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Role of Cu/Zn-Superoxide Dismutase in Xenobiotic Activation. I. Chemical Reactions Involved in the Cu/Zn-Superoxide Dismutase-Accelerated Oxidation of the Benzene Metabolite 1,4-Hydroquinone

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SUMMARY

Cu/Zn-superoxide dismutase (Cu/Zn-SOD) has been shown to modulate the autoxidation of a variety of phenolic compounds, including 1,4-hydroquinone (HQ), a benzene-derived metabolite. The acceleration of autoxidation of HQ by Cu/Zn-SOD results in the production of 1,4-benzoquinone (BQ). It has been proposed that the chemical mechanism involved in the Cu/Zn-SOD-catalyzed autoxidation of HQ may be occur through either its conventional activity as a superoxide:superoxide oxidoreductase or as a semiquinone:superoxide oxidoreductase. However, which of the above two mechanisms is responsible for the Cu/Zn-SOD-accelerated oxidation of HQ has not been resolved experimentally. In this study, with ESR spectroscopy we investigated further the chemical reactions involved in the SOD-accelerated oxidation of HQ. In phosphate-buffered saline (PBS), HQ underwent a slow autoxidation to BQ, which was accelerated by Cu/Zn-SOD, Mn-SOD, or Fe-SOD with a similar efficiency. In contrast, among free metals, only Cu(II) strongly mediated the oxidation of HQ to BQ. Mn(II) exhibited a slight capacity to oxidize HQ, whereas neither Fe(II) nor Fe(III) was capable of modulating the autoxidation of HQ. The presence of either form of SOD also dramatically enhanced the formation of semiquinone anion radicals ($SQ^{\cdot}$) from HQ. The SOD-accelerated oxidation of HQ was also accompanied by the generation of H_2O_2. In PBS containing bovine serum albumin (BSA) (PBS/BSA), HQ did not undergo autoxidation to $SQ^{\cdot}$, and as such the presence of SOD was unable to induce the formation of either $SQ^{\cdot}$ or BQ or the consumption of O_2. The addition of 10 μM BQ to HQ (100 or 1000 μM) in PBS/BSA resulted in the formation of $SQ^{\cdot}$ and initiated a slow rate of oxidation of HQ to BQ. In this case, the presence of Cu/Zn-SOD strongly accelerated the oxidation of HQ to $SQ^{\cdot}$ and BQ and the utilization of O_2. Furthermore, the enhancement by Cu/Zn-SOD of the generation of $SQ^{\cdot}$ or BQ from HQ in PBS/BSA was extensively inhibited under anaerobic conditions. The enhancement of $SQ^{\cdot}$ generation from HQ by all three forms of SOD does not support the possibility that Cu/Zn-SOD can oxidize $SQ^{\cdot}$ to BQ. Taken together, this study demonstrates that unlike free copper, Cu/Zn-SOD does not directly interact with HQ to cause its oxidation to BQ. Rather, the autoxidation of HQ to $SQ^{\cdot}$ is a prerequisite for the enhancing capacity of Cu/Zn-SOD, and the dismutation of superoxide anion radicals generated from the $SQ^{\cdot}$ in the presence of O_2 appears to be the underlying mechanism responsible for the enhancement by Cu/Zn-SOD of the oxidation of HQ.

Cu/Zn-SOD, a homodimeric metalloenzyme that catalyzes the dismutation of superoxide anion radicals to O_2 and H_2O_2 (1), has been demonstrated to play an important role in protecting aerobic cells against oxygen toxicity (2–4). Recently, Cu/Zn-SOD has been shown to be capable of accelerating the autoxidation of a number of phenolic chemicals, including 1,2-naphthohydroquinone, 5-hydroxyl-1,4-naphthohydroquinone, 3-glutathionyl-5-hydroxyl-1,4-naphthohydroquinone, 6-methyl-1,4-hydroquinone, and the benzene-derived HQ (5–7).

HQ can spontaneously oxidize to BQ through a semiquinone anion radical ($SQ^{\cdot}$) intermediate (8). A role for transition metals, such as copper, in the autoxidation of HQ has been described previously (9). In addition to transition metals, SOD was reported to be capable of stimulating the autoxidation or the horseradish peroxidase/H_2O_2-mediated oxidation of HQ (6, 10). The kinetics of the Cu/Zn-SOD-

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ABBREVIATIONS: Cu/Zn-SOD, Cu/Zn-superoxide dismutase; Mn-SOD, manganese-superoxide dismutase; Fe-SOD, iron-superoxide dismutase; HQ, 1,4-hydroquinone; BQ, 1,4-benzoquinone; $SQ^{\cdot}$, semiquinone anion radical; PBS, phosphate-buffered saline; BSA, bovine serum albumin; BCS, bathocuproinedisulfonic acid; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance.

accelerated oxidation of HQ has been further described by Eyer (7). It was observed that Cu/Zn-SOD dramatically enhanced the autoxidation rate of HQ by more than 2 orders of magnitude and that the stimulatory effect was first-order relative to the Cu/Zn-SOD concentration (7). Two potential mechanisms have been proposed to explain the capacity of Cu/Zn-SOD to accelerate the autoxidation of HQ to BQ. One involves the conventional action of Cu/Zn-SOD as a superoxide:superoxide oxidoreductase (6) and the other involves action through Cu/Zn-SOD functioning as a semiquinone:superoxide oxidoreductase (7), an activity of SOD originally proposed by Cadenas *et al.* (11). In the later mechanism, it was proposed that Cu/Zn-SOD was initially reduced by $SQ^{\cdot-}$ with BQ formation (7). In a subsequent step, the reduced enzyme could be reoxidized by a superoxide radical that is formed during autoxidation of the second $SQ^{\cdot-}$ (7). This later function of Cu/Zn-SOD may be plausible because of the high reduction potential of SOD-Cu^{+}/SOD-Cu^{2+} (0.26–0.41 V) and the considerable lower reduction potential of BQ/$SQ^{\cdot-}$ (0.78 mV) (7). Which of the above two actions of Cu/Zn-SOD is involved in the acceleration of the autoxidation of HQ to BQ has not, however, been resolved experimentally. Moreover, the formation of $SQ^{\cdot-}$ during the Cu/Zn-SOD-accelerated oxidation of HQ and the potential biological effects resulting from this enzyme/xenobiotic interaction have not been investigated. We have used ESR spectroscopy to further examine the reactions involved in the SOD-accelerated oxidation of HQ. Through direct measurement of the formation of $SQ^{\cdot-}$, this study provides strong evidence supporting the proposed role of Cu/Zn-SOD as a superoxide:superoxide oxidoreductase in accelerating the autoxidation of HQ to BQ. Biological effects resulting from this enzyme/xenobiotic interaction are described in Part II (12).

