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SCAVENGING EFFECT OF BERBAMINE ON ACTIVE OXYGEN RADICALS IN PHORBOL ESTER-STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES

JU HAISONG,* LI XIAOJIE, ZHAO BAOLU, HAN ZHEWU* and XIN WENJUAN†
Institute of Biophysics, Academia Sinica, Beijing 100080, China

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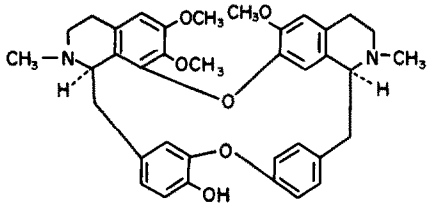
Abstract—The scavenging effect of berbamine (Ber) on active oxygen radicals was studied, using a spin-trapping technique and a chemiluminescence (CL) method in phorbol myristate acetate (PMA) stimulated polymorphonuclear leukocytes (PMN) and in four cell-free superoxide ($O_2^{\cdot-}$) or hydroxyl radical ($OH^{\cdot}$) generating systems. Ber (0.1 to 0.3 mM) effectively reduced active oxygen radicals in PMN stimulated with PMA, but had no obvious effect on oxygen consumption during the respiratory burst of PMN, measured with spin probe oxymetry. Ber (0.3 mM) prominently inhibited the CL response of PMA-stimulated PMN. The agent remarkably quenched $O_2^{\cdot-}$ in xanthine/xanthine oxidase and irradiation riboflavin systems and $OH^{\cdot}$ in the Fenton reaction. Its scavenging action on $O_2^{\cdot-}$ was stronger than that of Vitamin E in the xanthine/xanthine oxidase system but the same as Vitamin E in the riboflavin system, and its action on $OH^{\cdot}$ was similar to that of Vitamin E.

Although polymorphonuclear leukocytes (PMN‡) offer the primary cellular defense against bacteria in the human body, a great deal of experimental evidence accumulated during the last 10 years indicates that PMN-derived oxygen radicals play an important role in many diseases, including emphysema, myocardial infarction, silicosis, radiation and immune complex injury [1, 2]. Hence, further study of the mechanisms of tissue injury by active oxygen radicals and the search for effective free radical scavengers will provide insight into the development of new therapeutic strategies for the treatment of such diseases. It has been reported that many traditional Chinese herbs show a potent antioxidant activity [3, 4]. Berbamine (Ber) is a bis-benzyl-isoquinoline alkaloid isolated from the traditional Chinese herb *Berberis porretii* S. The structure of Ber is shown in Scheme I.



Berbamine

Scheme I.

Recent studies have demonstrated that Ber has anti-myocardium infarction, anti-arrhythmia [5] and anti-silicosis effects, and is useful in the prevention and treatment of radiation-induced leukopenia [6]. However, the mechanisms of the action of Ber are still unknown. It has been reported that cepharanthine, a bis-benzyl-isoquinoline alkaloid, shows inhibitory action on membrane lipid peroxidation and suppressive action on the formation of $O_2^{\cdot-}$ in guinea pig PMN [7]. We therefore inferred that Ber might have antioxidant activity. The inhibitory effect of Ber on lipid peroxide formation in an *in vitro* system was demonstrated recently in our laboratory (unpublished data).

The spin-trapping technique involves the addition reaction of very reactive short-lived free radicals ($O_2^{\cdot-}$ and $OH^{\cdot}$) to a diamagnetic compound, spin trap (such as DMPO), to produce relatively long-lived free radical products, spin adducts (such as DMPO-OOH and DMPO-OH), which can be easily studied by electron spin resonance (ESR). In favorable cases, the resulting ESR spectrum allows the identification of the original reactive radicals [8]. In the present study, the scavenging effect of Ber on oxygen radicals was studied by using an electron spin resonance spin-trapping technique and a chemiluminescence (CL) method.

