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Electron spin resonance study on the permeability of superoxide radicals in lipid bilayers and biological membranes

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The permeability of lipid bilayers and biological membranes to superoxide free radicals was examined by using superoxide dismutase (SOD)-loaded lipid vesicles and SOD-loaded erythrocyte ghosts. After exposing SOD lipid vesicles and SOD ghosts to enzymatically produced superoxide radicals and using spin-trapping and electron spin resonance (ESR) techniques, we found that SOD entrapped within erythrocyte ghosts effectively scavenges external $O_2^{\bullet-}$ while SOD inside the lipid bilayers has no effect. These results confirm that $O_2^{\bullet-}$ is able to cross through a biological plasma membrane but not across a pure lipid bilayer. The data provide instruction as to how and where anti-oxidant therapy is to be approached relative to the site of oxygen free radical production.

Superoxide radical; Permeability, Lipid vesicle; Erythrocyte ghost; Electron spin resonance

1. INTRODUCTION

Since oxygen free radicals are thought to play an important role in direct cellular injury, superoxide dismutase (SOD) has been considered to be an important enzyme to strengthen cellular defence. However, because of the compartmentalized production of $O_2^{\bullet-}$ and the localized distribution of SOD in cells [1–3], the successful use of exogenous SOD to eliminate $O_2^{\bullet-}$ in situ requires a thorough knowledge of the permeability of cell membranes to oxygen free radicals.

To date, reports of the permeation of lipid bilayers to $O_2^{\bullet-}$ are not conclusive. Takahashi et al. reported that the lipid bilayers were not permeable to $O_2^{\bullet-}$ generated inside lipid vesicles by the illumination of flavin mononucleotide [4], while Rumyantseva et al. found that enzymatically produced $O_2^{\bullet-}$ could cross a lipid bilayer to cause ferricyanide-ferrocyanide transformation inside lipid vesicles [5]. Evidence for the permeability of biological membranes to $O_2^{\bullet-}$ is limited to one available example of erythrocyte membrane [6]. By using xanthine oxidase-loaded lipid vesicles and chemically defined reaction, Lynch and Fridovich reported that erythrocyte membrane was permeable to $O_2^{\bullet-}$ possibly via an anion channel.

In order to clarify the permeability of $O_2^{\bullet-}$, a precise method for detecting and differentiating free radical species appears indispensable. In our laboratory, we have recently applied electron spin resonance (ESR) and spin trapping techniques, a more direct and sensitive method, to study the permeability of $O_2^{\bullet-}$. We have loaded phosphatidylcholine lipid vesicles and resealed erythrocyte ghosts with SOD. Following the incubation of SOD vesicles or SOD ghosts with an external source of $O_2^{\bullet-}$, the permeation of membranes to $O_2^{\bullet-}$ was determined by measuring retained external concentration of $O_2^{\bullet-}$ using both the ESR techniques as well as a chemically defined reaction. The results show that SOD trapped inside lipid vesicles did not scavenge external $O_2^{\bullet-}$ suggesting that lipid bilayers are not permeable to $O_2^{\bullet-}$. In contrast, SOD-loaded ghosts are able to scavenge external $O_2^{\bullet-}$ as effectively as free SOD supporting that the erythrocyte membranes are permeable to $O_2^{\bullet-}$.

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2. MATERIALS AND METHODS

2.1. *Preparation of SOD-loaded lipid vesicles*

Lipid vesicles were prepared according to described technique [7]. 5 mg of Cu/Zn SOD from yeast in 3 ml of 50 mM phosphate buffer, pH 7.4, was vortexed with 200 mg of N_2-dried egg phosphatidylcholine to form multilamellar vesicles (MLV). After placing a polycarbonate membrane (25 mm diameter, 0.1 μm pore size) at the bottom of a liposome extrusion apparatus, the MLV underwent a 5-cycle extrusion process. The final extruded mixture of SOD vesicles and free SOD was separated by Sephadex G-100 chromatography.

