MEASUREMENT OF SUPEROXIDE RADICALS FORMED BY ACTIVATED HUMAN NEUTROPHILS BY ACCUMULATION OF STABLE NITROXYL RADICALS, RECORDED BY THE EPR METHOD

A. N. Osipov, L. S. Vartanyan, O. A. Azizova, and Yu. A. Vladimirov*

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Their ability to form superoxide radicals ($O_2^{\cdot-}$) under the influence of various activators is a parameter of the functional activity of neutrophils. In this connection it is interesting to develop new and adequate methods of determination of $O_2^{\cdot-}$, generated by phagocytic cells. In recent years several investigations have been published in which it was suggested to use the reaction of $O_2^{\cdot-}$ with hydroxylamines (HA) of the oxazolidine [5, 6], imidazoline, and piperidine series [1-3], with the formation of stable nitroxyl radicals (NR), recordable by the EPR method, to determine the rate of formation of $O_2^{\cdot-}$ in different systems.

In [5, 6] bases of HA of the oxazolidine series, readily oxidized in air, were used. The use of hydrochlorides instead of bases in the case of HA of the imidazoline and piperidine series gives an essential advantage: hydrochlorides of HA in the crystalline state are stable for several months when kept at room temperature and do not require reduction before the experiment [2]. The velocity constants of the reaction of $O_2^{\cdot-}$ with different HA are closely similar, and are of the order of 10^3 M^{-1} sec^{-1}.

EXPERIMENTAL METHOD

Neutrophils were obtained from donated blood as in [4] and kept in Hanks' medium, buffered with 100 mM tris-HCl, pH 7.4, at 0°C. Phorbol myristate acetate (PMA) was used as the principal activator, but in some specially discussed cases, a formylated tripeptide was used (formyl-methionyl-leucyl-phenylalanine). The reaction mixture contained: HA 3.9 mM, diethylenetriaminepentaacetate (DETAPAC) 0.1 mM, PMA 0.27 μM, Hanks' solution, and neutrophils. The reaction was started by addition of PMA, after which the reaction mixture was quickly transferred into the cuvette of an EPR-spectrometer and NR formation was recorded during activation of the neutrophils. The reaction was carried out in a flat quartz cuvette at 37°C. EPR spectra were recorded on a "Varian E-4" EPR radiospectrometer (USA). The hydrochloride of HA 1-oxy-2,2,6,6-tetramethyl-4-oxopiperidine and the corresponding NR 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl were synthesized and generously provided by L. A. Krinitskaya, to whom the authors are grateful.

The EPR spectrum of NR is a triplet with a_N = 16 G, a g-factor of 2.00050, and a line half-width (H_{pp}) of 0.4 G [3]. The concentration of the radicals was determined as the intensity of the second component of the spectrum. A solution of the radical of known concentration served as the standard. PMA was obtained from "Sigma," USA, DETAPAC from "Koch-Light" England, superoxide dismutase (SOD) from the Institute of Biological Chemistry, Academy of Sciences of the Armenian SSR, and luminol from LKB, Sweden. All solutions were made up in bidistilled water and DETAPAC was added, for HA in an aqueous medium at pH 7.4 is readily oxidized in the presence of traces of heavy metals.

*Academician of the Academy of Medical Sciences of the USSR.

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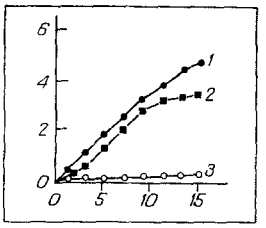

Fig. 1. Kinetic curves of NR formation from HA by activated neutrophils (1), accumulation of reduced cytochrome *c* (2), and self-oxidation of HA in the absence of cells (3). Abscissa, time (in min); ordinate, $O_2^{\cdot-}$ concentration $\times 10^5$ (in M).

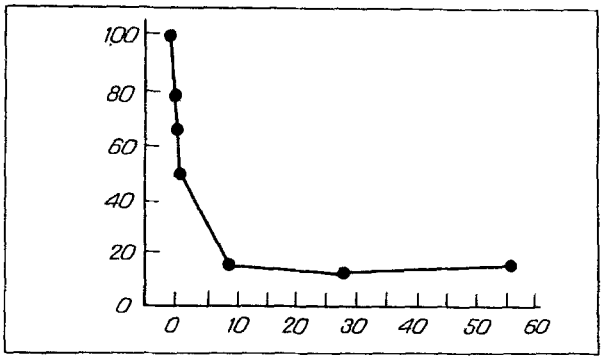

Fig. 2. Effect of SOD on velocity of NR formation from HA in the presence of neutrophils activated by PMA. Abscissa, SOD concentration (in nM); ordinate, velocity of NR formation (in %).

