We examined the effect of bicarbonate on the peroxidase activity of copper-zinc superoxide dismutase (SOD1), using the nitrite anion as a peroxidase probe. Oxidation of nitrite by the enzyme-bound oxidant results in the formation of the nitrogen dioxide radical, which was measured by monitoring 5-nitro-γ-tocopherol formation. Results indicate that the presence of bicarbonate is not required for the peroxidase activity of SOD1, as monitored by the SOD1/H_{2}O_{2}-mediated nitration of γ-tocopherol in the presence of nitrite. However, bicarbonate enhanced SOD1/H_{2}O_{2}-dependent oxidation of tocopherols in the presence and absence of nitrite and dramatically enhanced SOD1/H_{2}O_{2}-mediated oxidation of unsaturated lipid in the presence of nitrite. These results, coupled with the finding that bicarbonate protects against inactivation of SOD1 by H_{2}O_{2}, suggest that SOD1/H_{2}O_{2} oxidizes the bicarbonate anion to the carbonate radical anion. Thus, the amplification of peroxidase activity of SOD1/H_{2}O_{2} by bicarbonate is attributed to the intermediary role of the diffusible oxidant, the carbonate radical anion. We conclude that, contrary to a previous report (Sankarapandi, S., and Zweier, J. L. (1999) J. Biol. Chem. 274, 1226–1232), bicarbonate is not required for peroxidase activity mediated by SOD1 and H_{2}O_{2}. However, bicarbonate enhanced the peroxidase activity of SOD1 via formation of a putative carbonate radical anion. Biological implications of the carbonate radical anion in free radical biology are discussed.

Recently, it was reported that the bicarbonate anion (HCO_{3}^{-}) is required for peroxidase activity and the peroxidase function of copper-zinc superoxide dismutase (SOD1) (1). The peroxidase activity of SOD1 was determined using the electron spin resonance (ESR) technique to monitor the oxidation of spin trap 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) to the DMPO-hydroxyl radical adduct (DMPO-OH). Additional evidence for enhanced peroxidase activity was obtained by measuring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) to ABTS^•• radical cation (1). The investigators concluded that the bicarbonate anion, anchored to arginine 141 at the active site of SOD1, facilitated the redox cleavage of H_{2}O_{2} that led to enhanced peroxidase activity (1). In the present study, this interpretation is challenged, and an alternate mechanism for HCO_{3}^{-}-mediated increase in SOD1 peroxidase activity is provided.

Nearly 25 years ago, Hodgson and Fridovich demonstrated that the “copper-bound hydroxyl radical,” SOD-Cu^{2+}-OH, which is generated in the reaction between SOD1 and H_{2}O_{2}, oxidizes several anionic ligands including formate, azide, and nitrite anions (2, 3). These small molecular weight anionic ligands are presumed to be oxidized by the oxidant formed at the active site of SOD1. The oxidizing potential of this putative SOD-Cu^{2+}-OH species is similar to that of the “free” hydroxyl radical, which is considerably higher than those associated with conventional peroxidases (4). The one-electron oxidation potential for the HCO_{3}^{-}/carbonate radical anion (CO_{3}^{2-}) couple is +1.59 V (5, 6), which makes oxidation of HCO_{3}^{-} by SOD-Cu^{2+}-OH to CO_{5}^{2-}-OH thermodynamically feasible (7, 8). CO_{5}^{2-}, although less reactive than the hydroxyl radical, is a more selective oxidant that may diffuse over a longer distance, thereby causing oxidative damage to distant biological targets (9, 10).

We measured the peroxidase activity of SOD1 under anaerobic conditions by using the nitrite anion (NO_{2}^{-}) as a peroxidase substrate (4). The oxidation of NO_{2}^{-} to the nitrogen dioxide free radical (NO_{2}^{•}) was measured by monitoring the formation of α-tocopheryl quinone (α-TQ) and 5-nitro-γ-tocopherol (NGTH) (4, 11, 14). Our results clearly demonstrate that the bicarbonate anion is not required for the peroxidase activity of SOD1; however, bicarbonate enhances oxidation of α- and γ-tocopherols in the presence and absence of the nitrite anion. In addition, bicarbonate provided protection against H_{2}O_{2}-dependent inactivation of SOD1. Taken together, these data suggest that SOD1 and H_{2}O_{2} are able to oxidize HCO_{3}^{-} to CO_{5}^{2-}, a more selective oxidant, that leads to amplification of the peroxidase activity of SOD1. Biological implications of the oxidation of HCO_{3}^{-}, a ubiquitous cellular and plasma component, in free radical biology are discussed.