Experimental Procedures

Materials. Cu/Zn-SOD (4500 units/mg protein) from bovine erythrocytes, Mn-SOD from *Escherichia coli*, Fe-SOD from *E. coli*, HQ, BQ, catalase from bovine liver, BSA, ferrous sulfate, ferric chloride, BCS, and DTPA were purchased from Sigma Chemical Co. (St. Louis, MO). The unit of SOD activity used was that defined by McCord and Fridovich (1). Cupric sulfate and manganous chloride were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Dulbecco's PBS (10 times concentrated, pH 7.4) was obtained from GIBCO (Grand Island, NY) and diluted by 10-fold with deionized water before use.

Measurement of oxidation of HQ to BQ. HQ utilization and BQ generation were determined at wavelengths of 289 and 247 nm, respectively, in a UV spectrophotometer (Beckman, DU-7) on mixing HQ with SOD or other chemicals in PBS at 37°. The concentration of BQ was calculated according to a BQ (>99%) standard curve (9).

Measurement of O_2 consumption and H_2O_2 generation. O_2 consumption was monitored with a Clark oxygen electrode (YSI-53, Yellow Springs, OH) on mixing HQ with SOD or other chemicals in air-saturated PBS at 37°. H_2O_2 generation was indirectly determined by O_2 production on adding catalase (500 units/ml) to the reaction mixture of HQ and SOD (9).

ESR measurement of semiquinone anion radicals. ESR spectra were recorded at room temperature with a spectrometer (model ER 300, IBM-Bruker) operating at X-band with a TM 110 cavity and TM flat cell as described previously (13). 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 microwave counter (model 575, EIP Instruments, San Jose, CA) and an NMR gaussmeter (model ER 035M, Bruker Instruments, Billerica, MA), respectively. ESR data collections were performed, and the digital spectral data were transferred to a personal computer for analysis using software developed in the Electron Paramagnetic Resonance Center. Spectral simulations were performed on the personal computer and directly matched with experimental data to extract the spectral parameters (14).

Determination of the binding of HQ by BSA in PBS. HQ was incubated in PBS containing 1 mg/ml BSA at 37° for 30 min, and then the free HQ was separated from BSA with a Centricon concentrator (membrane molecular weight cutoff is 10,000) (Amicon, Beverly, MA). The HQ concentrations before and after filtration were determined as described above.

Results

Cu/Zn-SOD-accelerated oxidation of HQ to BQ, utilization of O_2, and generation of H_2O_2. Acceleration by Cu/Zn-SOD of the autoxidation of HQ has been described previously (6, 7). Eyer (7) focused on the chemical kinetics of the Cu/Zn-SOD-accelerated autoxidation of HQ to BQ. To set the stage for the ESR experiments, we measured the formation of BQ, the utilization of O_2, and the concomitant generation of H_2O_2 during the SOD-accelerated oxidation of HQ in PBS at 37°. As shown in Fig. 1A, HQ and BQ exhibit UV absorbance spectra with peak absorbancies at 289 and 247 nm, respectively. On mixing 100 μM HQ with 1000 units/ml Cu/Zn-SOD, the absorbance of HQ at 289 nm decreased with a corresponding increase in absorbance at 247 nm (Fig. 1B), indicating that the interaction of HQ with Cu/Zn-SOD in PBS results in increased conversion of HQ to BQ. As shown in Fig. 2A, HQ underwent autoxidation slowly to BQ in PBS, whereas in the presence of Cu/Zn-SOD, the oxidation of HQ to BQ was dramatically accelerated. The acceleration of oxidation of HQ by Cu/Zn-SOD was dependent on the concentration of SOD present. The concentrations of SOD used in this study are within the ranges found in various tissues (15) and are similar to those used in experiments by others (5–7). The Cu/Zn-SOD-accelerated oxidation of HQ was also accompanied by the utilization of O_2 (Fig. 2B) and the concomitant generation of H_2O_2 (Fig. 2C). An induction period for the acceleration of autoxidation of HQ by Cu/Zn-SOD was observed, being ~1.5 min after mixing HQ with Cu/Zn-SOD in either the generation of BQ or the utilization of O_2 (Fig. 2). This induction period was not significantly affected by varying the concentration of Cu/Zn-SOD present.

Comparison of the capacity of Cu/Zn-SOD, Mn-SOD, or Fe-SOD and Cu(II), Mn(II), Fe(II), or Fe(III) to enhance oxidation of HQ to BQ. Because the capacity of either Mn-SOD or Fe-SOD to accelerate the oxidation of HQ has not been studied, we next examined the effect of Mn-SOD or Fe-SOD on the autoxidation of HQ. As shown in Fig. 3A, all three forms of SOD exhibited a similar capacity to accelerate the oxidation of HQ to BQ. Previously, Cu(II) has been shown to strongly mediate the oxidation of HQ to BQ through the $SQ^{\cdot-}$ intermediate (9, 13). As shown in Fig. 3B, Mn(II) exhibited only a slight capacity to oxidize HQ to BQ, whereas neither Fe(II) nor Fe(III) enhanced the oxidation of HQ to BQ. In contrast to SOD, the Cu(II)- or Mn(II)-catalyzed oxidation of HQ to BQ did not exhibit any induction period.

