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Role of Cu/Zn-Superoxide Dismutase in Xenobiotic Activation. II. Biological Effects Resulting from the Cu/Zn-Superoxide Dismutase-Accelerated Oxidation of the Benzene Metabolite 1,4-Hydroquinone

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SUMMARY

Cu/Zn-superoxide dismutase (SOD)-accelerated oxidation of the benzene metabolite 1,4-hydroquinone (HQ) results in the enhanced formation of semiquinone anion radicals, electrophilic 1,4-benzoquinone (BQ), and H_2O_2. We selected bone marrow stromal cells and øX-174 double-stranded plasmid DNA as model systems to investigate the cytotoxicity and DNA cleaving activity of the Cu/Zn-SOD-mediated activation of HQ. The addition of either Cu/Zn-SOD or Mn-SOD to the primary bone marrow stromal cell cultures significantly enhanced HQ-induced cytotoxicity, which could be completely prevented by adding reduced glutathione (GSH) or dithiothreitol but not by adding catalase. Incubation of the plasmid DNA with the HQ/Cu/Zn-SOD system resulted in the induction of single- as well as double-strand breaks, which could be inhibited by catalase and the Cu(I) chelators, bathocuproinedisulfonic acid (BCS) and GSH. Although Mn-SOD could enhance HQ-induced cytotoxicity to stromal cells, the activation of HQ by Mn-SOD did not contribute to the induction of DNA strand breaks. Similar to the HQ/Cu(II) and H_2O_2/Cu(II) systems, the DNA strand breaks mediated by HQ/Cu/Zn-SOD could not be effectively inhibited by the hydroxyl radical scavengers, including dimethylsulfoxide, mannitol, and 5,5-dimethyl-1-pyrroline *N*-oxide, but could be protected by sodium azide. Low-temperature electron spin resonance experiments showed that incubation of Cu/Zn-SOD with HQ resulted in the release of copper from the Cu/Zn-SOD, which could be prevented by catalase. α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN)/spin-trapping studies demonstrated that the interaction of HQ with Cu/Zn-SOD, but not with Mn-SOD, resulted in the significant formation of POBN-CH_3 adduct in the presence of dimethylsulfoxide, suggesting the production of hydroxyl radical or its equivalent from this enzyme/xenobiotic interaction. The formation of the POBN-CH_3 adduct from the HQ/Cu/Zn-SOD could be inhibited by catalase, BCS or GSH, indicating the important role for H_2O_2 and Cu(I) in the production of reactive oxygen species. Addition of human myeloperoxidase to the HQ/Cu/Zn-SOD synergistically enhanced the formation of BQ from HQ. This enhancement could be abolished by catalase. Taken together, these results demonstrate that activation of HQ by either Cu/Zn-SOD or Mn-SOD results in cytotoxicity to primary bone marrow stromal cells through the formation of electrophilic BQ. Interaction of HQ with Cu/Zn-SOD causes oxidative damage to Cu/Zn-SOD, leading to the release of copper from the enzyme. The further reaction between the released copper and H_2O_2 generates reactive oxygen species that participate in the induction of strand breaks in plasmid DNA. The H_2O_2 generated from the Cu/Zn-SOD-accelerated oxidation of HQ can also be utilized by myeloperoxidase resulting in additional conversion of HQ to BQ.

Oxygen radical damage has been implicated in a wide range of disease processes, such as inflammatory diseases, amyotrophic lateral sclerosis, ischemia/reperfusion injury, and xenobiotic-induced toxicity (1–6). Cu/Zn-SOD catalyzes the dismutation of superoxide free radical to H_2O_2 and molecular O_2 (7) and plays an essential role in protecting aerobic cells against oxygen toxicity (8–11). A previous study by

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ABBREVIATIONS: Cu/Zn-SOD, Cu/Zn-superoxide dismutase; Mn-SOD, manganese-superoxide dismutase; HQ, 1,4-hydroquinone; $SQ^{\cdot-}$, semiquinone anion radical; BQ, 1,4-benzoquinone; GSH, reduced glutathione; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethylsulfoxide; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; ROS, reactive oxygen species; MPO, myeloperoxidase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; BCS, bathocuproinedisulfonic acid; DTT, dithiothreitol; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ESR, electron spin resonance.

Hodgson and Fridovich (12) demonstrated that Cu/Zn-SOD can undergo oxidative inactivation by its own reaction product, H_2O_2. They proposed that the enzyme-bound Cu(II) could be reduced to Cu(I) by H_2O_2, followed by a Fenton-type reaction of the Cu(I) with additional H_2O_2. This would generate Cu(II)—$OH^{\cdot}$ or its ionized equivalent, Cu(I)—$O^{\cdot-}$, which could then oxidatively attack an adjacent histidine and thus inactivate the enzyme (12). Consistent with this concept, with ESR and the spin traps DMPO and *N-tert*-butyl-α-phenylnitrone, Yim *et al.* (13) recently demonstrated that Cu/Zn-SOD, but not Mn-SOD, catalyzes the formation of hydroxyl radicals from H_2O_2 in pH 7.6 bicarbonate buffer. Sato *et al.* (14) have shown that the interaction of Cu/Zn-SOD with H_2O_2 causes the destruction of ligands for Cu(II) in Cu/Zn-SOD, resulting in free copper release from the oxidatively damaged enzyme (14). A more recent study by Uchida and Kawakishi (15) provided direct evidence that 2-oxo-histidine was generated in the Cu/Zn-SOD exposed to H_2O_2 and that its generation was selective at His^{118} of the active site of the enzyme. The peroxidative activity of Cu/Zn-SOD was also observed to exert deleterious effects on living cells. For example, Scott *et al.* (16, 17) found that *Escherichia coli* variants with elevated intracellular SOD activity exhibited an increased sensitivity to paraquat, hyperoxia, and ionizing radiation. Overproduction of human Cu/Zn-SOD in transfected mouse L-cells and human HeLa cells demonstrated an increase in lipid peroxidation (18). Similarly, a study by Amstad *et al.* (19) showed that mouse epidermal cell clones with increased levels of Cu/Zn-SOD had low intracellular reduced glutathione and more DNA single-strand breaks when exposed to a superoxide-generating system. Cu/Zn-SOD at higher concentrations was also reported to be toxic in isolated heart subjected to ischemia/reperfusion (20). Recently, Peled-Kamar *et al.* (21) observed thymic abnormalities and enhanced apoptosis of thymocytes and bone marrow cells in transgenic mice overexpressing Cu/Zn-SOD. Because both H_2O_2 and lipid peroxidation were elevated in the thymocytes of transgenic Cu/Zn-SOD mice, they proposed that the above defects may be caused by an increased oxidative damage associated with the overexpression of Cu/Zn-SOD in cells.

Another potential adverse effect associated with Cu/Zn-SOD may result from its capacity to accelerate the autoxidation of phenolic chemicals, generating reactive intermediates that cause cytotoxicity and genotoxicity. As demonstrated in Part I (22), the SOD-accelerated oxidation of HQ results in the enhanced formation of $SQ^{\cdot-}$, BQ, and H_2O_2. BQ has been implicated as a reactive species for both HQ-induced toxicity to bone marrow stromal cells and the hematotoxicities associated with benzene exposure (23–27). Because $SQ^{\cdot-}$, BQ, and H_2O_2 are formed during the Cu/Zn-SOD-accelerated oxidation of HQ and the biological effects resulting from this enzyme-xenobiotic interaction have not been investigated, we selected primary bone marrow stromal cells and øX-174 double-stranded plasmid DNA as model systems to investigate the cytotoxicity and DNA cleaving activity of the Cu/Zn-SOD-mediated activation of HQ. With ESR spectroscopy, we further examined the oxidative damage to this enzyme and the formation of ROS from the interaction of HQ with Cu/Zn-SOD. Because MPO has been implicated in the activation of HQ (28), we also investigated the combined effects of Cu/Zn-SOD and MPO on the oxidative activation of HQ.

