer from guanine to cytosine, and from adenine to thymine residues, resulting in DNA damage at the level of these additional residues [33].

In Down syndrome, the activity of CuZn-SOD is high because the gene for CuZn-SOD resides on chromosome 21. Down syndrome patients usually develop Alzheimer disease [34]. Increased levels of 8-oxodG and other oxidized DNA bases have been observed in the brains of Alzheimer patients [2,34,35]. The levels of SODs are increased in the brain tissues of Alzheimer patients [36], and overexpression of CuZn-SOD creates chronic oxidative stress in the transgenic neurons [37]. Satgé et al. and Hasle et al. have reported that the frequency of some tumors such as leukemias, lymphomas and germ cell tumors were high in Down syndrome patients [8,9]. The mechanisms of carcinogenesis in Down syndrome could be explained by our findings: SODs enhance metal-mediated DNA damage induced by H_2O_2.

We conclude that SODs may increase the frequency of mutations due to oxidative DNA damage in cells, increasing carcinogenic potential.

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