Effects of copper chelators BCS and DTPA on Cu/Zn-SOD-accelerated oxidation of HQ. Because copper chela-

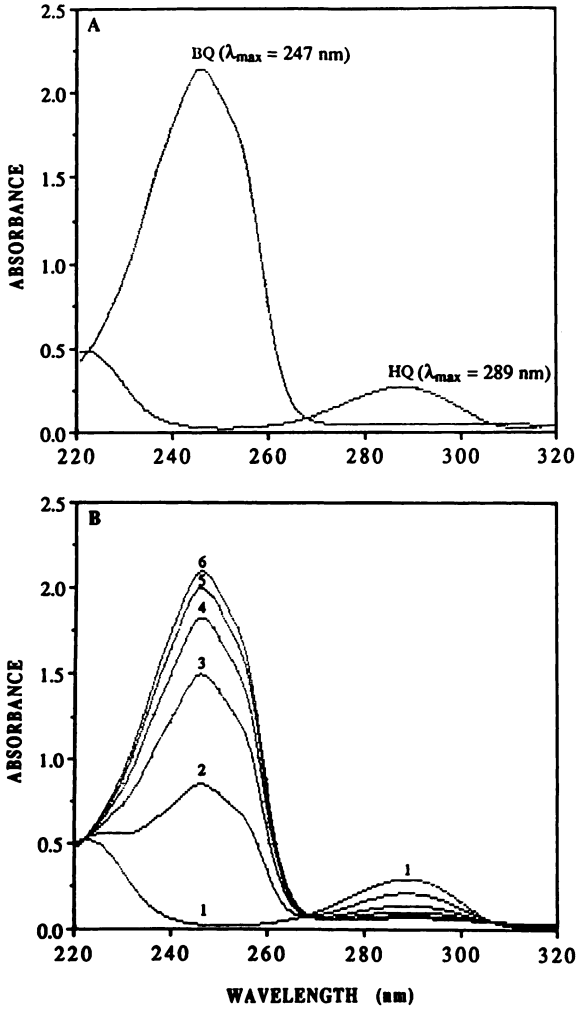

Fig. 1. A, Ultraviolet spectra of HQ (100 μM) and BQ (100 μM) in PBS. B, Formation of BQ during the Cu/Zn-SOD-accelerated oxidation of HQ in PBS at 37°. The reaction was initiated by mixing 100 μM HQ with 1000 units/ml Cu/Zn-SOD. Scans were recorded every 4 min. *Curve 1* was recorded within 5–10 sec after mixing HQ with Cu/Zn-SOD.

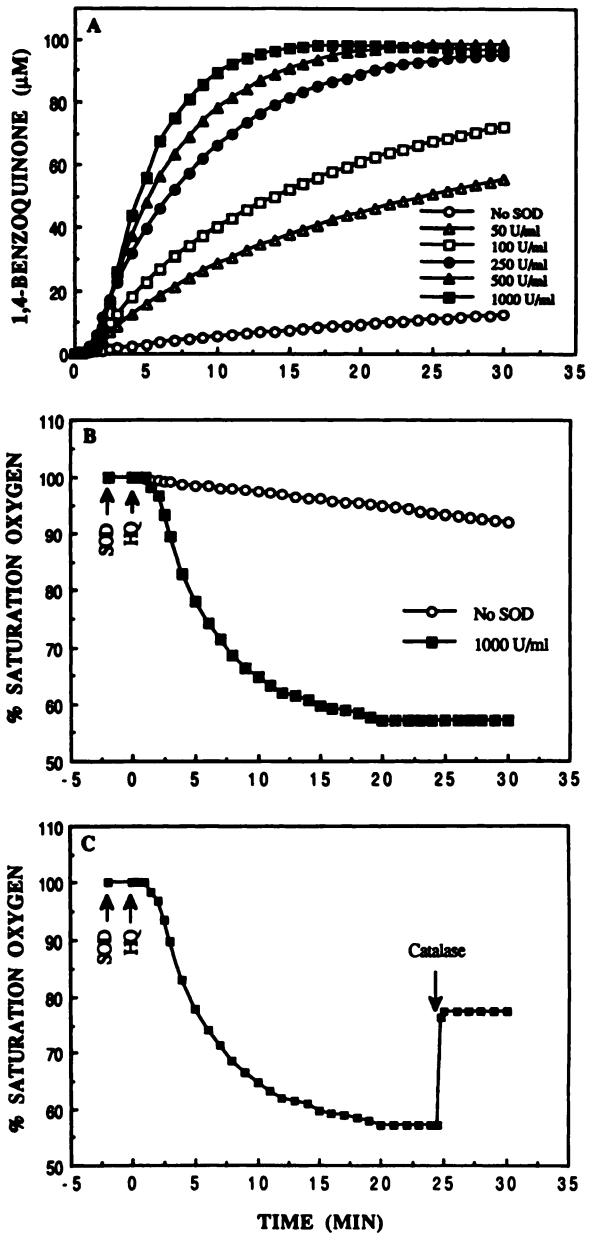

Fig. 2. BQ formation (A), O_2 consumption (B), and H_2O_2 generation (C) during the Cu/Zn-SOD-accelerated oxidation of HQ. The reactions were initiated by the addition of HQ to Cu/Zn-SOD in PBS at 37°. In A, B, and C, HQ concentration was 100 μM. In C, catalase (500 units/ml) was added to the mixture at the indicated time after incubation of HQ and Cu/Zn-SOD (1000 units/ml) in PBS at 37°.

tors BCS and DTPA have been demonstrated to strongly inhibit the Cu(II)-mediated oxidation of HQ (9, 16), we also examined whether these metal chelators could prevent Cu/Zn-SOD-accelerated oxidation of HQ. The autoxidation of HQ to BQ was inhibited by BCS and even more strongly by DTPA (Fig. 4). However, in contrast to its dramatic inhibition on Cu(II)-mediated oxidation of HQ (9), the presence of BCS did not significantly inhibit the Cu/Zn-SOD-accelerated oxidation of HQ to BQ (Fig. 4). Likewise, the Cu/Zn-SOD-accelerated oxidation of HQ to BQ was slowed by only ~3 min in the presence of DTPA. At 18 min after mixing HQ with Cu/Zn-SOD in the presence of DTPA, all of the HQ was converted to BQ (Fig. 4).