Experimental Procedures

Materials. Cu/Zn-SOD (4500 units/mg protein) from bovine erythrocytes, Mn-SOD from *E. coli*, HQ, catalase from bovine liver, HQ, BCS, GSH, DTT, DTPA, DMSO, mannitol, sodium azide, DMPO, and POBN were purchased from Sigma Chemical Co. (St. Louis, MO). The unit of SOD used here was as defined by McCord and Fridovich (7). The DMPO was further purified by double distillation. Dulbecco's PBS (10 times concentrated, pH 7.4), RPMI 1640 (No. 430-1800), FBS, horse serum, penicillin/streptomycin, 2-mercaptoethanol, and trypsin were obtained from GIBCO (Grand Island, NY). The concentrated PBS was diluted by 10-fold with deionized water before use. øX-174 RF I double-stranded plasmid DNA was purchased from New England Biolabs (Beverly, MA). Forty-eight-well tissue culture plates and other tissue culture plasticware were products of Costar (Cambridge, MA). Human MPO was obtained from Calbiochem (Lo Jolla, CA).

Animals. Male DBA/2J mice (25–30 g) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in an air-conditioned room with a light period from 6:00 a.m. to 6:00 p.m. Purina laboratory chow (Richmond, IN) and water were available *ad libitum*.

Primary adherent stromal cell culture. Mice were euthanized via cervical dislocation, and their femurs and tibias were removed. Bone marrow cells were flushed with RPMI 1640 medium from the femurs and tibias by with a 23-gauge needle and pooled in RPMI 1640 medium. The procedure used to establish primary adherent stromal cell cultures was described previously (29). For cell toxicity assays, stromal cells were trypsinized and replated in 48-well tissue culture plates at an initial concentration of 1×10^5 cells/well with each well containing 1 ml of cell suspension.

Chemical toxicity to stromal cells. For HQ toxicity assays, 13–18-day-old primary cells were plated in 48-well tissue culture plates. At 24 hr later, medium was removed and replaced with fresh medium containing various chemicals. After incubation, cell survival was assessed by crystal violet staining as described by Zhu *et al.* (29). Cell survival was expressed as percentage of control absorbance.

Assay for DNA strand breaks. DNA strand breaks were measured by the conversion of supercoiled øX-174 RF I double-stranded DNA to open circular and linear forms as described previously (30). Briefly, 0.2 μg of DNA was incubated with HQ, Cu/Zn-SOD, or other chemicals in PBS at 37° at a final volume of 24 μl in 1.5-ml brown microfuge tubes. After incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA and electrophoresed in a horizontal slab gel apparatus (Owl Scientific, MA) in Tris/acetate gel buffer. After electrophoresis, gels were then stained with 0.5 μg/ml solution of ethidium bromide for 30 min, followed by another 30 min of destaining in water, and exposed to UV light at 254 nm. The gels were then photographed, and the negatives were scanned with a Beckman DU-7 spectrophotometer at 561 nm. The percentage of DNA in each form was calculated by integrating the area under the peaks.

Low-temperature ESR detection of copper in Cu/Zn-SOD and ESR measurement of radicals. For detection of copper, Cu/Zn-SOD treated with HQ or H_2O_2 for 90 min at 37° in PBS was dialyzed against deionized water for 24 hr at 4°. Then, 200 μl of this dialyzed sample was transferred to a 3.5-mm (i.d.) quartz ESR tube under ambient conditions, which was then subjected to ESR spectroscopy (model ER 300, IBM-Bruker) operating at X-band with a TM 110 cavity at 77 K under the following instrumental conditions: modulation frequency, 100 kHz; modulation amplitude, 3.98 G; scan time, 60 sec; receiver gain, 1×10^5; microwave power, 20 mW; and microwave frequency, 9.3 GHz. For the measurement of radicals, ESR spectra were recorded at room temperature with samples loaded in a TM flat cell as described previously (31). Briefly, the spectrometer settings were modulation frequency, 100 kHz; modulation amplitude, 0.5 G; scan time, 30 sec; microwave power, 20 mW; and microwave frequency, 9.78 GHz. The microwave frequency and magnetic field were precisely measured with a source-locking micro-

wave counter (model 575, EIP Instruments, San Jose, CA) and an NMR gaussmeter (model ER 035 M, Bruker Instruments, Billerica, MA), respectively. ESR data collection and analysis were performed with a personal computer using software developed in the Electron Paramagnetic Resonance Center (The Johns Hopkins University School of Medicine, Baltimore, MD). Spectral simulations were performed and directly matched with experimental data to extract the spectral parameters (32).

Statistical analysis. Student's *t* test was used. Statistical significance was considered at $p < 0.05$.

Results

Stromal cytotoxicity resulting from the Cu/Zn-SOD-mediated activation of HQ. Because BQ has been suggested to be a reactive electrophilic species contributing to HQ-induced bone marrow stromal cell injury (23, 26, 27), we determined whether extracellularly added Cu/Zn-SOD could enhance HQ-induced cytotoxicity to stromal cells. In the absence of extracellularly added Cu/Zn-SOD, incubation of stromal cells with 25, 50, or 100 μM HQ for 5 hr did not result in significant cytotoxicity at this time (Fig. 1A). However, in the presence of 500 units/ml Cu/Zn-SOD, HQ induced significant decreases in cell survival. In this system, Cu/Zn-SOD alone did not induce any change in cell viability (data not shown). In data not shown, the presence of Mn-SOD also dramatically enhanced HQ-induced toxicity to stromal cells. The Cu/Zn-SOD-enhanced cytotoxicity of HQ also exhibited a SOD concentration-dependency (Fig. 1B), which is consistent with the observation that Cu/Zn-SOD-accelerated oxidation of HQ to BQ also shows a SOD concentration-dependent relationship (22). The concentrations of SOD used in this study are within the ranges found in various tissues (33) and are similar to those conducted in experiments by others (12–15). The Cu/Zn-SOD-mediated activation of HQ generates $SQ^{\cdot}$, BQ, and H_2O_2 (22). To identify which of these species is responsible for the enhanced cytotoxicity, the effects were examined of GSH, DTT, or catalase on Cu/Zn-SOD-enhanced HQ-induced cytotoxicity to stromal cells. The data in Fig. 2 showed that the addition of 1 mM GSH or 0.5 mM DTT to the cultures just before the addition of HQ and Cu/Zn-SOD completely blocked the cytotoxicity induced by HQ in the presence of Cu/Zn-SOD, whereas supplementation of the extracellular medium with catalase did not provide any protection.

