MEASUREMENT OF OXYGEN CONSUMPTION OF HUMAN NEUTROPHILS BY SPIN PROBE

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UDC 577.334

KEY WORDS: oxygen; spin probe; neutrophils

The antimicrobial and cytotoxic activity of phagocytic cells is determined by their ability to undergo activation, during which the metabolism of the cells is transformed. Activation of phagocytes takes place as a result of their interaction with immune complexes [8], bacteria [7], and zymosan particles [14], or under the influence of soluble activators, including chemotaxic oligopeptides [11], concanavalin A [6], and others. Activation of neutrophils and macrophages is accompanied by a "respiratory burst," including increased production of NADPH as a result of intensification of the hexose monophosphate shunt (HMPS) [12], increased oxygen consumption [5], and superoxide radical formation [5] as a result of increased activity of NADPH oxidase [2]. Determination of the characteristics of the "respiratory burst" enables the functional state of the neutrophils to be judged.

The aim of this investigation was to develop a method of measuring oxygen consumption in a suspension of neutrophils with the aid of the spin probe 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxyl (CTPO). The suggested method of measuring oxygen concentration is based on determination of parameters of the EPR spectrum of the probe, which depend on the level of spin exchange between the oxygen molecule and the spin probe molecule. Interaction of the probe with an oxygen molecule widens the lines of the superhyperfine structure of the spectrum and leads to reduction of their amplitude. The principle of determination of the oxygen concentration was suggested previously in [1, 9, 13].

EXPERIMENTAL METHOD

Heparinized blood from healthy blood donors was used to obtain neutrophils. The neutrophils were isolated by sedimentation during centrifugation of a Ficoll-Verografin density gradient (1.078 g/cm^3) [4]. Contaminating erythrocytes were removed by hypotonic lysis. The cell suspension contained 93% of neutrophils with a viability of over 95%. The neutrophils were activated by zymosan particles and opsonized in heterologous serum as in [10]. The rate of oxygen consumption was measured with the aid of a CTPO probe generously provided by L. A. Krinitskaya, of the Institute of Chemical Physics, Academy of Sciences of the USSR.

The verografin was obtained from "Chirana," the Ficoll-400 from "Sigma," and the xanthine oxidase, xanthine, and cytochrome C from "Serva." Xanthine oxidase activity was determined as the degree of reduction of cytochrome C on a "Beckman" spectrophotometer (USA). EPR spectra were recorded on a "Varian E-4" radiospectrometer (USA).

EXPERIMENTAL RESULTS

Figure 1 shows the EPR spectrum of the central component of CTPO in solution containing different oxygen concentrations, and it can be seen that as the oxygen concentration in the solution falls, resolution of the superhyperfine structure of the probe increases. Lowering the oxygen concentration reduces spin exchange between CTPO and oxygen, and thereby narrows the lines of the superhyperfine structure and increases their intensity. The change in the EPR spectrum of CTPO with a change in oxygen concentration can be characterized by the value of α, equal to the ratio of the sum of components of the superhyperfine structure to twice the amplitude of the signal (Fig. 1).

Department of Biophysics, N. I. Pirogov Second Moscow Medical Institute. Laboratory of the Biophysical Bases of Pathology, Research Institute of Physicochemical Medicine, Ministry of Health of the RSFSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR Yu. A. Vladimirov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 11, pp. 561-563, November, 1989. Original article submitted December 25, 1988.