MATERIALS AND METHODS

Materials. Phorbol myristate acetate (PMA), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (CTPO) and xanthine were purchased from the Sigma Chemical Co. A stock solution of PMA was made in acetone at a concentration of 50 μg/mL and stored in aliquots at −20°. Immediately before use, PMA was diluted with PBS to the desired concentration. DMPO was

* Present address: Inner Mongolia Medical College, Department of Pharmacology, Huhehot, China.
† Corresponding author.
‡ Abbreviations: PMN, polymorphonuclear leukocytes; Ber, berbamine; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; CL, chemiluminescence; PMA, phorbol myristate acetate; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; HBSS, Hanks' Balanced Salt Solution; DETAPAC, diethylenetriaminepentaacetic acid; and SOD, superoxide dismutase.

purified by active charcoal before use. No impurity signals were found on the ESR spectra. CTPO was first dissolved in a small amount of ethanol and diluted further with PBS. Vitamin E and luminol were purchased from the E. Merck Co. Luminol was prepared as 1 mM stock solution in dimethyl sulfoxide (DMSO) and diluted fresh daily in HBSS. Xanthine oxidase was obtained from the Institute of Biochemistry, Academia Sinica. Hydrogen peroxide was obtained from Beijing Chemical Agent Manufactory. All other reagents were of analytical grade from China. Berbamine, a white crystal (fat soluble compound with m.p. 268°–270°), was supplied by the Institute of Applied Ecology, Shenyang, China.

Isolation of PMN. Fresh venous blood of healthy adult donors was obtained from the Central Blood Station of the Beijing Red Cross. Sodium citric acid was used as the anticoagulant. PMNs were isolated by Ficoll–Hypaque density gradient centrifugation according to the method of Markert *et al.* [9]. The remaining erythrocytes were removed by hypotonic lysis, and PMNs were resuspended in HBSS to a concentration of 5×10^7 PMN/mL. The viability of PMN was more than 95% as determined by the exclusion of trypan blue [10].

Measurement of active oxygen radicals [11]. A mixture containing 1.7×10^7 PMN/mL, 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC), and 100 ng/mL PMA (PMA was added at time = 0) was incubated at 37° for 2 min; then DMPO was added to a final concentration of 90 mM and mixed well. Reaction mixtures were transferred to a quartz capillary, fitted into the cavity of an ESR spectrometer (Varian E-109 ESR spectrometer). Samples were measured at 4 min after PMA was added. ESR instrument settings were: microwave power, 15 mW, X band; central field, 3240 G; modulation frequency, 100 kHz with an amplitude of 1.0 G; sweep time, 50 G/min; time constant, 0.128; and temperature 25°.

O_2^- and OH· assay. A solution containing 0.32 mM xanthine, 0.16 mM DETAPAC, and 80 mM DMPO was mixed well, and then xanthine oxidase (0.1 units/mL, final concentration) was added immediately. Samples were measured 3 min after XO was added [12]. The second O_2^- generating system was a mixture containing 0.3 mM riboflavin, 5 mM EDTA and 0.1 M DMPO which was transferred to a quartz capillary and put into the cavity of an ESR spectrometer. After the samples were irradiated by xenon lamp (power, 1 kW; distance 70 cm) for 30 sec, the ESR spectrum was measured immediately [11]. The third O_2^- generating system was an alkaline DMSO system as described by Hyland *et al.* [13]. Test tubes containing 0.3 mM luminol and different doses of Ber were kept in an ice bath for 20 min. Then, alkaline DMSO (DMSO containing 1% water and 5 mM NaOH) was added. The luminol-enhanced CL was measured by a liquid scintillation counter. The Fenton reaction was used to generate OH· [8]. The assay mixture contained 1% hydrogen peroxide, 100 μM ferrous ammonium sulfate and 0.1 M DMPO. Samples were measured 4 min after DMPO was added. ESR instrument settings were the same as described above.

Measurement of PMN oxygen consumption. Oxygen consumption was determined by spin probe oxymetry according to the method of Lai *et al.* [14] and modified by our laboratory [11]. The effects of Ber on oxygen consumption by PMN were evaluated by using the same method. ESR instrument settings were: microwave power, 1 mW; sweep time, 0.37 G/min; and modulation amplitude, 0.05 G; other conditions were the same as described above.