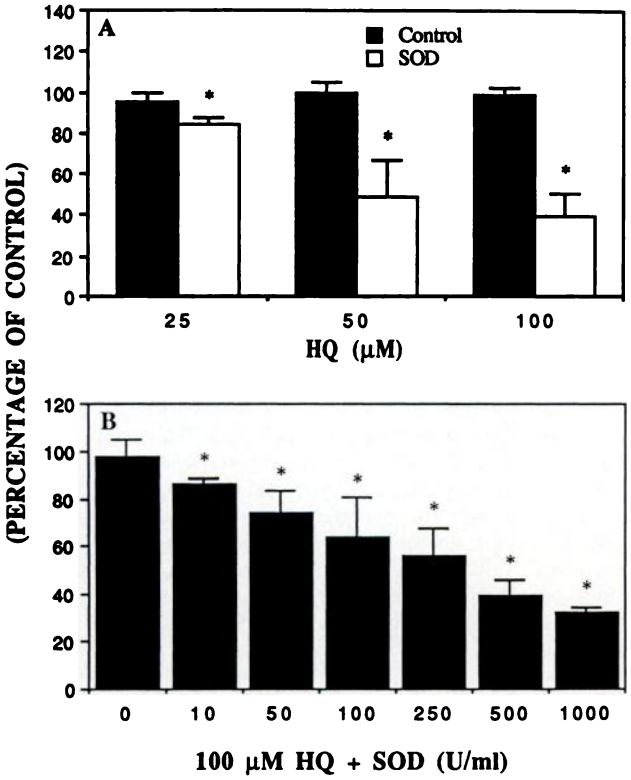

Fig. 1. Cu/Zn-SOD-enhanced cytotoxicity of HQ to bone marrow stromal cells. Cells were incubated with the indicated concentrations of HQ in the absence or presence of 500 units/ml Cu/Zn-SOD (A) or with 100 μM HQ in the absence or presence of various concentrations of Cu/Zn-SOD (B) at 37° for 5 hr in RPMI 1640 medium supplemented with 7.5% FBS and 7.5% horse serum. After this 5-hr incubation period, the cell viability was determined as described in Experimental Procedures. Values represent mean ± standard error of percentage of control absorbance from three separate experiments. *, Significantly different from control (A) or HQ alone (B) by Student's *t* test, $p < 0.05$.

Induction of DNA strand breaks by HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD. Induction of single-strand breaks to supercoiled double-strand plasmid DNA leads to the formation of open circular DNA, whereas the formation of a linear form of DNA is indicative of double-strand breaks (30). In agarose gel electrophoresis, supercoiled DNA migrates faster than its linear form, whereas open circular DNA that has lost all of its superhelicity migrates appreciably slower than either the supercoiled or the linear form. As shown in Figs. 3 and 4, interaction of HQ with Cu/Zn-SOD induced the formation of both open circular and linear forms from the supercoiled øX-174 DNA. Increasing the concentration of HQ resulted in greater conversion of supercoiled DNA to open circular and linear forms. Incubation of DNA with Cu/Zn-SOD plus 1 mM H_2O_2 for 90 min also induced the formation of both open circular- and linear-form DNA. Neither HQ nor H_2O_2 alone induced any significant induction of DNA strand breaks. Cu/Zn-SOD at 1000 units/ml induced only a slight conversion of supercoiled DNA to open circular form. The induction of DNA strand breaks by HQ/Cu/Zn-SOD also exhibited a SOD concentration-dependency (Fig. 4). The extent of DNA strand breaks induced by 1000 units/ml Cu/Zn-SOD plus 1 mM HQ or 1 mM H_2O_2 showed a time-dependent relationship (Fig. 5). The strand breaks induced by H_2O_2/Cu/Zn-SOD occurred at least 30 min earlier than did those induced by HQ/Cu/Zn-SOD, which may be because the formation of H_2O_2 from the HQ/Cu/Zn-SOD system is time dependent (22). In data not shown, incubation of plasmid DNA with Mn-SOD also resulted in induction of DNA strand breaks. However, the addition of either HQ or H_2O_2 to Mn-SOD did not significantly potentiate the DNA strand breaks.

Effects of copper chelators and reactive oxygen scavengers on the induction of DNA strand breaks by HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD. Activation of HQ by Cu/Zn-SOD results in enhanced formation of $SQ^{\cdot}$, BQ, and H_2O_2 (22). Interaction of Cu/Zn-SOD with H_2O_2 has also been shown to result in the formation of hydroxyl radicals and the release of copper from the enzyme (13, 14). Due to the formation of H_2O_2 from the Cu/Zn-SOD-mediated activation of HQ, ROS could also be produced and participate in the induction of DNA strand breaks. Therefore, we next examined the possible roles for H_2O_2, copper, and ROS in the induction of DNA strand breaks by HQ/Cu/Zn-SOD. As shown in Fig. 6A, the presence of catalase but not heat-inactivated catalase extensively inhibited the HQ/Cu/Zn-

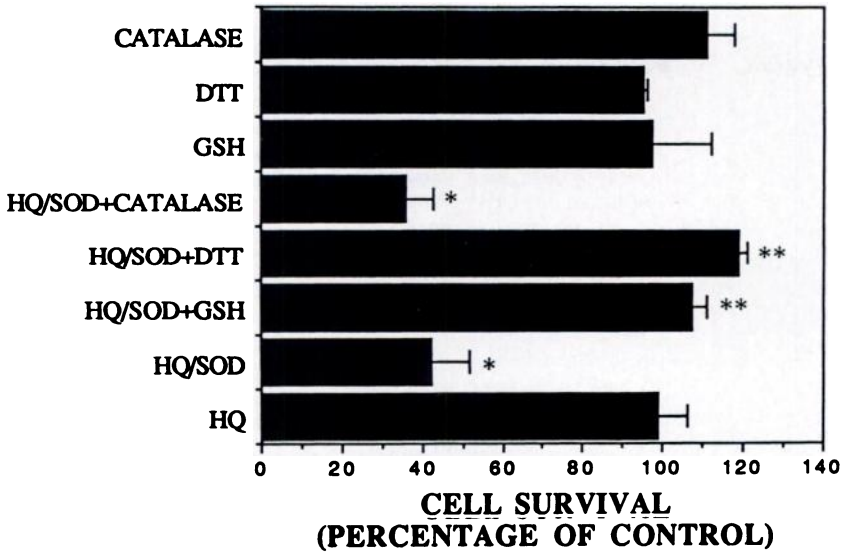

Fig. 2. Effects of GSH, DTT, or catalase on the Cu/Zn-SOD-enhanced cytotoxicity of HQ to bone marrow stromal cells. The cells were incubated with 100 μM HQ and 500 units/ml Cu/Zn-SOD in the absence or presence of 1 mM GSH, 0.5 mM DTT, or 500 units/ml catalase at 37° for 5 hr in RPMI 1640 medium supplemented with 7.5% FBS and 7.5% horse serum. After this 5-hr incubation period, the cell viability was determined as described in Experimental Procedures. Values represent mean ± standard error of percentage of control absorbance from three separate experiments. *, Significantly different from HQ alone by Student's *t* test, $p < 0.05$. **, Significantly different from HQ/SOD by Student's *t* test, $p < 0.05$.

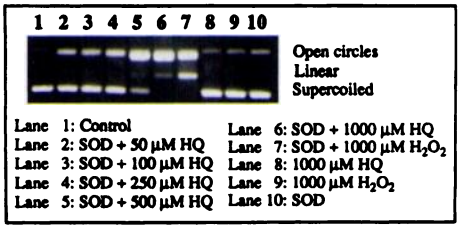

Fig. 3. Agarose gel, stained with ethidium bromide, of øX-174 RF I plasmid DNA incubated with the indicated concentrations of HQ or H_2O_2 in the presence of 1000 units/ml Cu/Zn-SOD in PBS at 37° for 90 min. The DNA strand breaks were determined by measuring the conversion of supercoiled DNA to open circular and linear forms. The sequence of addition to DNA was Cu/Zn-SOD followed by HQ or H_2O_2.