2.2. *Preparation of sealed SOD-loaded ghosts*

Hemoglobin-depleted erythrocyte ghosts were prepared according to established methods [8–10]. Freshly drawn human blood was washed four times in isotonic buffer (0.9% KCl), the buffy coat was carefully removed after each centrifugation. The washed erythrocytes (50% hematocrit) were lysed in 30 volumes of a hypotonic hemolysis buffer (4 mM $MgSO_4$, 3.8 mM CH_3COOH, pH 4) for 5 min with stirring. The erythrocyte ghosts were then collected and washed with washing buffer (4 mM $MgSO_4$, 1.2 mM CH_3COONa, 2 mM NaH_2PO_4/Na_2HPO_4, pH 7) until the ghosts had a grayish-white color. SOD-loaded ghosts were prepared by mixing SOD with the ghosts in

the washing buffer and restoring the tonicity to 100 mM. The SOD-loaded ghosts were fully resealed by incubation at 37°C for 45–60 min.

2.3. *Electron spin resonance (ESR)*

Trace metals present in the buffer solution used for ESR experiments were removed by passing all buffers through a chelating ion-exchange membrane. For the determination of the permeation of superoxide radicals, 1 ml of 50 mM phosphate buffer, pH 7.8, contains 4 mM xanthine, 100 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 0.05 mM DETAPAC, and SOD loaded-vesicles or SOD loaded-ghosts. The generation of $O_2^{\cdot-}$ was initiated by the addition of a sufficient amount of xanthine oxidase (6 nM). Typical Bruker ESP 300 Spectrometer settings were: field set, 3477 G; modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9.67 GHz; scan time, 1.2 min; cavity mode, TE_{120}.

2.4. *Analytical procedures*

SOD activity was measured using a ferricytochrome *c* reduction assay [11]. Lipids were extracted by the method of Bligh and Dyer [12], and lipid phosphorus was measured as described [13].

3. RESULTS

3.1. *Permeability of lipid bilayers to superoxide radicals*

In order to determine whether $O_2^{\cdot-}$ passing through the lipid bilayer, intact and deoxycholate (DOC)-lysed SOD vesicles were incubated with xanthine/xanthine oxidase. The samples were taken from the mixture during the incubation course to measure the retained superoxide radical concentration by $O_2^{\cdot-}$-dependent cytochrome *c* reduction. During the 15-min incubation, O_2 concentrations in the control system and SOD-loaded vesicle system exhibited the same trend characterized by a gradual decrease of ΔA from 0.022 to 0.012 at 550 nm (Fig. 1). This phenomenon could be attributed to the spontaneous degradation of $O_2^{\cdot-}$ rather than to the effect of intravesicular SOD. In contrast, the addition of DOC- lysed SOD vesicles in the xanthine/xanthine oxidase system completely blocked $O_2^{\cdot-}$-dependent cytochrome *c* reduction within 4 min. These results imply that permeation of the lipid membrane to superoxide radicals is negligible.

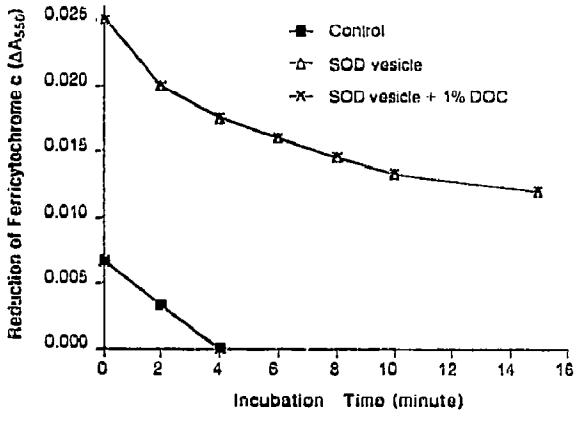

Fig. 1. Accessibility of SOD enclosed in lipid vesicles to extravesicular $O_2^{\cdot-}$. The control medium contained 0.05 mM xanthine, 6 nM xanthine oxidase, and 0.1 mM DETAPAC in 50 mM phosphate pH 7.8. The samples were taken at regular intervals to examine the remaining extravesicular $O_2^{\cdot-}$ by the addition of 0.01 mM ferricytochrome *c*.