CL measurement. Luminol-dependent CL was measured in an LKB-1250 Luminometer according to the method of Shult *et al.* [15]. The PMN (1×10^6/mL) suspensions were preincubated at 37° for 45 min and maintained in suspension by frequent gentle agitation. PMN (3.1×10^5/mL) in HBSS were mixed with 60 μM luminol to a final volume of 1.5 mL. To activate the system, PMA (42 ng/mL, final concentration) was added at time = 0. Samples were mixed homogeneously, and light emission was recorded for 20 min at 4-min intervals. The intensity of CL was expressed in millivolts.

Preparation of drugs and measurement. Ber and Vitamin E were dissolved in DMSO and diluted to the desired concentration with appropriate buffer solution according to the experimental system. Ber and Vitamin E were added into each test system in order to evaluate the scavenging effect of Ber on oxygen radicals. For control, the same volume buffer solution was added instead of the drugs. The resulting ESR signal intensity was measured and compared with the control.

RESULTS

The ESR spectrum of the control with all components except PMN is shown in Fig. 1a. Since the DMPO-OOH signal decayed slowly with time, the spectrum of DMPO adducts was measured under the same condition (at 4 min after PMA was added), and as shown in Fig. 1b, DMPO-OH was not produced by the Ber solution.

When PMNs were incubated with PMA at 37° for 2 min and DMPO was added to the mixture, the ESR signal appeared (Fig. 2a). The scavenging effect of Ber on active oxygen radicals was examined by the same procedure in the absence or the presence of the drug. As shown in Fig. 2, b and c, Ber effectively reduced active oxygen radicals at concentrations of 0.1 and 0.3 mM, its action being stronger than that of Vitamin E.

CTPO is a paramagnetic compound, and its ESR spectrum is shown in Fig. 3b. A broadening effect was observed, as shown in Fig. 3a, if higher concentrations of O_2 were present. This was due to the increase of spin–spin interaction of CTPO and oxygen molecules. Therefore, the oxygen concentration can be measured by the degree of broadening of superhyperfine ESR lines. In this study, oxygen concentration was estimated by the K parameter; the larger the K parameter, the higher the oxygen consumption. The K parameter was calculated according to the formula: $K = (b + c)/2a$ [14]. As presented in Fig. 4, the K parameter of PMN reached steady values at 12 min after PMA stimulation. Ber had no obvious effect on oxygen consumption by PMN.

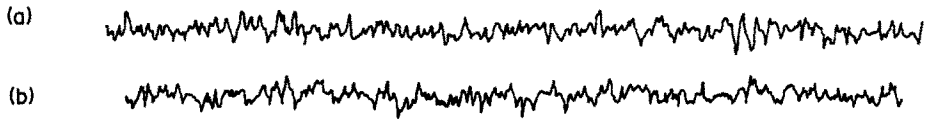


Fig. 1. Electron spin resonance spectrum of 0.1 M DMPO (a) and 0.3 mM Ber (b) in PBS solution containing 0.1 mM DETAPAC and 100 ng/mL PMA.