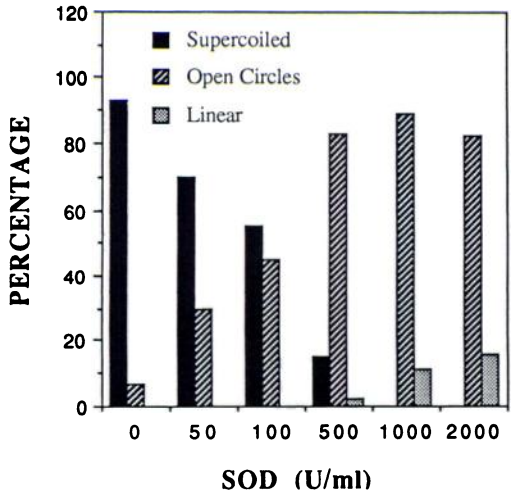

Fig. 4. DNA strand breaks induced by HQ in the presence of varying concentrations of Cu/Zn-SOD. DNA was incubated with 1 mM HQ in the presence of the indicated concentrations of Cu/Zn-SOD in PBS at 37° for 90 min. The percentage of each form of DNA was calculated as described in Experimental Procedures. The sequence of the addition to DNA was Cu/Zn-SOD followed by HQ.

SOD-induced DNA strand breaks, indicating that H_2O_2 is critical for the induction of DNA strand breaks. The presence of the Cu(I) chelators BCS and GSH or the Cu(II) chelator DTPA also extensively inhibited the DNA strand breaks induced by either HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD, indicating the critical role for Cu(II)/Cu(I) redox in the induction of DNA strand breaks by these two systems. In contrast to the inhibition of DNA strand breaks, the Cu/Zn-SOD-accelerated oxidation of HQ to BQ could not be inhibited by BCS and only slightly slowed by DTPA (22). Because both H_2O_2 and copper redox are critical for the DNA cleaving activity of the HQ/Cu/Zn-SOD, ROS generated from the further interaction of H_2O_2, and copper may be the ultimate reactive species that cause DNA strand breaks. Accordingly, we determined the effects of hydroxyl radical and singlet oxygen scavengers on the HQ/Cu/Zn-SOD- as well as H_2O_2/Cu/Zn-SOD-induced DNA strand breaks. As shown in Fig. 6A, all of the hydroxyl radical scavengers examined, DMSO, mannitol, and DMPO, did not significantly inhibit the DNA strand breaks induced by HQ/Cu/Zn-SOD. In contrast, the presence of sodium azide, a chemical that is frequently viewed as a singlet oxygen quencher, extensively inhibited the DNA strand breaks. Likewise, the DNA cleaving capacity of H_2O_2/Cu/Zn-SOD was extensively diminished by sodium azide but not by the hydroxyl radical scavengers (Fig. 6B).

Copper release from Cu/Zn-SOD treated with HQ or H_2O_2. The DNA strand breaks induced by either HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD could be inhibited by the copper chelator BCS, GSH, or DTPA. These metal chelators are unlikely to directly chelate the copper in the Cu/Zn-SOD. Therefore, a direct chelation of the copper released from the oxidatively damaged Cu/Zn-SOD seems to be responsible for their inhibitory effects on DNA strand breaks induced by these two systems. Accordingly, a low-temperature ESR technique was used to examine the modifications of copper binding in Cu/Zn-SOD treated with HQ or H_2O_2. Cu/Zn-SOD incubated with 1 mM HQ or 1 mM H_2O_2 in PBS for 90 min and then dialyzed against deionized water for 24 hr was subjected to low-temperature ESR detection. As shown in Fig. 7 (A and B), the spectra of the untreated Cu/Zn-SOD were composed of two components, $g_{//}$ and $g_{\perp}$. The $g_{//}$ component has the hyperfine structure characteristic of Cu(II) with I = 3/2. This structure was diminished by ~85% in HQ-treated Cu/Zn-SOD (Fig. 7C), indicating the release of copper from Cu/Zn-SOD after incubation with HQ. The decrease of the Cu(II)

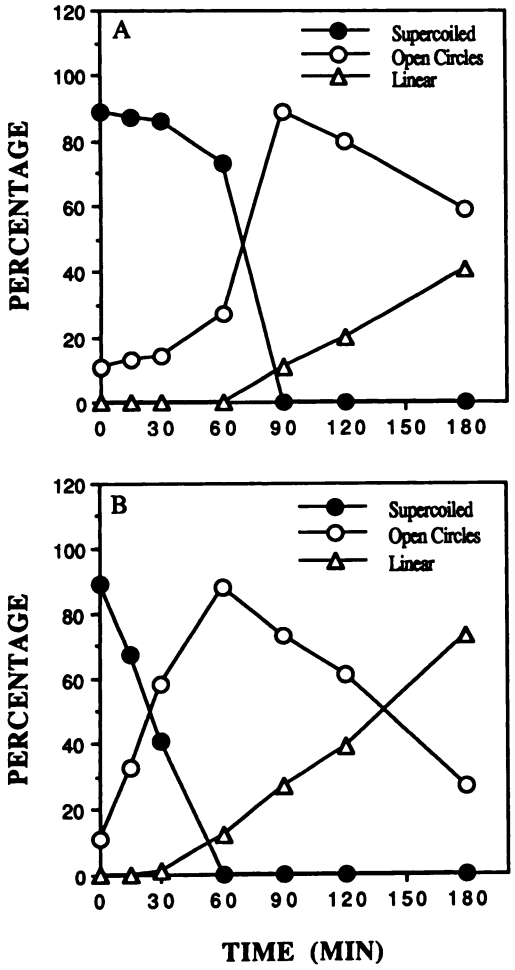

Fig. 5. Time course of DNA strand breaks induced by HQ plus Cu/Zn-SOD (A) or H_2O_2 plus Cu/Zn-SOD (B). DNA was incubated with 1000 units/ml Cu/Zn-SOD plus 1 mM HQ (A) or plus 1 mM H_2O_2 (B) in PBS at 37° for the indicated times, and the percentage of each form of DNA was determined as described in Experimental Procedures. The sequence of the addition to DNA was Cu/Zn-SOD followed by HQ or H_2O_2.