Enhancement by SOD of the generation of $SQ^{\cdot}$ from HQ. It was proposed that interaction of semiquinone radical with Cu/Zn-SOD could lead to the oxidation of semiquinone radical to quinone (7, 17). Using ESR spectroscopy, we also examined the effects of the three forms of SOD on the $SQ^{\cdot}$ generation from HQ. DTPA did not completely prevent the autoxidation of HQ to BQ (Fig. 4). Similarly, in PBS containing DTPA, 1 mM HQ underwent autoxidation, resulting in the formation of a 1:4:6:4:1 quintet ESR spectrum with a_H = 2.3 G (Fig. 5), which can be assigned to the $SQ^{\cdot}$ by reference to the splitting constant reported previously (18). The presence of Cu/Zn-SOD, Mn-SOD, or Fe-SOD dramatically enhanced the formation of $SQ^{\cdot}$. The efficiencies of all three forms of SOD in accelerating the oxidation of HQ to $SQ^{\cdot}$ were similar (Fig. 5). Inactivation of SOD by heating prevented such an enhancement of $SQ^{\cdot}$ generation from HQ in PBS containing DTPA (data not shown).

Autoxidation is required for the Cu/Zn-SOD-accelerated oxidation of HQ. In the absence of HQ autoxidation, Cu(II) has been demonstrated to directly cause the one-electron oxidation of HQ to $SQ^{\cdot}$ (13). Because the autoxidation of HQ in PBS could not be completely prevented by BCS or

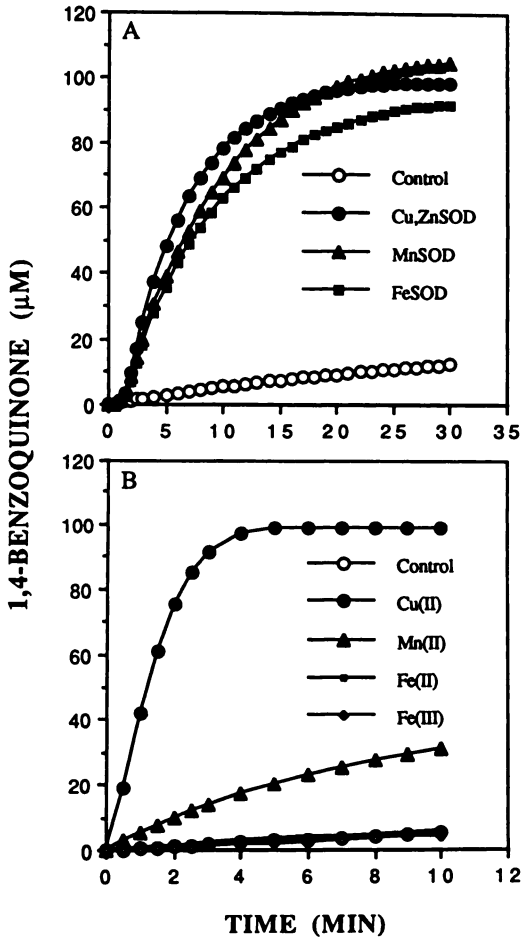

Fig. 3. A, BQ formation during Cu/Zn-SOD-, Mn-SOD-, or Fe-SOD-accelerated oxidation of HQ. B, BQ formation during Cu(II)-, Mn(II)-, Fe(II)-, or Fe(III)-catalyzed oxidation of HQ. The reactions were initiated by the addition of HQ (100 μM) to 1000 units/ml Cu/Zn-SOD, Mn-SOD, or Fe-SOD in PBS at 37° (A) or to 10 μM Cu(II), Mn(II), Fe(II), or Fe(III) in PBS at 37° (B).

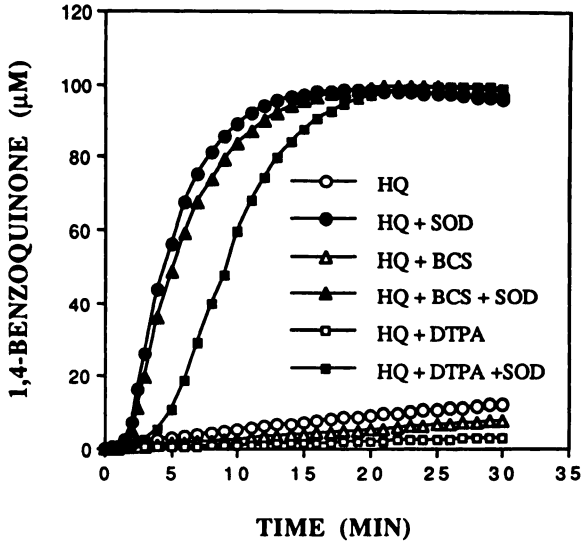

Fig. 4. Effects of BCS and DTPA on the Cu/Zn-SOD-accelerated oxidation of HQ. The reactions were initiated by the addition of HQ (100 μM) to 1000 units/ml Cu/Zn-SOD in the presence of 50 μM BCS or DTPA in PBS at 37°.

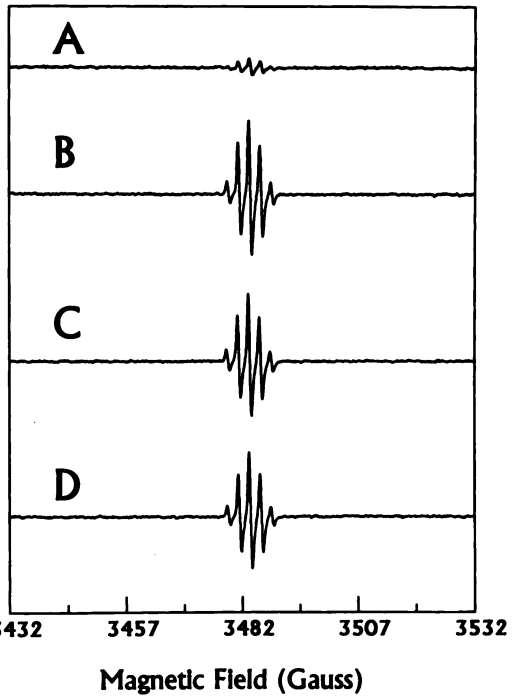

Fig. 5. Generation of $SQ^{\cdot}$ from HQ in the presence of various types of SOD. A, 1 mM HQ in PBS containing 50 μM DTPA. B, As in A, but with 1000 units/ml Cu/Zn-SOD. C, As in A, but with 1000 units/ml Mn-SOD. C, As in A, but with 1000 units/ml Fe-SOD. On adding HQ, the ESR spectra were repetitively acquired for 5 min under the instrument conditions described in Experimental Procedures. Spectra represent averaged ESR signals of 10 scans of 30 sec, with receiver gain being 1×10^5.