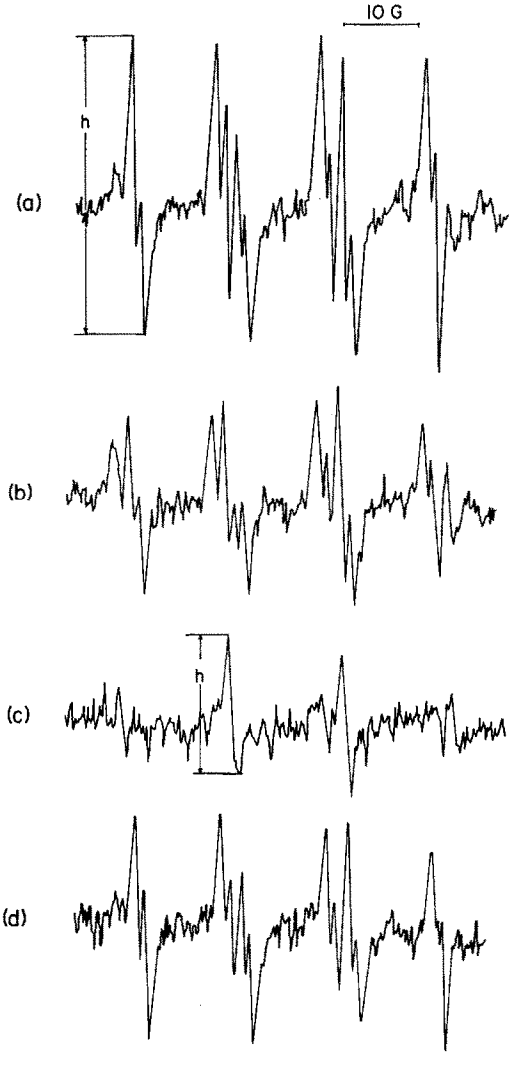


Fig. 2. Electron spin resonance spectra of the DMPO adduct of oxygen radicals in the respiratory burst of PMA-stimulated human polymorphonuclear leukocytes. Key: (a) control; (b) Ber, 0.1 mM; (c) Ber, 0.3 mM; and (d) Vitamin E, 0.3 mM. For control, PBS was added instead of free radical scavengers.

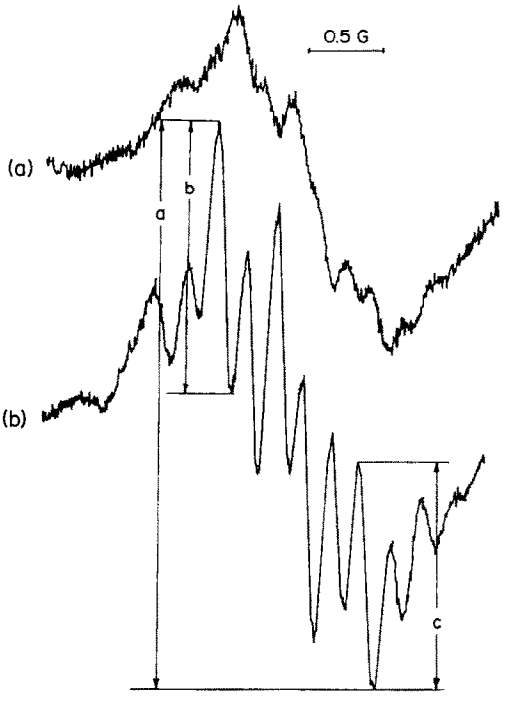


Fig. 3. Component of M(O) from the ESR spectrum of the spin probe CTPO (a) at the beginning and (b) at the end of the respiratory burst of human polymorphonuclear leukocytes stimulated with PMA. ESR settings were: central field 3240 G; microwave power, 1 mW; modulation frequency, 100 kHz with an amplitude of 0.05 G.

The effect of Ber on O_2^- and OH$^\cdot$ was examined by performing the same reaction in the absence or the presence of the drug in three cell-free oxygen radical generating systems. The relative intensity of the ESR signal was estimated according to the height of the spectrum of the DMPO adducts. The percent inhibition of the oxygen radicals by the drugs was calculated by the following formula:

$$\frac{h_o - h_x}{h_o} \times 100\%.$$

Here h_o represents the height of the first peak of the control sample in the ESR spectra of DMPO-OOH in Fig. 2a and the height of the second peak of the control sample in the ESR spectra of DMPO-OH in Fig. 2c; h_x represents the height of the first peak of DMPO-OOH and the height of the second peak of DMPO-OH in corresponding ESR spectra, respectively, after the drugs were added [11]. In both the Xan/XO and riboflavin systems, Ber exerted a remarkable scavenging effect on O_2^-. Its scavenging action on O_2^- was stronger than that of Vitamin E in the Xan/XO system (Table 1), but the same as Vitamin E in the riboflavin system (Table 2). In the alkaline DMSO system, Ber showed a potential and