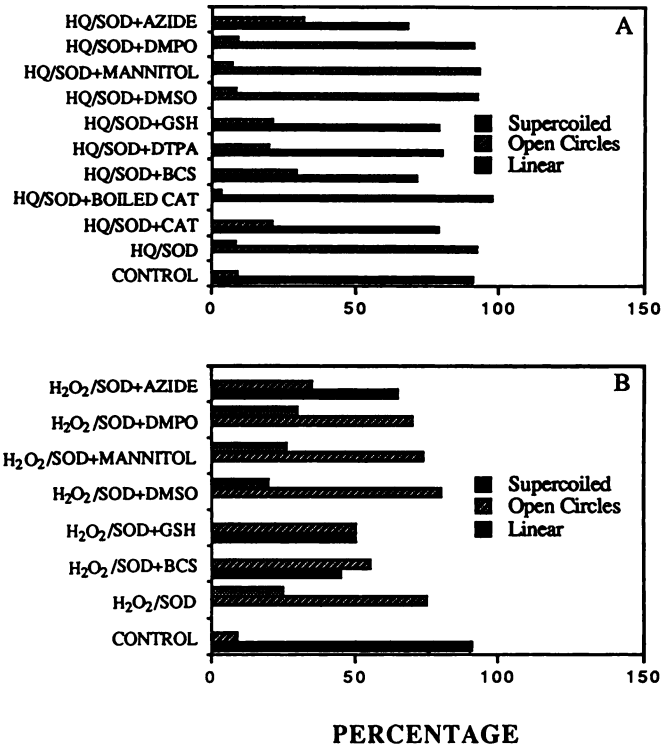

Fig. 6. Effects of metal chelators and reactive oxygen scavengers on the DNA strand breaks induced by HQ/Cu/Zn-SOD (A) or H_2O_2/Cu/Zn-SOD (B). DNA was incubated with 1000 units/ml Cu/Zn-SOD plus 1 mM HQ (A) or plus 1 mM H_2O_2 (B) in the absence or presence of 200 units/ml catalase, 50 μM BCS, 50 μM DTPA, 1 mM GSH, 0.5 M DMSO, 50 mM mannitol, 50 mM DMPO, or 50 mM sodium azide in PBS at 37° for 90 min. The percentage of each form of DNA was determined as described in Experimental Procedures. The sequence of the addition to DNA was inhibitors, Cu/Zn-SOD, and HQ or H_2O_2.

ESR signal in HQ-treated Cu/Zn-SOD could be prevented by catalase (Fig. 7D), suggesting that H_2O_2 derived from the Cu/Zn-SOD-accelerated oxidation of HQ is responsible for the oxidative damage to the copper site of Cu/Zn-SOD treated with HQ. Indeed, the Cu(II) ESR signal was also diminished in H_2O_2-treated Cu/Zn-SOD (data not shown). In data not shown, incubation of Cu/Zn-SOD with HQ also resulted in the loss of the catalytic activity of SOD as detected by the cytochrome *c* reduction assay using hypoxanthine and xanthine oxidase as a superoxide-generating system (7).

Formation of ROS from the Cu/Zn-SOD-mediated activation of HQ. DMPO/spin-trapping studies have demonstrated that the interaction of Cu/Zn-SOD with H_2O_2 leads to the formation of hydroxyl radicals (13, 14). Using POBN in conjunction with DMSO, we also previously observed that hydroxyl radical or its equivalent is formed from the copper-mediated oxidation of HQ (31). In this study, we used POBN as a spin trap in conjunction with DMSO to examine ROS formation from the Cu/Zn-SOD-mediated activation of HQ. As shown in Fig. 8, incubation of Cu/Zn-SOD with HQ in the presence of POBN plus DMSO resulted in the detection of a nitroxide with hyperfine splitting constants (a_N = 16.1 G, a_H = 2.6 G) consistent with those reported previously for the methyl adduct of POBN, POBN—CH_3 (34). In data not shown, incubation of Cu/Zn-SOD with H_2O_2 in the presence of POBN/DMSO also resulted in the formation of a POBN—CH_3 adduct. The ESR signal for the POBN—CH_3 adduct generated from H_2O_2/Cu/Zn-SOD was also stronger than that formed from HQ/Cu/Zn-SOD (data not shown). These observations suggest that the Cu/Zn-SOD-mediated activation of HQ generates hydroxyl radical or an equivalent reactive intermediate. However, Mn-SOD-accelerated oxidation of HQ resulted in a much weaker ESR signal for POBN—CH_3 adduct. The intensity of the ESR signal for the $SQ^{\cdot-}$ in the HQ/Mn-SOD system was similar to that in the HQ/Cu/Zn-SOD system (Fig. 8).

Inhibition by catalase, BCS, or GSH of the ROS formation from the Cu/Zn-SOD-mediated activation of HQ. As indicated in Fig. 6, both H_2O_2 and copper are critical for the induction of DNA strand breaks by the HQ/Cu/Zn-SOD. To further examine the role of copper and H_2O_2 in the formation of ROS, the effects of catalase, BCS, or GSH on the formation of ROS were determined. The presence of catalase extensively inhibited the formation of POBN—CH_3 adducts from the HQ/Cu/Zn-SOD system, whereas the formation of $SQ^{\cdot-}$ was enhanced by catalase (Fig. 9B). The presence of BCS also resulted in ~50% inhibition of the generation of the

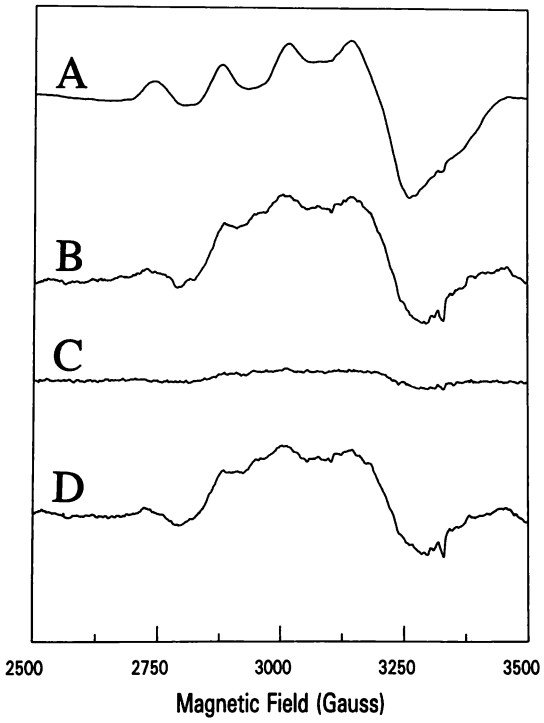

Fig. 7. ESR spectra at 77 K of Cu/Zn-SOD treated with or without HQ. Cu/Zn-SOD was incubated with 1 mM HQ in the absence or presence of catalase in PBS at 37° for 90 min and then dialyzed against deionized water at 4° for 24 hr before ESR detection. A, 12,500 units/ml Cu/Zn-SOD. B, 1000 units/ml Cu/Zn-SOD. C, 1000 units/ml Cu/Zn-SOD treated with 1 mM HQ. D, 1000 units/ml Cu/Zn-SOD treated with 1 mM HQ in the presence of 200 units/ml catalase. Spectra represent averaged ESR signals of 20 scans, with scales being 19 for A and 17 for B, C, and D, respectively. The spectra of untreated Cu/Zn-SOD were composed of two components, $g_{//}$ and $g_{\perp}$. The $g_{//}$ component has the hyperfine structure characteristic of Cu(II) with I = 3/2. Other ESR experimental conditions are described in details in Experimental Procedures.