DTPA, the enhancement of oxidation of HQ by SOD observed in this study may be due to either the direct oxidation of HQ by the copper site in Cu/Zn-SOD or the simple acceleration of the autoxidation of HQ. To examine whether a direct interaction between Cu/Zn-SOD and HQ results in the oxidation of HQ to $SQ^{\cdot}$, PBS containing 1 mg/ml BSA was chosen as a reaction buffer because HQ does not undergo autoxidation in the PBS/BSA as determined by the consumption of HQ and the utilization of O_2 (13). Indeed, no $SQ^{\cdot}$ ESR signal was observed to be generated from the HQ in PBS/BSA (Fig. 6A). Furthermore, the addition of Cu/Zn-SOD to the HQ in PBS/BSA failed to initiate the generation of $SQ^{\cdot}$ (Fig. 6B), whereas the addition of Cu(II) could induce the formation of $SQ^{\cdot}$ from HQ in PBS/BSA (Fig. 6C). Also, incubation of 100 μM HQ in PBS/BSA at 37° for 30 min did not result in any change in the free HQ concentration (data not shown), indicating that BSA does not bind HQ. These results strongly suggest that the autoxidation of HQ is a prerequisite for SOD to mediate the enhancement of oxidation of HQ. Because BQ has been suggested to react with HQ, generating $SQ^{\cdot}$, and to initiate the autoxidation of HQ to BQ (7), 10 μM BQ was added to 1 mM HQ in PBS/BSA. As shown in Fig. 7, the addition of 10 μM BQ to 1 mM HQ in PBS/BSA resulted in the formation of $SQ^{\cdot}$. In this case, the presence of Cu/Zn-SOD strongly accelerated the further formation of $SQ^{\cdot}$. Furthermore, incubation of HQ with Cu/Zn-SOD in PBS/BSA for 30 min did not exhibit any autoxidation of HQ as determined by the absorbance changes at 289 and 247 nm and the utilization of O_2 (Fig. 8). However, the addition of 10 μM BQ to the HQ (100 μM) in the presence of Cu/Zn-SOD in PBS/BSA resulted in marked decrease in absorbance at 289 nm (Fig.

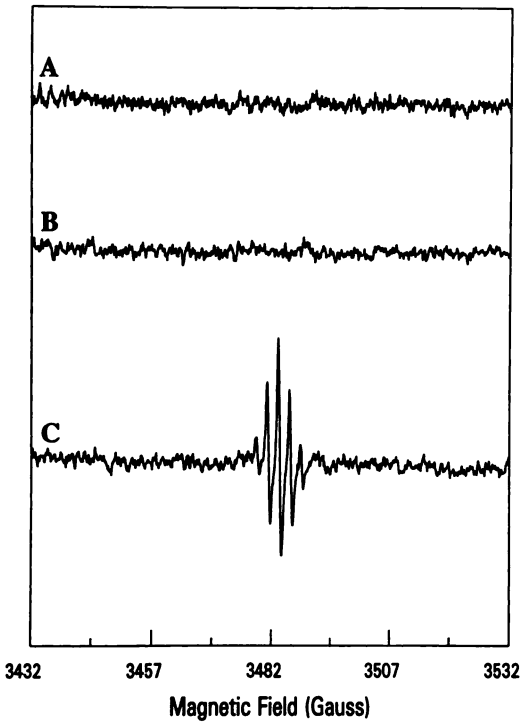

Fig. 6. Generation of $SQ^{\cdot-}$ from HQ in the presence of Cu/Zn-SOD or Cu(II) in PBS containing BSA. A, 1 mM HQ in PBS containing 1 mg/ml BSA. B, As in A, but with 1000 units/ml Cu/Zn-SOD. C, As in A, but with 10 μM Cu(II). On adding HQ, the ESR spectra were repetitively acquired for 5 min under the instrument conditions described in Experimental Procedures. Spectra represent averaged ESR signals of 10 scans of 30 sec, with receiver gain being 2×10^5.

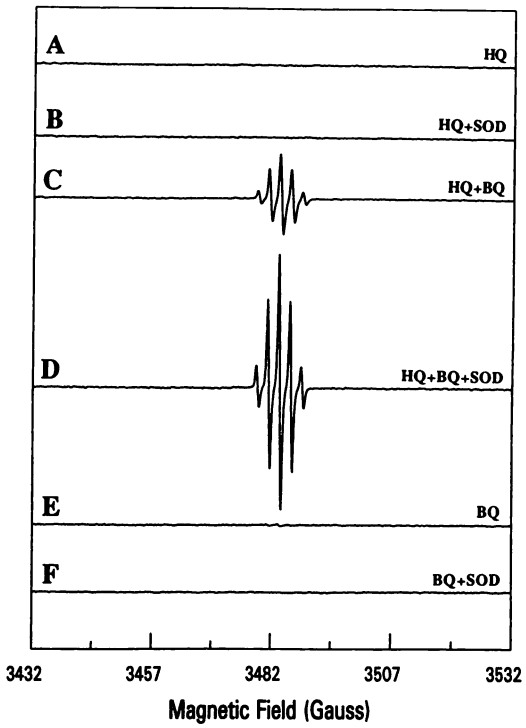

Fig. 7. Cu/Zn-SOD-accelerated BQ-initiated autoxidation of HQ in PBS containing BSA. A, 1 mM HQ in PBS containing 1 mg/ml BSA. B, As in A, but with 1000 units/ml Cu/Zn-SOD. C, As in A, but with 10 μM BQ. D, As in C, but with 1000 units/ml Cu/Zn-SOD. E, 10 μM BQ in PBS containing 1 mg/ml BSA. F, As in E, but with 1000 units/ml Cu/Zn-SOD. On adding HQ (A, B, C, and D) or BQ (E and F), the ESR spectra were repetitively acquired for 5 min under the instrument conditions described in Experimental Procedures. Spectra represent averaged ESR signals of 10 scans of 30 sec with receiver gain being 5×10^4.