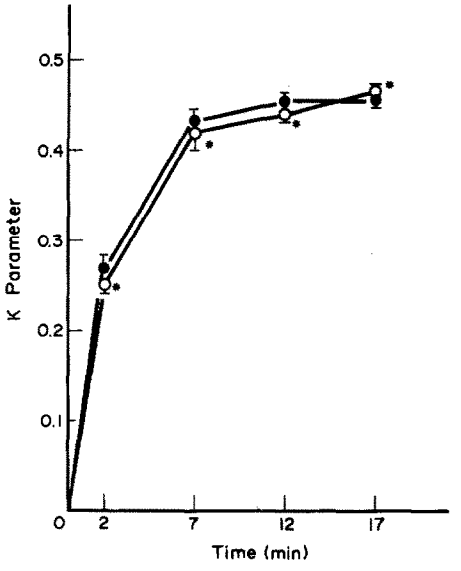


Fig. 4. Effect of berbamine on oxygen consumption during the respiratory burst of human polymorphonuclear leukocytes stimulated with PMA. Key: (●) control, and (○) Ber, 0.1 mM. For control, PBS was added instead of Ber. Each point is the mean ± SD of three determinations. *P > 0.05.

Table 1. Effects of berbamine on O_2^- in the xanthine/xanthine oxidase system

Group	Dose (mM)	Signal intensity (mm)	% Inhibition
Control		99.7 ± 1.2	0
Berbamine	0.2	73.0 ± 1.0*†	26.8 ± 1.3
	0.6	53.3 ± 2.9*‡	46.5 ± 2.7
Vitamin E	0.6	89.7 ± 3.5	9.4 ± 1.7

Each value is the mean ± SD of three replicate experiments.
* P < 0.01 vs control.
† P < 0.05 vs Vitamin E.
‡ P < 0.01 vs Vitamin E.

Table 2. Effects of berbamine on O_2^- in the irradiated riboflavin system

Group	Dose (mM)	Signal intensity (mm)	% Inhibition
Control		96.3 ± 8.5	0
Berbamine	0.2	72.9 ± 5.7*†	24.3 ± 2.4
Vitamin E	0.2	75.0 ± 5.2	22.0 ± 1.8

Each value is the mean ± SD of three separate experiments.
* P < 0.01 vs control.
† P > 0.05 vs Vitamin E.

dose-dependent scavenging action on O_2^- (Table 3). The Fenton reaction is a classical method for the generation of $OH^·$. In this system, Ber elicited an inhibitory action similar to that of Vitamin E (Table 4).

In the luminol-enhanced CL system, the oxidation of luminol by active oxygen radicals generates an excited aminophtalate anion that relaxes to the ground state with the production of light emission. Hence, it is a sensitive and accurate method for the determination of PMN oxidative metabolism [16]. As shown in Fig. 5, maximal light emission was achieved at 12 min after the activation of PMN. Ber prominently inhibited the chemiluminescence of PMN.

DISCUSSION

The use of spin trapping by Green *et al.* to observe the production of oxygen radicals in stimulated neutrophils may be the first use of the spin-trapping technique in intact cells [10]. PMN is one of the main sources of the production of oxygen radicals in the human body. To evaluate the antioxidant activity, the scavenging effects of Ber on active oxygen radicals in PMA-stimulated PMN were investigated. Ber exhibited a markedly suppressive action on ESR signal, but had no effect on the oxygen consumption by the actived PMN. This finding further confirmed that the reduction of active oxygen radicals was not due to inhibition of the respiratory burst of PMN but to the scavenging effect of the drug on active oxygen radicals. Since the ESR spectra of DMPO-trapped active oxygen radicals in the PMN system consist of an overlapping spectra of DMPO-OOH and DMPO-OH [11], it is difficult to quantitatively study the scavenging effects of the drug. To quantitatively study the effect of Ber, three O_2^- generating systems and one $OH^·$ generating system were employed. Our results clearly show that Ber was an effective scavenger of O_2^- and $OH^·$. In the Xan/XO system, which exists in the human body, a potential and concentration-dependent scavenging effect of Ber on O_2^- was obtained. The scavenging effect of Ber on O_2^- was stronger than that of Vitamin E in the Xan/XO system, but the same as Vitamin E in the riboflavin system, while the effect on $OH^·$ was similar to that of Vitamin E. Our results demonstrated that Ber displayed scavenging effects on active oxygen radicals in both cell and cell-free systems.