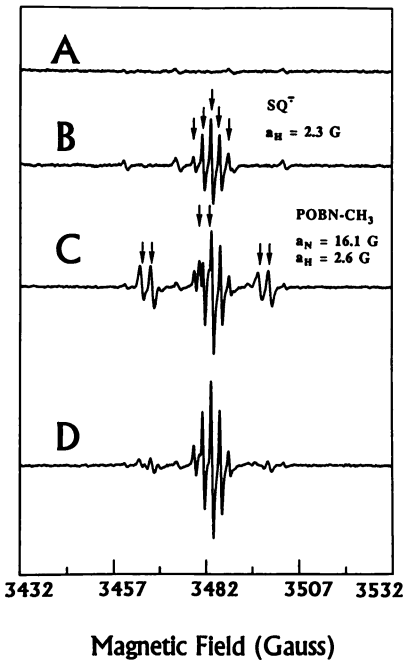

Fig. 8. Generation of POBN—CH_3 adduct from the reaction of HQ with SOD in the presence of POBN/DMSO. SOD was incubated with HQ in PBS at 37° for 60 min. After the addition of POBN and DMSO, the incubation was continued at 37° for another 30 min. ESR detection of the POBN—CH_3 adduct formation was carried out immediately after this 30-min incubation. A, 1000 units/ml Cu/Zn-SOD, 50 mM POBN, and 0.5 M DMSO. B, 1 mM HQ, 50 mM POBN, and 0.5 M DMSO. C, As in A, but with 1 mM HQ. D, As in B, but with 1000 units/ml Mn-SOD. The ESR spectra were repetitively acquired for 5 min under the instrument conditions as described in Experimental Procedures. Spectra represent averaged ESR signals of 10 scans of 30 sec, with receiver gain being 1×10^5.

POBN—CH_3 adduct without significantly affecting the formation of $SQ^{\cdot-}$ (Fig. 9C). Both $SQ^{\cdot-}$ and POBN—CH_3 formation was extensively blocked by GSH (Fig. 9D).

Combined effects of MPO and Cu/Zn-SOD on the oxidation of HQ to BQ. MPO/H_2O_2 can oxidize HQ to BQ, and as such has been suggested to be involved in the activation of HQ in bone marrow cells, which are relatively rich in MPO (28). In this study, because the Cu/Zn-SOD-accelerated oxidation of HQ generates H_2O_2, we also examined whether MPO can utilize this H_2O_2 to further induce activation of HQ to BQ. As shown in Fig. 10A, either Cu/Zn-SOD or MPO alone accelerated the autoxidation of HQ to BQ, whereas in the presence of both Cu/Zn-SOD and MPO, the oxidation of HQ to BQ was dramatically enhanced, with all of the HQ converted to BQ at 7 min after mixing HQ with SOD and MPO in PBS. The enhancement by MPO of the formation of BQ from the HQ/Cu/Zn-SOD system was abolished in the presence of catalase, indicating that H_2O_2 is needed by MPO to enhance the oxidation of HQ to BQ. The combined effects of Cu/Zn-SOD and MPO on the oxidation of HQ to BQ were synergistic (Fig. 10B). Previously, Sawada *et al.* (35) also reported that the rate of BQ formation from HQ plus horseradish peroxidase/H_2O_2 was increased in the presence of SOD.

Discussion

Benzene is a human leukemogen (36, 37). The majority of benzene metabolism occurs in liver, where cytochrome P-450 oxidizes benzene to phenol, catechol, HQ, 1,2,4-benzenetriol, and *trans,trans*-muconaldehyde (36, 37). After benzene exposure, these primary metabolites, particularly HQ and phenol, have been demonstrated to accumulate in the bone marrow, where their further metabolic activation and the subsequent interactions with the bone marrow cell populations result in cellular and molecular alterations leading to benzene-induced hematotoxicities (28, 36–39). Although the exact activation mechanism of HQ remains to be determined, it is generally considered that its oxidative activation to BQ and/or other reactive species in target tissues or cells is required for eliciting toxicity.

In Part I, we demonstrated that Cu/Zn-SOD is capable of accelerating the oxidation of HQ, producing $SQ^{\cdot-}$, BQ, and H_2O_2 (22). To assess whether the Cu/Zn-SOD-accelerated oxidation of HQ could contribute to cellular toxicity, we selected primary bone marrow stromal cells to test this possibility. Bone marrow stromal cells have been identified as target cells of benzene-derived metabolites, particularly HQ (40, 41). The addition of Cu/Zn-SOD (or Mn-SOD) to the cultures significantly enhanced HQ-induced cytotoxicity to stromal cells (Fig. 1), indicating that Cu/Zn-SOD could induce the activation of HQ in the culture system. The ineffectiveness of catalase on the Cu/Zn-SOD-enhanced HQ-induced

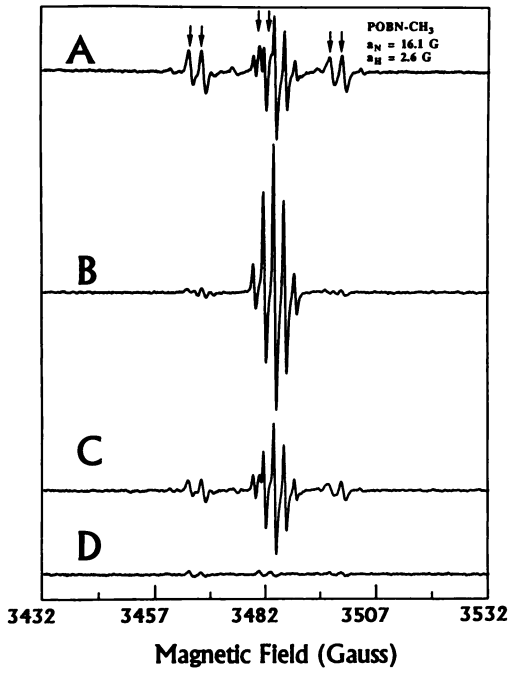

Fig. 9. Effects of catalase, BCS, or GSH on the formation of POBN—CH_3 adduct from the reaction of HQ with Cu/Zn-SOD in the presence of POBN/DMSO. Cu/Zn-SOD was incubated with HQ in PBS at 37° for 60 min. After the addition of POBN and DMSO, the incubation was continued at 37° for another 30 min. ESR detection of the POBN—CH_3 adduct formation was carried out immediately after this 30-min incubation. A, 1 mM HQ, 1000 units/ml Cu/Zn-SOD, 50 mM POBN, and 0.5 M DMSO. B, As in A, but with 200 units/ml catalase. C, As in A, but with 50 μM BCS. D, As in A, but with 1 mM GSH. The ESR spectra were repetitively acquired for 5 min under the instrument conditions as described in Experimental Procedures. Spectra represent averaged ERS signals of 10 scans of 30 sec, with receiver gain being 1×10^5.