**EXPERIMENTAL PROCEDURES**

SOD1 (bovine) was purchased from Roche Molecular Biochemicals. 1,2-Dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC) was purchased from Avanti Polar Lipids (Alabaster, AL). α-Tocopherol (α-TH), γ-tocopherol (γ-TH), and H_{2}O_{2} were obtained from Sigma.

**Synthesis of Oxidation Products—α-TQ** was synthesized as previously reported (11, 15). NGTH was synthesized by incubating γ-TH (2

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**ROLE OF CARBONATE ANION RADICAL**

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Printed in U.S.A.

EXPERIMENTAL PROCEDURES

SOD (bovine) was purchased from Roche Molecular Biochemicals. 1,2-Dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC) was purchased from Avanti Polar Lipids (Alabaster, AL). α-Tocopherol (α-TH), γ-tocopherol (γ-TH), and H_{2}O_{2} were obtained from Sigma.

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**Received for publication, June 18, 1999, and in revised form, July 20, 1999**
Bicarbonate and Peroxidase Activity

**Fig. 1.** HPLC analysis of α-TH, γ-TH, and their oxidation and nitration products. α-TH, γ-TH, NGTH, and α-TQ were incorporated into large unilamellar liposomes and analyzed by HPLC. Typical HPLC traces show the result of the retention times of authentic standards (20 μM each) of NGTH (A), γ-TH (B), α-TQ (C), and α-TH (D).

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**Results**

**HPLC Analysis of α-TH, γ-TH, and Their Oxidation and Nitration Products**—α-TH and γ-TH were used as probes to monitor the peroxidase activity of SOD1 in the presence of H₂O₂ (4). In order to limit CO₂ contamination, all solutions were degassed and stored in a nitrogen glove box. After a minimum of 48 h, experiments were performed in the nitrogen glove box. A buffer concentration of 200 mM phosphate was used to prevent changes in pH after the addition of bicarbonate solutions. The pH was determined both before and after the termination of all reactions to ensure that the bicarbonate effect was not due to pH changes.

Fig. 1 shows typical HPLC traces obtained when analyzing large unilamellar DLPC liposomes containing α-TH, γ-TH, NGTH, or α-TQ (structures shown in insets). Fluorescence detection (λ<sub>ex</sub> = 275 nm, λ<sub>em</sub> = 320 nm) was used to detect both γ-TH (B) and α-TH (D) with retention times of 13.7 and 15.9 min, respectively. NGTH (A), the nitration product of γ-TH, was observed to have a retention time of 18.0 min at 290 nm. α-TQ (C), the two-electron oxidation product of α-TH, was observed to have a retention time of 11.4 min at 266 nm. All peaks were verified using authentic standards.

**The Effect of HCO<sub>3</sub>⁻ on SOD1/H₂O₂-mediated Oxidation/Nitration of Tocopherols**—The time course of SOD1/H₂O₂/NO₂⁻-mediated α-TH depletion is shown in Fig. 2A. α-TH was incubated with SOD1 (1 mg/ml), H₂O₂ (1 mM), and NO₂⁻ (1 mM) in
phosphate buffer (200 mM, pH 7.4) containing DTPA (100 μM). Approximately 10 μM α-TH was consumed during the first hour of incubation, after which tocopherol depletion occurred at a slower rate (5 μM during the next hour and 6 μM over the next 3 h). This result can be explained in terms of the reaction between α-TH and NO2 by SOD1/H2O2 (4). The addition of NO2 to this system markedly enhanced α-TH oxidation and α-TQ formation. When 3 mM HCO3 was added to the solution, little change in α-TH consumption occurred during the first hour. However, by the fourth hour of incubation, a small increase in consumption was observed. With increasing HCO3 concentration, a significant increase in α-TH consumption (Fig. 2A) and α-TQ formation (Fig. 2B) was observed at each time point.