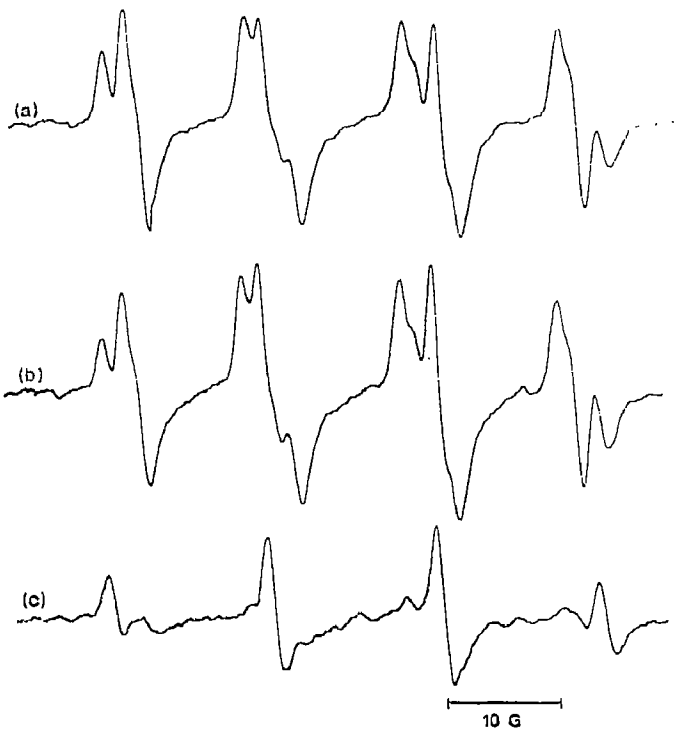

Fig. 2. ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate buffer, (b) SOD vesicles (2 μmol lipid phosphorus/ml) and (c) DOC relysed SOD vesicles (2 μmol lipid phosphorus/ml).

Spin trapping and electron spin resonance offered additional evidence for the conclusion. Fig. 2a shows a typical spectrum generated by xanthine/xanthine oxidase. Fig. 2b was obtained following the reaction of xanthine with xanthine oxidase in the presence of SOD-loaded vesicles. Intact SOD-loaded vesicles did not result in the decrease of ESR signals compared with Fig. 2a indicating no influx of $O_2^{\cdot-}$ through the lipid bilayer. Fig. 2c shows that DOC-lysed SOD vesicles effectively scavenged the superoxide radicals, resulting in depletion of ESR signals. Note that the remaining signals in Fig. 2c represent the hydroxyl radicals and not superoxide

Table I

Determination of leakage of SOD ghosts

Time (min)	Cytochrome *c* reduction (ΔA 550 nm)
0	0.0247
15	0.0243
25	0.0247
35	0.0246

SOD ghosts were incubated at 25°C, then after sedimentation of SOD ghosts, SOD activity in supernatant was determined by a cytochrome *c* reduction assay.

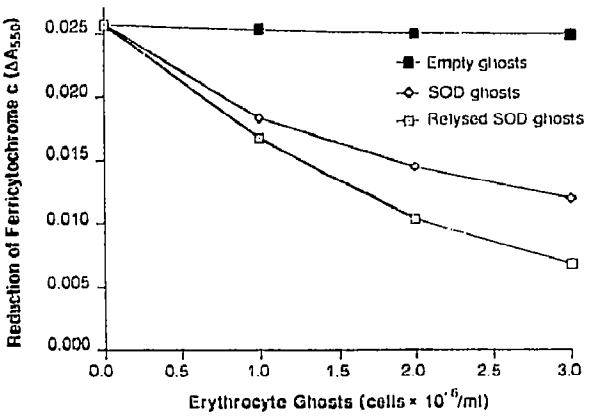

Fig. 3. Accessibility of SOD enclosed in the erythrocyte membrane to extracellular $O_2^{\bullet-}$. The incubation medium was the same as described in Fig. 1. The reaction was started by the addition of 6 nM xanthine oxidase.