It has been reported that both O_2^- scavenger (superoxide dismutase) and $OH^·$ scavenger (sodium benzoate) show inhibitory effects on the CL of activated PMN. The results imply that O_2^- and $OH^·$ contribute to the generation of CL [15]. Although the mechanism of the inhibiting effect of Ber on PMN CL is not known, it is reasonable to speculate that the scavenging effect on O_2^- and $OH^·$ may play an important role. What mechanism is responsible for the scavenging action on O_2^- and $OH^·$? We have no direct evidence at the present time. Some researchers suggest that the phenol hydroxyl group of some antioxidants plays a critical role in the scavenging action [17]. There is one phenol hydroxyl in Vitamin E. The scavenging action of Vitamin E on O_2^- was in at least two steps: first,

Table 3. Scavenging effect of berbamine on O_2^- in the alkaline/DMSO system

Group	Dose	Chemiluminescence (cpm × 10^4)	% Inhibition
Control		542 ± 31	0
Berbamine	1 μM	376 ± 18*	30.6 ± 2.9
	10 μM	103 ± 24*	81.0 ± 1.1
	100 μM	17 ± 2*	96.8 ± 0.4
SOD	66 ng/mL	273 ± 21*	50.8 ± 1.6

Each value is the mean ± SD of four determinations. Chemiluminescence was measured by using a Beckman LS-5801 Liquid Scintillation Counter.
* $P < 0.01$ vs control.

Table 4. Effects of berbamine on OH· in the Fenton reaction

Group	Dose (mM)	Signal intensity (mm)	% Inhibition
Control		91.0 ± 1.0	0
Berbamine	0.6	66.9 ± 3.6*†	26.5 ± 3.3
Vitamin E	0.6	69.7 ± 4.4	23.5 ± 4.1

Each value is the mean ± SD of three separate experiments.
* $P < 0.01$ vs control.
† $P > 0.05$ vs Vitamin E.

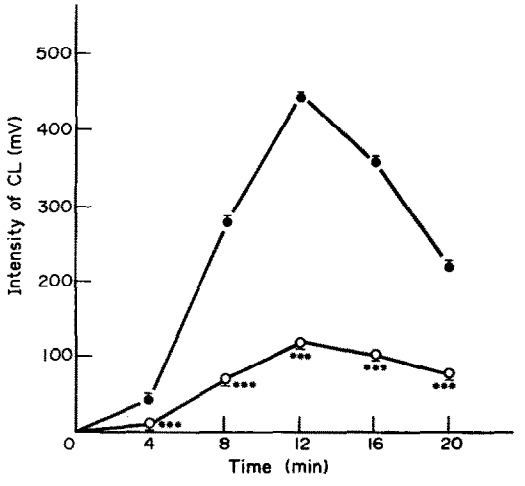


Fig. 5. Chemiluminescent response of PMA-stimulated human polymorphonuclear leukocytes in the absence (●) or the presence (○) of 0.3 mM Ber. For control, HBSS was added instead of Ber. Each point is the mean ± SD of three determinations. ***$P < 0.01$.

superoxide quenching and then development of a phenoxy radical [18]. To confirm the mechanism of the scavenging action of Ber on oxygen radicals, further study is needed.

The agents (superoxide dismutase, catalase, Vitamin E and ibuprofen) that can scavenge free radicals or prevent free radical formation have been used to protect against ischemia/reperfusion induced myocardium injury [19, 20]. Ber may provide a beneficial effect on some of the oxygen free radical-linked diseases.

In conclusion, the scavenging effects of Ber on active oxygen radicals were established in different *in vitro* systems. Ber is a new oxygen radical scavenger.

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