Fig. 2C (control, t = 0 h) shows the effect of the presence or absence of NO2 and HCO3 on SOD1/H2O2-mediated oxidation of α-TH depletion. Approximately 60 μM γ-TH was incubated with SOD1 (1 mg/ml) in the presence of H2O2 (1 mM). After 4 h (control, t = 4 h), α-TH levels remained constant. The addition of NO2 (1 mM) resulted in the consumption of approximately 20 μM α-TH, and the formation of 15 μM α-TQ, the two-electron oxidation product of α-TH. This clearly demonstrates that the peroxidase activity of SOD1/H2O2 is not dependent on the presence of HCO3. However, the presence of both NO2 and HCO3 resulted in the consumption of 46 μM α-TH, with a further increase in α-TQ formation. When HCO3 (20 mM) alone was added, approximately 20 μM α-TH was consumed; however, in this case, α-TQ formation was negligible. Further investigation is required to fully characterize the product(s) formed from the oxidation of α-TH by the SOD1/H2O2/HCO3 system.

The time course of SOD1/H2O2/NO2-mediated γ-TH depletion is shown in Fig. 3A. γ-TH was incubated with SOD1 (1 mg/ml), H2O2 (1 mM), and NO2 (1 mM) in phosphate buffer (200 mM, pH 7.4) containing DTPA (100 μM). Approximately 15 μM γ-TH was consumed during the first hour of incubation, after which tocopherol depletion occurred at a slower rate (10 μM during the next 3 h). As before (cf. Fig. 2A), the addition of HCO3 caused a significant increase in γ-TH consumption at each time point.

The effect of HCO3 on SOD1/H2O2/NO2-mediated NGTH formation is shown in Fig. 3B. γ-TH was incubated with SOD1 (1 mg/ml), H2O2 (1 mM), and NO2 (1 mM) in phosphate buffer (200 mM, pH 7.4) containing DTPA (100 μM). During the first hour of incubation, approximately 4 μM NGTH was formed. The addition of HCO3 had little effect on NGTH formation.

Fig. 3C shows the effect of the presence or absence of NO2 and HCO3 on α-TH depletion and NGTH formation after a 4-h incubation in the presence of SOD1 (1 mg/ml) and H2O2 (1 mM). Approximately 5 μM α-TH was consumed over a 4-h period, possibly due to NO2 contamination (as evidenced by a slight increase in the formation of NGTH, control t = 4 h). In the presence of added NO2 (1 mM), 20 μM γ-TH was consumed, and 10 μM NGTH was formed. In the presence of HCO3 (20 mM), 20 μM γ-TH was also consumed, but NGTH formation was negligible. When both NO2 and NO2 were present, approximately 90% of γ-TH was consumed, with a concomitant formation of approximately 8 μM NGTH. This paradoxical result (i.e. enhanced γ-TH depletion and decreased NGTH formation) can be explained if CO3 also caused the oxidation of NGTH.

These reactions suggest that HCO3 is not required for SOD1/H2O2/NO2-mediated nitration of γ-TH. The presence of HCO3, however, enhanced the oxidation of γ-TH and possibly NGTH.

ESR Detection of Tocopheroxyl Radicals: Enhancement by HCO3—To probe the involvement of tocopheroxyl radical during HCO3-enhanced oxidation of tocopherols, we used direct ESR. The addition of H2O2 (10 mM) to an incubation mixture containing α-TH (2 mM) and SOD1 (10 mg/ml) produced a
Fig. 4. HCO$_3^-$-enhanced formation of $\alpha$-T and NGT$^*$ radicals during the oxidation of $\alpha$-TH and NGTH by SOD1/H$_2$O$_2$. DLPC liposomes containing $\alpha$-TH or NGTH (3.5 mM each) were incubated in the presence of SOD1 (10 mg/ml) and H$_2$O$_2$ (10 mM) in phosphate buffer (200 mM, pH 7.4) at ambient temperature. A, a, ESR spectrum of $\alpha$-T generated in the presence of SOD1 and H$_2$O$_2$; b, ESR spectrum generated in the presence of 25 mM bicarbonate; c, simulation of the spectrum using the spectral parameters for $\alpha$-T as described under "Results." B, spectra $\alpha$–c correspond to the conditions used in panel A, $\alpha$–c, using NGTH in place of $\alpha$-TH. Spectrometer conditions were as follows: time constant, 0.25 s; microwave power, 40 milliwatts; scan range, 100 G; scan time, 1 min; modulation amplitude, 0.5 G. DLPC liposomes containing NGTH (50 mM) were incubated in the presence of SOD1 (1 mg/ml), H$_2$O$_2$ (1 mM) in the absence (Ca) and presence (Cb) of HCO$_3^-$ (25 mM). D, traces a and b correspond to the conditions used in panel C, a and b, using NGTH in place of $\alpha$-TH.