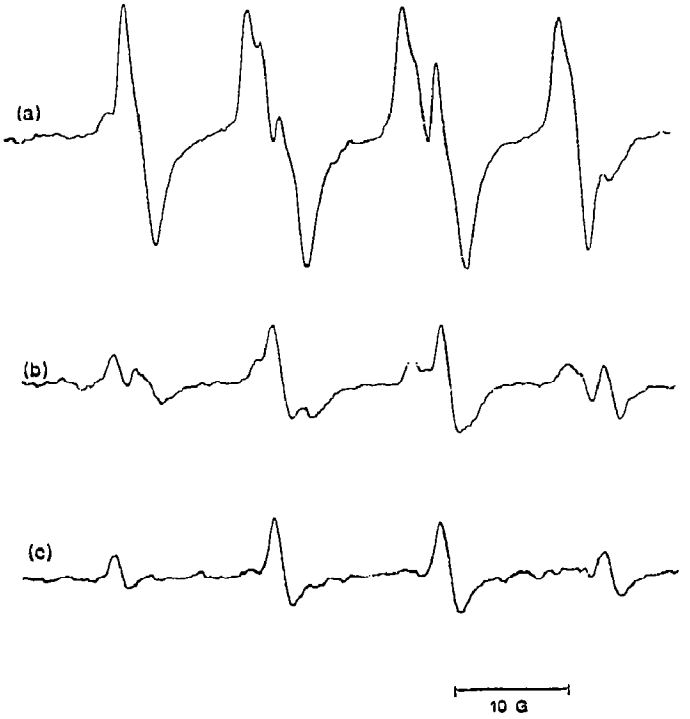

Fig. 4. ESR obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) empty erythrocyte ghosts, (b) intact SOD ghosts (2×10^6 cells/ml) and (c) hypotonic relysed SOD ghosts (2×10^6 cells/ml).

radicals. DOC alone was without any effect. These ESR data are in agreement with the cytochrome *c* reduction assay experiments confirming that superoxide radicals are unable to cross the lipid bilayer.

3.2. *Characteristics of SOD ghosts*

SOD-loaded ghosts prepared as described in section 2 were 'intact' and impermeable to SOD molecules as shown in Table I. There was also no non-specific binding of SOD to the erythrocyte membrane, which was demonstrated by mixing empty ghosts with SOD, followed by thorough washing. No SOD activity was found in the empty ghosts.

3.3. *Permeability of biological membranes to superoxide radicals*

Fig. 3 shows that empty erythrocyte ghosts did not influence $O_2^{\bullet-}$-dependent cytochrome *c* reduction while SOD ghosts or hypotonic relysed SOD ghosts were effective in a dose-dependent pattern. The addition of 2.3 $\times 10^6$, 4×10^6, and 6×10^6 SOD ghosts to the reaction system decreased $O_2^{\bullet-}$-dependent cytochrome *c* reduction by 29%, 42% and 53%, respectively, implying that $O_2^{\bullet-}$ has diffused through the 'intact' erythrocyte membranes and interacted with entrapped SOD.

Additional ESR data are shown in Fig. 4. Typical DMPO-OOH and DMPO-OH peaks in spectrum (a) represented the reaction of xanthine with xanthine oxidase in the presence of empty erythrocyte ghosts. However, replacement of empty ghosts with SOD ghosts resulted in significant depletion of spin trapping adduct signals (Fig. 4, spectrum b). This inhibitory effect was similar to that of hypotonic relysed SOD-loaded ghosts (Fig. 4, spectrum c) strongly supporting that $O_2^{\bullet-}$ is able to cross the erythrocyte membrane and be quenched by entrapped SOD.

4. DISCUSSION

One of the complexities of successfully using SOD as a therapeutic agent is that the enzyme has to be delivered or targeted promptly or it will be cleared rapidly from the circulation [14]. Some investigators have tried to protect native SOD from inactivation by encapsulating SOD within a lipid bilayer membrane. The product, SOD liposome, has advantages in enhancing half-life and intracellular delivery of SOD [15,16]. However, a major complication of the SOD liposome is that the non-polar lipid bilayer prevents SOD from direct acting on oxygen free radicals. It has to be ascertained whether liposomal entrapped SOD still has the ability to scavenge external $O_2^{\bullet-}$ as effectively as the free SOD. In our experiments, we have shown that SOD, once encapsulated into the lipid vesicles, cannot catalyze the dismutation of superoxide radicals generated outside the vesicles unless the lipid bilayer is disrupted by detergents. This leads to two important points: (a) the lipid bilayer is likely impermeable to the superoxide radicals and (b) SOD liposomes, although improving certain properties of SOD, are unlikely to be effective scavengers of superoxide radicals until the enzyme is released.

Our data indicate clearly that superoxide radicals can diffuse freely across the erythrocyte membrane. This is confirmed by the depletion of spin trapping adduct signals on ESR spectra and the suppression of cytochrome

c reduction, following the reaction of SOD ghosts with external $O_2^{\bullet-}$. Since SOD is membrane impermeable and most oxygen free radicals are locally produced [17,18], the finding that the biological membrane is permeable to superoxide radicals is of particular relevance to these new ideas: (a) SOD-enriched erythrocytes may be used as physiological scavengers of $O_2^{\bullet-}$ in blood; (b) it may not be essential for the SOD to be administered directly to the site of $O_2^{\bullet-}$ production as long as the superoxide radical can pass through the necessary membrane barriers to react the exogenous (extracellular) scavengers.

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