8A) and of utilization of O_2 (Fig. 8C) and in an increase in absorbance at 247 nm (Fig. 8B). As shown in Fig. 8 (A and D), the addition of 10 μM BQ to HQ in PBS/BSA initiated a slow rate of decrease in absorbance at 289 nm and a corresponding increase in absorbance at 247 nm, indicating a slow rate of oxidation of HQ to BQ. This slow rate of autoxidation initiated by the addition of BQ appears to be a prerequisite for the Cu/Zn-SOD-mediated enhancement of oxidation of HQ in PBS/BSA.

Inhibition of Cu/Zn-SOD-accelerated oxidation of HQ to BQ under anaerobic conditions. To assess the role of O_2 in the Cu/Zn-SOD-accelerated oxidation of HQ in PBS/BSA plus 10 μM BQ, we next examined the capacity of Cu/Zn-SOD to mediate the enhancement of oxidation of HQ to BQ in PBS/BSA plus 10 μM BQ system under anaerobic conditions. As shown in Fig. 9, mixing 10 μM BQ with 100 μM HQ in the presence of Cu/Zn-SOD in PBS/BSA initiated a dramatic increase in the absorbance at 247 nm, whereas flushing the reaction buffer with N_2 before adding BQ completely prevented the increase in absorbance at 247 nm for the first 7 min after adding BQ. A longer incubation resulted in a gradual increase in absorbance at 247 nm, which may be due to the leaking of air into the reaction buffer.

Discussion

At physiological pH, HQ undergoes autoxidation to BQ in phosphate buffer or cell culture medium. Previously, Cu/Zn-SOD has been shown to accelerate HQ autoxidation to BQ in phosphate buffer (6, 7). Two possible mechanisms have been proposed to explain the capacity of Cu/Zn-SOD to accelerate the oxidation of HQ to BQ. One involves the dismutation of superoxide derived from $SQ^{\cdot-}$ by Cu/Zn-SOD (reaction 3), resulting in a shift of the reaction equilibrium to the right (reactions 1 and 2).

$$HQ + O_2 \rightleftharpoons SQ^{\cdot-} + O_2^{\cdot-} + 2H^+ \quad (1)$$

$$SQ^{\cdot-} + O_2 \underset{k}{\rightleftharpoons} BQ + O_2^{\cdot-} \quad (2)$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{Cu/ZnSOD}} H_2O_2 + O_2 \quad (3)$$

Particularly in eq. 2, in view of the rate constant k being $10^9\ M^{-1}\ sec^{-1}$, Sawada *et al.* (10) proposed that the reaction of $SQ^{\cdot-}$ with O_2 was not appreciable only because its back reaction is much faster. However, the presence of SOD may eliminate the back reaction, thereby stimulating the oxidation of HQ, producing BQ and H_2O_2 (6, 10). The other proposed mechanism is that Cu/Zn-SOD catalyzes the oxidation of $SQ^{\cdot-}$ by superoxide through the following reactions (reactions 4–6).

$$HQ + O_2 \rightleftharpoons SQ^{\cdot-} + O_2^{\cdot-} + 2H^+ \quad (4)$$

$$SOD\text{-}Cu^{++} + SQ^{\cdot-} \rightarrow SOD\text{-}Cu^{+} + BQ \quad (5)$$

$$SOD\text{-}Cu^{+} + O_2^{\cdot-} + 2H^+ \rightarrow SOD\text{-}Cu^{++} + H_2O_2 \quad (6)$$