seven-line ESR spectrum (Fig. 4A, a) characteristic of the $\alpha$-T radical. In the presence of HCO$_3^-$, there was a marked increase in signal intensity (Fig. 4A, b). Due to a decreased signal-to-noise ratio, a high modulation amplitude was used that restricted our ability to resolve couplings from the methyl protons at carbon-8 and methylene protons at carbon-4. The computer simulation of the $\alpha$-T radical is shown in Fig. 4A, c ($\alpha$CH$_3$ (3H) = 5.66 G; $\alpha$CH$_2$ (3H) = 4.49 G) (20, 22, 23). When either SOD1 or H$_2$O$_2$ was excluded, there was no detectable ESR signal (data not shown).

Incubation of DLPC liposomes containing NGTH with SOD1/H$_2$O$_2$ produced a four-line spectrum (Fig. 4B, a) with a 1:3:3:1 intensity ratio, typical for an electron interacting with three equivalent protons. The addition of 25 mM HCO$_3^-$ resulted in the enhancement of this signal (Fig. 4B, b). The computer simulation of this spectrum (dotted lines) is shown in Fig. 4B, c ($\alpha$CH$_3$ (3H) = 4.35 G). At this high modulation, the nitrogen coupling could not be resolved.

DLPC liposomes containing $\alpha$-TH (60 $\mu$M) were incubated with SOD1 (1 mg/ml) and H$_2$O$_2$ (1 mM). Fig. 4C shows HPLC traces of samples incubated in the absence (Fig. 4C, a, 58 ± 1 $\mu$M $\alpha$-TH) or presence (Fig. 4C, b, 39 ± 2 $\mu$M $\alpha$-TH) of HCO$_3^-$ (25 mM) after a 4-h incubation at 37 °C. Incubation of DLPC liposomes containing NGTH (25 $\mu$M) with SOD1 (1 mg/ml) and H$_2$O$_2$ (1 mM) at 37 °C for 4 h resulted in a reduction in NGTH concentration to 20 ± 1 $\mu$M in the absence of HCO$_3^-$ (Fig. 4D, a) and to 14 ± 1 $\mu$M in the presence of HCO$_3^-$ (25 mM, Fig. 4D, b).

The ESR and HPLC results clearly demonstrate that the HCO$_3^-$ anion enhanced SOD1/H$_2$O$_2$-mediated oxidation of tocopherols via a radical-mediated pathway. It is likely that an oxidant, possibly the CO$_3^-$ radical derived from the SOD1/H$_2$O$_2$/ catalyzed oxidation of HCO$_3^-$ anion, is responsible for the increased oxidation of tocopherols.

**Lipid Peroxidation by SOD1/H$_2$O$_2$/HCO$_3^-$—SOD1/H$_2$O$_2$/HCO$_3^-$ and SOD1/H$_2$O$_2$/NO$_2^-$-mediated lipid peroxidation were monitored by measuring conjugated diene formation at 234 nm in $\alpha$-lecithin liposomes containing DTPA (100 $\mu$M). Conjugated diene formation was minimal during the incubation of liposomes in the presence of SOD1 (200 $\mu$g/ml) and H$_2$O$_2$ (1 mM) in the absence of either HCO$_3^-$ or NO$_2^-$ (Fig. 5). The addition of NO$_2^-$ (1 mM) resulted in an increase in absorbance at 234 nm, corresponding to the formation of conjugated dienes as a function of time. HCO$_3^-$ (25 mM) significantly enhanced conjugated diene formation in the presence of NO$_2^-$.