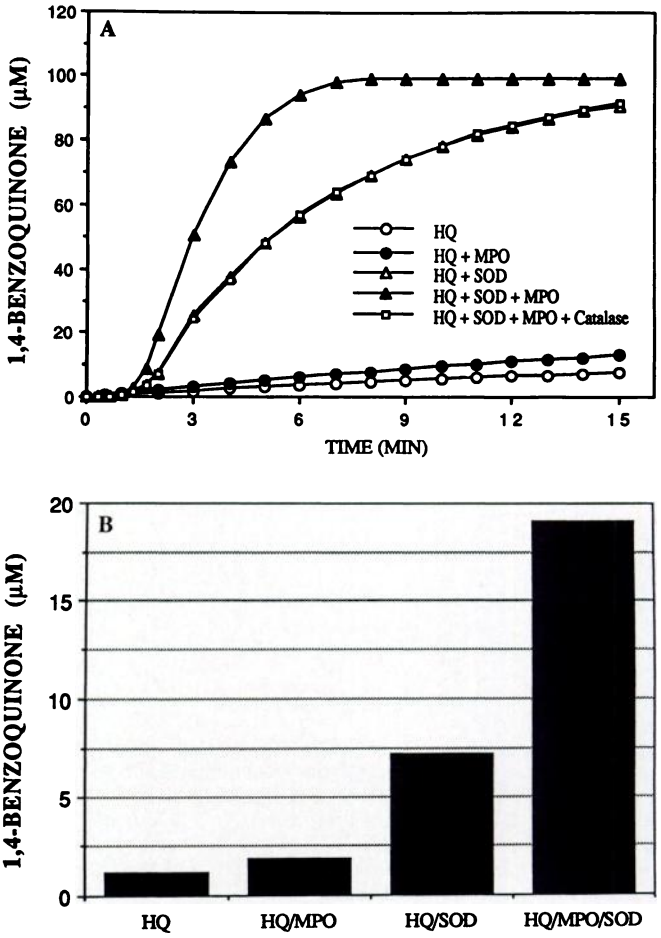

Fig. 10. Combined effects of Cu/Zn-SOD and MPO on the oxidation of HQ to BQ in PBS at 37°. A, the reactions were initiated by the addition of HQ (100 μM) to Cu/Zn-SOD (500 units/ml) or MPO (0.05 units/ml) or Cu/Zn-SOD plus MPO in the absence or presence of catalase (500 units/ml). B, As in A, but with the BQ formation measured at 2 min after the addition of HQ.

cytotoxicity suggests that H_2O_2 is not involved in the enhanced cytotoxicity and further implicates BQ as the reactive species that causes cytotoxicity. Indeed, the addition of BQ to DBA/2-derived stromal cells is highly cytotoxic (27). Although the interaction of BQ with GSH was reported to produce $SQ^{\cdot-}$ (42), we previously demonstrated that the presence of GSH or DTT completely blocks BQ-induced stromal cell injury (43). This observation suggests that $SQ^{\cdot-}$ does not contribute significantly to the stromal cytotoxicity observed under our experimental conditions. A complete protection against Cu/Zn-SOD-enhanced HQ-induced cytotoxicity was also observed in the presence of either GSH or DTT (Fig. 2). There are two possibilities for this protection: (i) GSH or DTT may form an adduct with the BQ produced, thereby protecting the cells from BQ-induced cytotoxicity, and (ii) GSH or DTT may block the Cu/Zn-SOD-accelerated oxidation of HQ to BQ. In data not shown, the consumption of O_2 during the Cu/Zn-SOD-accelerated oxidation of HQ could be inhibited by GSH or DTT in PBS.

Interaction of Cu/Zn-SOD and H_2O_2 was previously reported to be capable of causing inactivation of α_1-protease inhibitor in phosphate buffer (14). This study demonstrated that interaction of either HQ or H_2O_2 with Cu/Zn-SOD also induced DNA strand breaks in øX-174 plasmid. In contrast to Cu/Zn-SOD, Mn-SOD alone exhibited significant DNA cleaving activity (data not shown). It remains unknown why Mn-SOD causes DNA cleavage. It may be due to the endonuclease contamination in Mn-SOD isolated from *E. coli*. However, the interaction between Mn-SOD and either HQ or H_2O_2 did not result in significant potentiation of the DNA strand breaks (data not shown). In this regard, it has been have shown that incubation of Mn-SOD with H_2O_2 does not produce hydroxyl radicals or cause enzyme inactivation (13). Previously, we demonstrated that the activation of HQ by Cu(II) induces DNA strand breaks in øX-174 plasmid, with H_2O_2 and Cu(II)/Cu(I) redox being two critical components (30). Cu/Zn-SOD-accelerated oxidation of HQ generates nearly the same concentration of H_2O_2 as the initial HQ concentration used (22, 44). Inhibition by catalase of the HQ/Cu/Zn-SOD-induced DNA strand breaks indicates the involvement of H_2O_2 in the induction of DNA strand breaks. BCS is a Cu(I)-specific chelator that can strongly bind Cu(I), forming a stable complex (45). DNA strand breaks induced by HQ/Cu(II) in øX-174 plasmid have been shown to be completely inhibited by BCS (30). The inhibition by BCS of the HQ/Cu/Zn-SOD- or H_2O_2/Cu/Zn-SOD-induced DNA strand breaks suggests that Cu(I) is involved in the induction of DNA strand breaks by these two systems. BCS does not inhibit the Cu/Zn-SOD-accelerated oxidation of HQ (22), which further indicates that direct chelation by BCS of cop-

per released from Cu/Zn-SOD is responsible for its inhibitory effects on DNA strand breaks. The DNA strand breaks induced by the HQ/Cu/Zn-SOD could also be blocked by DTPA, which can chelate Cu(II), forming a stable redox-inert complex (30). Because GSH can chelate Cu(I), forming a stable GSH/Cu(I) complex (46), the protection by GSH against HQ/Cu/Zn-SOD- or H_2O_2/Cu/Zn-SOD-induced DNA strand breaks may occur as a result of blocking the interaction of Cu(I) with H_2O_2. Because the oxygen utilization resulting from the Cu/Zn-SOD-accelerated oxidation of HQ could be inhibited by GSH (data not shown), the protection by GSH on the HQ/Cu/Zn-SOD-induced DNA cleavage may also be due to its inhibition of the Cu/Zn-SOD-accelerated oxidation of HQ and the production of H_2O_2.

Cu(II) or H_2O_2 alone does not induce strand cleavage in isolated DNA (47, 48). The locally formed hydroxyl radical or its equivalent from the interaction of DNA base-bound copper with H_2O_2 has been suggested to participate in the site-specific DNA damage observed in the presence of both copper and H_2O_2 (47–49). To examine the involvement of ROS in the HQ/Cu/Zn-SOD- or H_2O_2/Cu/Zn-SOD-induced DNA strand breaks, the effects of several reactive oxygen scavengers were examined. DMSO, mannitol, and DMPO are generally regarded as hydroxyl radical scavengers, whereas azide is frequently viewed as a singlet oxygen quencher (14, 47, 50). The ineffectiveness of hydroxyl radical scavengers on both HQ/Cu/Zn-SOD- and H_2O_2/Cu/Zn-SOD-induced DNA cleavage (Fig. 6) suggests that free hydroxyl radicals, if generated, do not appear to be involved in the observed DNA strand breaks in either system. Results of our previous studies in the HQ/copper system (30, 31) and those of others in the H_2O_2/copper system (47, 51) suggested that copper first binds to DNA and then the DNA-bound copper reacts with HQ or H_2O_2, forming a bound hydroxyl radical or an equivalent oxidant, possibly a copper/oxygen complex that probably mediates the site-specific DNA cleavage. Because the copper is released from HQ- or H_2O_2-treated Cu/Zn-SOD, it may also bind to plasmid DNA, forming a DNA/copper complex. Through a similar mechanism as that described above, a DNA-bound oxidant may be formed and participate in the induction of the plasmid DNA strand breaks. Because the oxidant is formed on the specific sites of DNA, it may elude the protective effects of ROS scavengers, including DMSO even though such an oxidant may be capable of oxidizing DMSO to $CH_3^{\cdot}$ in the spin-trapping experiments (52). In contrast to the hydroxyl radical scavengers examined, azide extensively inhibited the DNA strand breaks induced by either HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD (Fig. 6). Azide has been previously demonstrated to protect Cu/Zn-SOD against inactivation by H_2O_2, possibly via its activity as an electron donor (12). As such, the inhibition by azide of the DNA cleavage induced by either HQ/Cu/Zn-SOD or H_2O_2/Cu/Zn-SOD may result from the prevention of the oxidative inactivation of the enzyme and the subsequent release of copper, as discussed later.