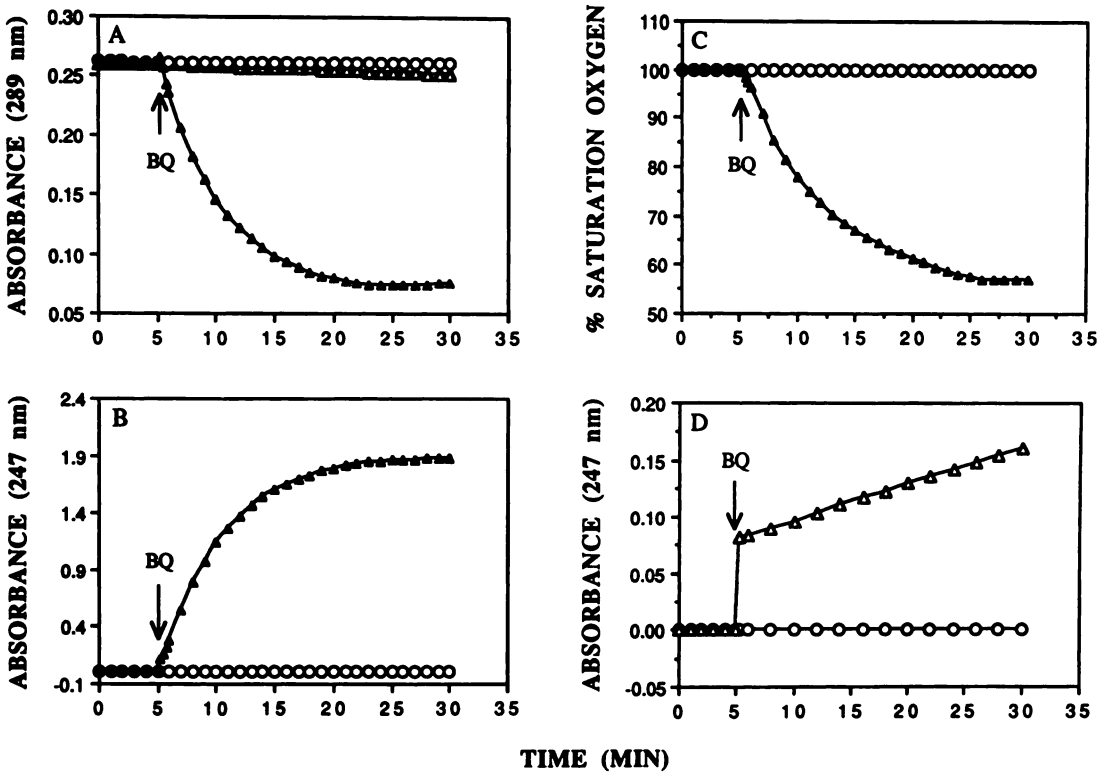

Fig. 8. A, HQ consumption during the Cu/Zn-SOD-accelerated BQ-initiated autoxidation of HQ. The incubation mixtures contained 100 μM HQ in the presence (○ and ▲) or absence (△) of 1000 units/ml Cu/Zn-SOD, with 10 μM BQ added at the indicated time (△ and ▲). B, BQ formation during the Cu/Zn-SOD-accelerated BQ-initiated autoxidation of HQ. The incubation mixtures contained 100 μM HQ in the presence (▲) or absence (○) of 1000 units/ml Cu/Zn-SOD, with 10 μM BQ added at the indicated time (▲). C, Oxygen utilization during the Cu/Zn-SOD-accelerated BQ-initiated autoxidation of HQ. The incubation mixtures contained 100 μM HQ in the presence (▲) or absence (○) of 1000 units/ml Cu/Zn-SOD, with 10 μM BQ added at the indicated time (▲). D, Additional BQ formation during BQ-initiated autoxidation of HQ. The incubation mixtures contained 100 μM HQ (○ and △), with 10 μM BQ added at the indicated time (△). All reactions in A–D were carried out in PBS containing 1 mg/ml BSA at 37°.

Another possibility is that HQ may directly react with the catalytic site of Cu/Zn-SOD, resulting in the formation of BQ and H_2O_2 (reactions 7–9).

$$\text{SOD-Cu}^{++} + \text{HQ} \rightleftharpoons \text{SOD-Cu}^{+} + \text{SQ}^{\cdot-} + 2\text{H}^{+} \quad (7)$$

$$\text{SQ}^{\cdot-} + \text{O}_2 \rightleftharpoons \text{BQ} + \text{O}_2^{\cdot-} \quad (8)$$

$$\text{SOD-Cu}^{+} + \text{O}_2^{\cdot-} + 2\text{H}^{+} \rightarrow \text{SOD-Cu}^{++} + \text{H}_2\text{O}_2 \quad (9)$$

However, past studies have not resolved which of these activities of Cu/Zn-SOD is involved in the oxidation of HQ to BQ (7).

This study demonstrates with the use of ESR spectroscopy that Cu/Zn-SOD strongly accelerates the oxidation of HQ, producing $SQ^{\cdot-}$ and, ultimately, BQ. This oxidation process is also accompanied by the enhanced utilization of O_2 and the concomitant generation of H_2O_2 (Fig. 2). The acceleration of the oxidation of HQ by Cu/Zn-SOD exhibited an induction period, being ~1.5 min after mixing HQ with Cu/Zn-SOD in both the generation of BQ and the utilization of O_2 (Fig. 2). This induction period was not significantly affected by varying the concentration of SOD, which is consistent with a previous observation (7). The ineffectiveness of increasing Cu/Zn-SOD concentration on reducing the induction period of both BQ generation and O_2 utilization does not support the possibility that Cu/Zn-SOD could directly react with $SQ^{\cdot-}$, causing its oxidation to BQ. If dismutation of superoxide is the underlying mechanism for Cu/Zn-SOD-accelerated oxidation of HQ, two other forms of SOD, i.e., Mn-SOD and Fe-SOD, should also be capable of enhancing the oxidation of HQ. Indeed, all three forms of SOD exhibited a similar capacity to accelerate oxidation of HQ to BQ, whereas the corresponding metal ions showed a dramatic difference in terms of the capacity to induce the oxidation of HQ to BQ (Fig. 3). SOD may be grouped into two families whose structural and evolutionary independence is shown by their unrelated amino acid sequences. Mn-SOD and Fe-SOD share a similar α/β fold and are structurally unrelated to the Greek key β-barrel fold of Cu/Zn-SOD (19, 20). These families of SOD catalyze the dismutation of superoxide with essentially identical efficiencies, as would be expected if they were selected on the basis of this activity. The equal efficiencies of acceleration by the three forms of SOD of oxidation of HQ to BQ (Fig. 3A) and $SQ^{\cdot-}$ (Fig. 5) do not appear to support a direct interaction between the copper site of Cu/Zn-SOD and $SQ^{\cdot-}$ or HQ. Similarly, both Cu/Zn-SOD and Mn-SOD have been shown to exert indistinguishable effects on the autoxidation of 1,4-naphthohydroquinone (21).

Previous studies have implicated that trace copper contamination in PBS is responsible for the autoxidation of HQ in PBS (9). To further examine whether transition metal contamination in PBS affects the SOD-mediated oxidation of HQ, we used BCS and DTPA, two potent copper chelators. Previously, these two metal chelators have been shown to prevent Cu(II)-mediated oxidation of HQ to BQ (9, 16). How-

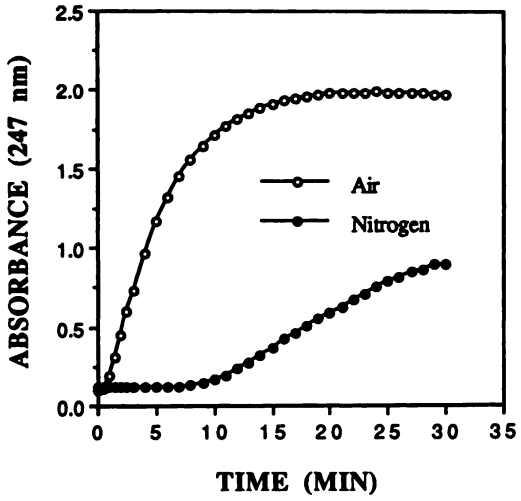

Fig. 9. Inhibition of the Cu/Zn-SOD-accelerated BQ-initiated autoxidation of HQ in PBS containing 1 mg/ml BSA at 37° under anaerobic conditions. The incubation mixtures contained 100 μM HQ, 1000 units/ml Cu/Zn-SOD, and 10 μM BQ under aerobic (○) and anaerobic (●) conditions. For creating anaerobic conditions, reaction buffers were flushed thoroughly with N_2 before the reactions were initiated. The reactions were initiated by the addition of 10 μM BQ at time 0.