**Protection of H$_2$O$_2$-mediated Inactivation of SOD1 by Bicarbonate—**Fig. 6A (inset) shows the rate of superoxide-dependent cyt $c$ reduction, monitored as an increase in absorbance at 550 nm (a). In the presence of SOD1, the increase in absorbance was inhibited (f). After incubation of SOD1 with H$_2$O$_2$ for 4 h, SOD1-mediated inhibition of cyt $c$ reduction was less effective (b). The presence of HCO$_3^-$ (c), NO$_2^-$ (d), or HCO$_3^-$ and NO$_2^-$ (e) prevented the inactivation of SOD1 by H$_2$O$_2$. SOD1 activity was calculated from the initial rate of cyt $c$ reduction and is shown as a percentage of the rates of cyt $c$ observed in the absence (a) or in the presence (f) of SOD1 ($n = 3 ± S.D.$).
Bicarbonate and Peroxidase Activity

The putative oxidant, SOD-Cu²⁺-OH, reacts with several electron donor compounds to form the corresponding radical while protecting the enzyme from oxidative inactivation. HCO₃⁻ partially protects against H₂O₂-mediated inactivation of SOD1 and inhibits copper release. This may be attributed to the oxidation of HCO₃⁻ to CO₃⁻ by the enzyme-bound oxidant (2–4).

SOD-Cu²⁺ + OH + HCO₃⁻ → SOD-Cu²⁺ + H₂O + CO₃⁻

**SCHEME 2**

Unlike the copper-bound hydroxyl radical at the active site, CO₃⁻ is a freely diffusible oxidant that can oxidize target molecules at a distance (9, 10). We attribute the increased peroxidase activity of SOD1 (observed in the presence of HCO₃⁻) to the oxidative reactions catalyzed by CO₃⁻.

Oxidative Reactions of CO₃⁻—In contrast to CO₂, a reducing radical formed from oxidation of the formate anion, CO₃⁻ is an oxidizing radical (28, 29). For example, CO₃⁻ reacts with O₂ to form O₂⁻, whereas CO₂ does not react with O₂. While CO₃⁻ reduces NO to NO₂, CO₂ oxidizes NO to NO₂ (30). CO₂ forms a conjugate acid and base form, and this radical is mostly unprotonated and exists as an anion at physiological pH levels (31, 32).

The HCO₃⁻-induced depletion of tocopherols in the presence of SOD1 and H₂O₂ reported in the present study can be explained based on the following reactions.

CO₃⁻ + α-TH → HCO₃⁻ + α-T⁻

**SCHEME 3**

NO₂⁻ can abstract the phenolic hydrogen atom from the tocopherol to form the tocopheroxyl radical (34). NO₂⁻ reacts with α-T to form the corresponding quinone α-TQ. Alternatively, NO₂⁻ reacts with the linoleate-type fatty acid (k = 10⁵ to 10⁶ M⁻¹ s⁻¹) to form the lipid-conjugated diene.

CO₂ can participate in electron transfer reactions, addition, or hydrogen abstraction reactions (9, 10). CO₂ reacts rapidly with tyrosine (Tyr-OH), tryptophan, and other phenolic compounds (k = 10⁷ to 10⁸ M⁻¹ s⁻¹) to form the corresponding phenoxyl radicals (35).

Tyr-OH + CO₂ → Tyr-O⁻ + HCO₃⁻

**SCHEME 6**

CO₂ directly reacts at the sulfur site of many sulfur-containing compounds such as glutathione (k = 5 × 10⁶ M⁻¹ s⁻¹) and cysteine (k = 5 × 10⁷ M⁻¹ s⁻¹) (35).

Reactions similar to those discussed above may explain the HCO₃⁻-induced increase in formation of DMPO-OH and ABTS radical cation (ABTS⁺) observed in the SOD1/H₂O₂ system (1).
The DMPO^• cation radical has been postulated as an intermediate (7, 36). The DMPO^• will undergo hydrolysis to form DMPO-OH. This proposal is in agreement with our previous spin trapping investigation in oxygen-17-enriched water and H_2O_2 (36). To determine the source of the oxygen atom in the DMPO-OH adduct, we used oxygen-17-enriched hydrogen peroxide ([1^17O]H_2O_2) and water ([1^17O]-H_2O). The reaction of SOD1 with [1^17O]-H_2O_2 in HCO_3^-/CO_2 buffer yielded 63% DMPO-[1^17O]OH and 37% DMPO-[1^16O]OH. In contrast, the relative concentrations of DMPO-[1^17O]OH and DMPO-[1^16O]OH formed in the Fenton reaction were 90 and 10%, respectively. Since the commercial [1^17O]H_2O_2 contained 89% 17O-labeled H_2O_2, nearly all of the DMPO-OH adduct formed in the Fenton reaction originated from H_2O_2. In contrast, nearly 35% of DMPO-OH arises from the incorporation of oxygen from water in the SOD1/H_2O_2 reaction. These results were further confirmed using a different spin trap, 5-diethoxypyrophosphoryl-5-methyl-1-pyrrole N-oxide (36). These results do not support the model previously proposed for HCO_3^-/DMPO-OH adduct hydroxylation of DMPO at the active site of SOD1 (1). Based on that model, one would have expected 100% incorporation of H_2O_2-derived oxygen into DMPO-OH.