Because BCS or DTPA does not significantly affect the Cu/Zn-SOD-accelerated oxidation of HQ (22), it is unlikely that BCS or DTPA can access the copper-binding site of Cu/Zn-SOD. As such, the inhibition by BCS or DTPA of either HQ/Cu/Zn-SOD- or H_2O_2/Cu/Zn-SOD-induced DNA cleavage suggests that the release of copper from the Cu/Zn-SOD is a critical step in the induction of DNA strand breaks by either system. Low-temperature ESR experiments further confirm that copper is released from the Cu/Zn-SOD treated with either HQ (Fig. 7) or H_2O_2 (data not shown). To provide direct evidence for the formation of ROS from the Cu/Zn-SOD-mediated activation of HQ, POBN/spin-trapping technique was used in this study. Detection of POBN—CH_3 adduct from the HQ/Cu/Zn-SOD in the presence of POBN plus DMSO suggests the formation of hydroxyl radical or its equivalent. ESR signal for such a POBN—CH_3 adduct is much lower in the HQ/Mn-SOD system (Fig. 8D), indicating that formation of ROS is dependent on the type of SOD used. Interaction of H_2O_2 with Cu/Zn-SOD, but not with Mn-SOD, has also been shown to result in the formation of DMPO—OH adduct when using DMPO as a spin trap (13). The slight POBN—CH_3 signal formed by HQ/Mn-SOD may result from the interaction of possible transition metal contamination in the reaction buffer with H_2O_2 generated from the Mn-SOD-accelerated oxidation of HQ. Consistent with the inhibitory effects on the DNA strand breaks, catalase extensively inhibited the formation of POBN—CH_3 adducts (Fig. 9B). Previously, BCS has been demonstrated to completely inhibit POBN—CH_3 formation from the copper-mediated oxidation of HQ (31). In the HQ/Cu/Zn-SOD system, BCS inhibited the POBN—CH_3 formation by ~50% (Fig. 9C) but nearly completely prevented the induction of DNA strand breaks (Fig. 6). The partial inhibition of the POBN—CH_3 formation by BCS indicates that in the HQ/Cu/Zn-SOD system POBN—CH_3 may also be formed through the interaction of the copper-binding site of Cu/Zn-SOD with H_2O_2 or produced by the hydroxyl radicals diffused out from the copper-binding site of the Cu/Zn-SOD. The formation of DMPO—OH adduct from the interaction of Cu/Zn-SOD with H_2O_2 in the presence of DMPO has also been shown to be partially inhibited by DTPA (14). Taken together, the above results suggest that interaction of Cu/Zn-SOD with H_2O_2 derived from the SOD-accelerated oxidation of HQ causes copper release from the

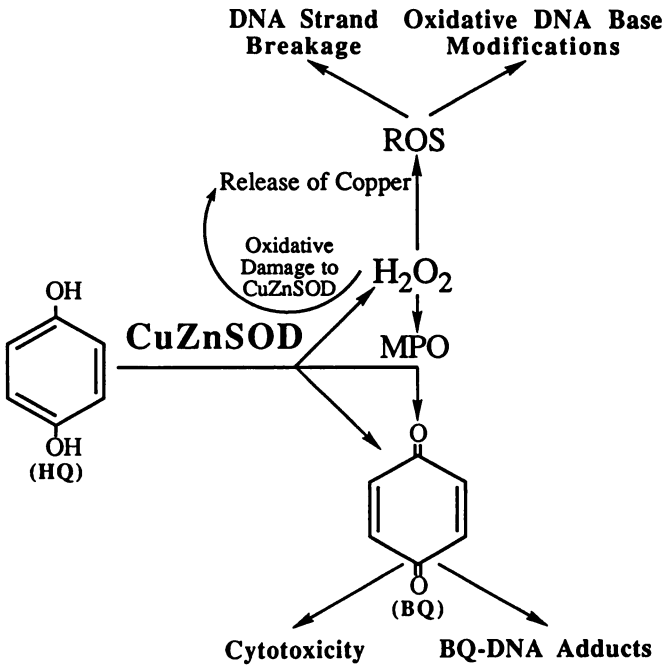

Fig. 11. Schematic of the Cu/Zn-SOD-mediated activation of HQ and the subsequent biological effects. BQ has been shown to form DNA adducts in either isolated DNA or cells (57).

oxidatively damaged enzyme. Although both the copper site in Cu/Zn-SOD and the free copper released from the oxidatively damaged Cu/Zn-SOD seem to be involved in the formation of ROS, the ROS generated from the reaction of the DNA-bound copper that was released from Cu/Zn-SOD with additional H_2O_2 appears to be the main reactive species that mediates the DNA strand breaks in øX-174 plasmid by the HQ/Cu/Zn-SOD.

Peroxidases, such as MPO and prostaglandin H synthetase, have been demonstrated to oxidize HQ in various *in vitro* systems (28). Because bone marrow cells are relatively rich in MPO, the MPO/H_2O_2 system has been implicated in the activation of HQ in bone marrow (28). The availability of H_2O_2 in a biological system will determine the ability of a peroxidase such as MPO to mediate the activation of HQ. Because the Cu/Zn-SOD-accelerated oxidation of HQ generates H_2O_2, it is possible that MPO further oxidizes HQ to BQ by utilizing the H_2O_2 generated by this reaction. Indeed, the addition of MPO to the mixture of HQ and Cu/Zn-SOD synergistically enhanced the rate of HQ oxidation to BQ (Fig. 10). This enhancement by MPO could be abolished by catalase, confirming that by utilizing the H_2O_2 generated from the HQ/Cu/Zn-SOD interaction, MPO can induce the further oxidative activation of HQ to BQ. Cu/Zn-SOD, a primary cytosolic enzyme, has been demonstrated to exist in high activity in bone marrow cells, including progenitor cells (53, 54). A recent study by Schattenberg *et al.* (55) has also shown that MPO mRNA is expressed in murine bone marrow progenitor cells and that both murine and human progenitor cells have marked peroxidase activity. Accordingly, the interaction between MPO and Cu/Zn-SOD may be relevant to the activation of HQ in bone marrow cells.

In summary, as depicted in Fig. 11, this study demonstrates that activation of HQ by either Cu/Zn-SOD or Mn-SOD causes cytotoxicity to bone marrow stromal cells through the formation of BQ. The Cu/Zn-SOD-accelerated oxidation of HQ resulted in the release of copper from the enzyme via the formation of H_2O_2. The released copper can bind to DNA, forming a DNA/copper complex (47, 56). The further interaction between the DNA-bound copper and H_2O_2 generated ROS that participated in the DNA strand breaks. Apart from its involvement in the formation of ROS and the inactivation of Cu/Zn-SOD, the H_2O_2 generated from the Cu/Zn-SOD-accelerated autoxidation of HQ can also be utilized by MPO to further mediate the activation of HQ to BQ. This synergistic interaction may be relevant to the activation of HQ in target cells, where both MPO and Cu/Zn-SOD are present. Although in the present study, we focused on the activation of the benzene-derived HQ by SOD, the acceleration by SOD of the autoxidation of other phenolic chemicals may also result in adverse biological effects.

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