ever, the Cu/Zn-SOD-accelerated oxidation of HQ to BQ could not be inhibited by BCS and could be slowed by only ~3 min in the presence of DTPA (Fig. 4). In contrast to free copper-mediated oxidation of HQ, the enhancement of HQ oxidation by Cu/Zn-SOD cannot be blocked by metal chelators. Although trace copper contamination has been implicated in the autoxidation of HQ in PBS, the presence of DTPA cannot completely prevent the autoxidation of HQ to BQ in PBS (Fig. 4). Indeed, HQ underwent autoxidation, generating $SQ^{\cdot-}$ in PBS in the presence of DTPA (Fig. 5). The distinct effects of BCS and DTPA on the Cu(II)-mediated oxidation of HQ and the Cu/Zn-SOD-accelerated oxidation of HQ are consistent with the concept that free copper and Cu/Zn-SOD catalyze oxidation of HQ via different mechanisms.

It was determined that a number of semiquinone radicals could reduce the copper of Cu/Zn-SOD, with rate constants varying from 1×10^6 to 2.5×10^8 M^{-1} sec^{-1} (17). Such a reaction could also be responsible for the capacity of Cu/Zn-SOD to accelerate the oxidation of HQ to BQ. If this were true, the formation of $SQ^{\cdot-}$ from HQ would be diminished in the presence of Cu/Zn-SOD. However, the presence of either Cu/Zn-SOD, Mn-SOD, or Fe-SOD dramatically enhanced the formation of $SQ^{\cdot-}$ from HQ (Fig. 5). Again, the efficiencies of all three forms of SOD in accelerating the oxidation of HQ to $SQ^{\cdot-}$ were similar. This observation strongly suggests that Cu/Zn-SOD is not capable of catalyzing the oxidation of $SQ^{\cdot-}$ by superoxide, forming BQ. Our ESR data provide direct evidence against the possible role of SOD as a semiquinone: superoxide oxidoreductase in accelerating the oxidation of HQ to BQ.

As mentioned above, the autoxidation of HQ in PBS to $SQ^{\cdot-}$ and BQ cannot be completely prevented by metal chelators BCS and DTPA (9, 13; Fig. 4). Thus, it was interesting to determine whether the autoxidation of HQ was a prerequisite for SOD to enhance the oxidation of HQ. We previously observed that in the presence of BSA, HQ does not undergo autoxidation in PBS, as determined by the consumption of HQ and the utilization of O_2 (13). In addition, no $SQ^{\cdot-}$ ESR signal was observed from the HQ in PBS/BSA (Fig. 6A). In the absence of initial $SQ^{\cdot-}$, the presence of Cu/Zn-SOD did not induce the formation of $SQ^{\cdot-}$ (Fig. 6B). In data not shown, incubation of HQ in PBS/BSA for 30 min did not result in any change in the free HQ concentration, indicating that BSA is not capable of binding HQ in PBS. These results suggest that Cu/Zn-SOD cannot directly reduce HQ to $SQ^{\cdot-}$ and further implicate that autoxidation of HQ is needed for the enhancing effect of SOD on oxidation of HQ to BQ. In contrast, free Cu(II) can directly oxidize HQ to $SQ^{\cdot-}$ in PBS/BSA (Fig. 6C). To further verify that autoxidation of HQ to $SQ^{\cdot-}$ is a prerequisite for the action of Cu/Zn-SOD, 10 μM BQ was added to 1 mM HQ in PBS/BSA to induce the formation of $SQ^{\cdot-}$ and initiate a slow rate of autoxidation of HQ. Previously, it has been proposed that reaction of HQ with BQ generates $SQ^{\cdot-}$ (22). In this study, with the use of ESR spectroscopy, we directly observed that adding BQ to HQ in PBS/BSA resulted in the formation of $SQ^{\cdot-}$ (Fig. 7). This interaction also initiated a slow rate of HQ consumption and BQ formation (Fig. 8). Under this condition, the presence of Cu/Zn-SOD strongly enhanced the formation of $SQ^{\cdot-}$, consumption of HQ, utilization of O_2, and generation of BQ (Figs. 7 and 8). These observations were additional proof that the autoxidation of HQ to $SQ^{\cdot-}$ is required for Cu/Zn-SOD to further accelerate the oxidation of HQ to BQ. The acceleration of the oxidation of HQ to BQ by Cu/Zn-SOD could be extensively inhibited under anaerobic conditions (Fig. 9). This further strengthens the theory that dismutation of superoxide derived from $SQ^{\cdot-}$ in the presence of O_2 appears to be the underlying mechanism of Cu/Zn-SOD-accelerated oxidation of HQ to BQ.

In summary, this study demonstrates that unlike free copper, Cu/Zn-SOD does not directly interact with HQ to cause its oxidation to BQ; a slow autoxidation of HQ to $SQ^{\cdot-}$ is a prerequisite for the action of SOD to accelerate the oxidation of HQ to $SQ^{\cdot-}$ and BQ and the formation of H_2O_2; and enhancement of the $SQ^{\cdot-}$ formation from HQ by all three forms of SOD does not support a direct interaction between the copper site of Cu/Zn-SOD and the $SQ^{\cdot-}$ leading to its oxidation to BQ. As such, dismutation of superoxide anion radicals generated from $SQ^{\cdot-}$ under aerobic conditions by SOD appears to be the underlying mechanism involved in the enhancing effect by SOD on the oxidation of HQ.

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