 0007-4888/89/0011-1572$12.50

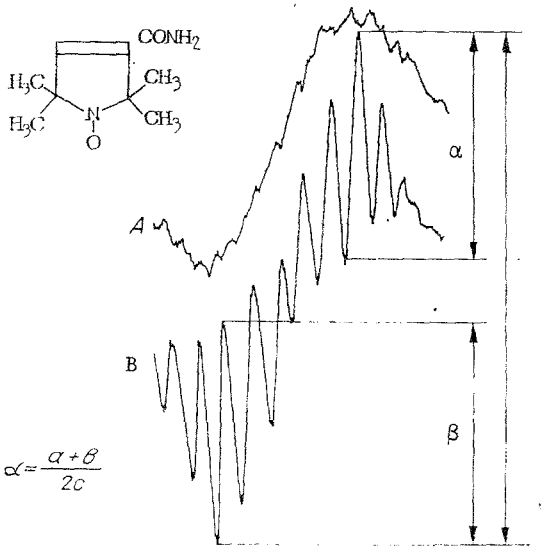

Fig. 1

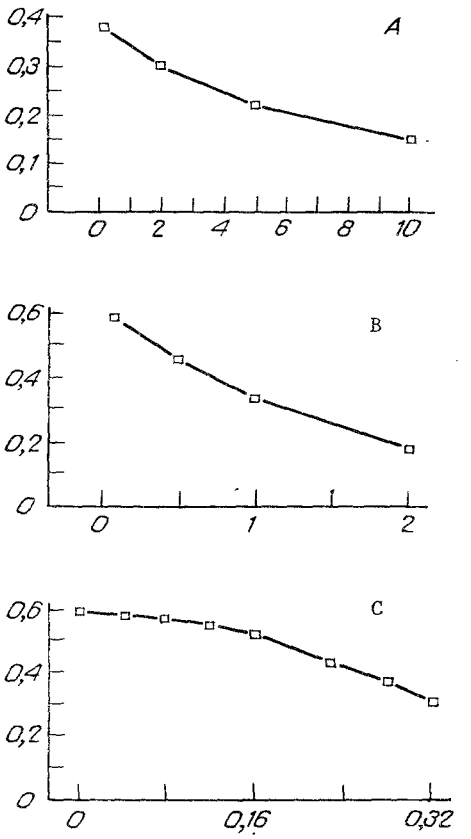

Fig. 2

Fig. 1. EPR spectrum of CTPO (0.1 mM) in Hanks' solution with different oxygen concentrations. A) Oxygen concentration 192 μmoles/liter (saturating oxygen concentration at 20°C); B) in the absence of oxygen. Determination of value of parameter α from EPR spectrum.

Fig. 2. Dependence of parameter α on CTPO concentration (B, × 10^4 (in mM) and on conditions of recording spectrum: shf power (A, in mW) and amplitude of modulation (C, in G). CTPO dissolved in Hanks' solution. Specimen exposed to a vacuum of 0.01 mm Hg at 37°C. Concentration of STPO for graphs A and C was 0.1 mM.

The sensitivity of the method can be increased and, consequently, the error of measurement of the parameter α reduced, if recording conditions are chosen in which the parameter α reaches its highest value. Figure 2 shows dependence of the parameter α of CTPO in the absence of oxygen on concentration of the probe and under different conditions of recording of the spectrum. Clearly the value of the parameter α rises with a fall in concentration of the probe, of the amplitude of modulation, and of the shf power applied. As a result of these measurements, recording conditions corresponding to the maximal value of the parameter α were chosen: concentration of probe 0.1 mM, amplitude of modulation 20 mG, shf power 0.3 mW.

The relationship between the value of the parameter α and oxygen concentration can be established, knowing the oxygen concentration and measuring the value of α. To calibrate the parameter α its values were measured in the presence of oxygen in a concentration corresponding to its saturating concentration in Hanks' solution at 37°C, namely 192 μmoles/liter, and in the absence of oxygen. At these values of oxygen concentration the value of the parameter α was 0.443 and 0.030, respectively.

The linear character of the relationship between the value of α and the oxygen concentration can be determined by using a system consuming oxygen at a constant rate. As such a system we chose the xanthine oxidase system. With a concentration of xanthine oxidase of 80 μg/ml and xanthine of 500 μM, the rate of oxygen utilization was constant at 1 mmole/min. These measurements showed that dependence of the parameter α on oxygen concentration is linear in character and can be described by the following equation.

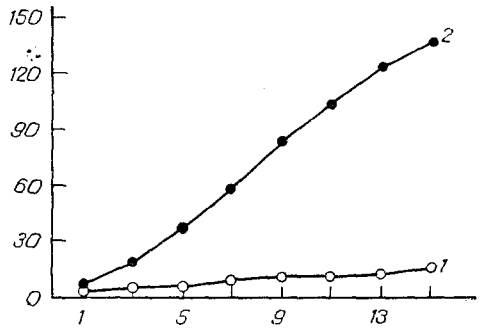

Fig. 3. Kinetic curves of oxygen utilization by peripheral blood neutrophils. 1) Resting cells; 2) cells activated by zymosan. Measurements made at 37°C, ratio of zymosan to cell 100:1, cell concentration 10^7/ml, probe concentration 0.1 mM. Abscissa, time (in min); ordinate, quantity of oxygen utilized (in mmoles/10^7 cells).

$$/O_2/ = 206 \quad \mu M - 0.465\alpha$$

Knowing the relationship between the parameter α and the oxygen concentration, the rate of utilization of oxygen by the cells can be measured. Typical curves of oxygen utilization by neutrophils before and after their activation by particles of opsonized zymosan are given in Fig. 3. They show that the kinetic curve of oxygen utilization by neutrophils after activation is linear in character between 2 and 12 min. This period corresponds to the peak rate of oxygen uptake. On average the maximal rate of oxygen uptake by the neutrophils before and after activation was 1.45 ± 0.21 and 11.28 ± 1.78 nmoles/min/10^7 cells, respectively. These results are in agreement with data in the literature obtained polarographically [3, 5].

The method of determining the rate of oxygen uptake with the aid of CTPO described above thus enables the oxygen concentration to be measured in a cell suspension with an accuracy of 1 μM, and in this case, moreover, the oxygen concentration is measured in the system both in the solution and in phagosomes and cells. However, besides the more accurate determination of the oxygen concentration, the method used also has other advantages over the polarographic method using a Clark's electrode: a) spin probes do not utilize oxygen, as is the case of a Clark's electrode so that it is possible to measure slow processes, coupled with oxygen consumption; b) the suggested method does not require mixing, which may damage or activate cells; c) while possessing equal sensitivity with the polarographic method, the suggested method needs a smaller volume of sample (50-100 μl) [13].

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