Detection of CO_3^− at Physiological pH—The CO_3^− radical formed during SOD1/H_2O_2-catalyzed oxidation of the ferrous ion has been spin-trapped with DMPO to give a DMPO-CO_3^− adduct, which gave a characteristic ESR spectrum (28). In contrast, the addition of SOD1 to an incubation mixture containing DMPO, H_2O_2, HCO_3^−, and DTPA enhanced DMPO-CO_3^− formation (1, 36). The DMPO-CO_3^− adduct was not stable enough to be detected at physiological pH levels. Recently, using a rapid mixing continuous flow ESR, CO_3^− production from peroxynitrite and carbon dioxide was detected at physiological pH levels (32, 37). It is conceivable that CO_3^− formed from SOD1/H_2O_2/HCO_3^− may be detected using similar experimental techniques.

Biological Implications—Hodgson and Fridovich (38) reported that the addition of carbonate anion to an aerobic xanthine/xanthine oxidase system produced luminescence. This effect was attributed to a dimerization of CO_3^− radical anion (39) formed from the reaction between 'OH and CO_3^− (33) as follows.

```
SCHEME 7

\[
\begin{align*}
\text{metal} & \quad \text{O}_2^• + \text{H}_2\text{O}_2 & \rightarrow & \text{OH}^• + \text{OH}^+ + \text{O}_2^- \\
\text{ions} & \\
\text{OH}^• + \text{CO}_3^− & \quad k=4 \times 10^9 \text{M}^{-1} \text{s}^{-1} & \rightarrow & \text{CO}_3^- + \text{OH}^• \\
2 \text{CO}_3^− & \quad k=10^5 \text{M}^{-1} \text{s}^{-1} & \rightarrow & \text{hv} + \text{products}
\end{align*}
\]
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In the pioneering studies of the peroxidase activity of SOD1, some experiments were performed in carbonate buffer (2, 3). It is likely that H_2O_2-induced inactivation of SOD1 was partially protected by the carbonate anion.

Bicarbonate has been shown to alter the reactivity of reactive nitrogen species (40–48). Peroxynitrite, a potent oxidant formed from a diffusion-controlled reaction between O_2^- and NO, has been proposed to react with CO_2 to generate an intermediate that consists of a caged radical pair, \text{CO}_2/\text{NO}_2^-.

The chemiluminescence detected in the reaction between ONOO^- and CO_2 was attributed to the formation of a carbonate anion radical (49). The CO_2-peroxynitrite reaction facilitated the nitration and oxidation of tyrosine (39). This scenario is similar to the bicarbonate-enhanced reactivity of SOD1/H_2O_2/NO_2^- reported in the present study.

HCO_3^- is abundant in biological systems. The plasma concentration of HCO_3^- is approximately 25 mM (50). Recently, an extracellular SOD1 has been identified in atherogenesis (51). The peroxidase activity of this extracellular SOD1 has been suggested to play an important role in the oxidative modification of lipid. This activity may be amplified in the presence of HCO_3^-.

In conclusion, in contrast to a previous report (1), evidence presented in this study strongly suggests that HCO_3^- is not an absolute requirement for eliciting the peroxidase activity of SOD1. However, HCO_3^- enhances the peroxidase activity of SOD1 by acting as a "sacrificial" electron donor and in so doing forms a CO_3^- radical intermediate, a selective yet potent biological oxidant.

The involvement of CO_3^- in biological oxidation seems more ubiquitous (56) and should be taken into consideration in future studies.

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