EXPERIMENTAL RESULTS

The kinetic curve (1) of NR formation in a system containing activated neutrophils is shown in Fig. 1. Clearly the process of NR formation has an induction period of 1-2 min, after which the reaction velocity reaches a constant value. In the absence of neutrophils HA is oxidized at a constant velocity, which is under 5% of that with the complete system. This velocity is $2 \cdot 10^{-9}$ mole/sec (curve 3). The kinetic curve of NR accumulation in Fig. 1 (1) was obtained by subtracting the kinetic curve of NR formation during self-oxidation of HA (curve 3) from the total kinetic curve of NR (not given in Fig. 1).

Comparison of the curve of NR formation (1) and of accumulation of reduced cytochrome c (2) shows that the velocities of the two reactions in the linear region of the kinetic curve are virtually identical for both methods.

The sensitivity of the reaction of NR formation to SOD is an indication that NR formation was induced by interaction between HA and $O_2^{\cdot-}$ radicals. It was shown that with SOD in a concentration of over 10^{-7} the reaction was inhibited by 90% or more (Fig. 2). Consequently, the kinetic curve of NR formation reflects $O_2^{\cdot-}$ formation in the system virtually completely.

The reversibility of the reaction of HA oxidation by $O_2^{\cdot-}$ radicals was studied, It was found that on incubation of independently synthesized NR with activated neutrophils for 20 min or more, there was no change in the intensity of the EPR signal. Consequently, the reaction of reduction of NR by activated neutrophils is not significant, and the observed kinetics of NR formation reflects the process of interaction between HA and $O_2^{\cdot-}$.

With the HA concentration used (3.9 mM) there is evidently complete interception of the $O_2^{\cdot-}$ radicals formed, for with a further increase in the HA concentration the velocity of NR formation remained virtually unchanged. On the basis of this fact, the absolute number of $O_2^{\cdot-}$ radicals formed in the system can be estimated.

Dependence of the velocity of NR formation on the concentration of neutrophils in the system was measured. The rate of $O_2^{\cdot-}$ formation was determined as the tangent of the angle of slope of the SOD-sensitive part of the kinetic curve of NR formation by activated neutrophils. Absolute values of the $O_2^{\cdot-}$ concentration were taken to be equivalent to the NR concentration (the SOD-sensitive part of the spectrum) with HA in a concentration of 3.9 mM. The velocity of $O_2^{\cdot-}$ formation by neutro-

phils was found to be linear in character with a change in the cell concentration from $4 \cdot 10^5$ to $6 \cdot 10^6$ in 1 ml, i.e., a change of 1.5 orders of magnitude. Thus HA can be used for the quantitative determination of $O_2^{\cdot-}$ formed by neutrophils after their activation. The relationship between the velocity of $O_2^{\cdot-}$ formation and the cell concentration is described by the equation:

$$[O_2^{\cdot-}] = 0.763 \cdot [\text{neutrophils}] + 0.09,$$

where the velocity of $O_2^{\cdot-}$ formation is expressed in μmoles/min, and the concentration of neutrophils in millions/ml. An attempt was made in [6] to use HA to determine the velocity of $O_2^{\cdot-}$ formation by whole cells. However, the authors cited were unable to obtain a linear relationship between the velocity of $O_2^{\cdot-}$ formation and the concentration of neutrophils in the sample. The reason was evidently that with an arbitrarily chosen ratio of concentrations of HA (0.5-1.0 mM) and cells ($1.25 \cdot 10^6$ cells/ml), complete interception of $O_2^{\cdot-}$ radicals could not be achieved.

The sensitivity of the suggested method of measuring $O_2^{\cdot-}$ formation was compared with that of the method of chemiluminescence, traditionally used, on the same sample of cells. The concentration of cells with which the rate of NR formation in the presence of PMA was 3 times higher than the rate of NR formation during self-oxidation of HA in the absence of cells was taken as the lower limit of sensitivity of the suggested method. This cell concentration was found to be 400,000 cells/ml (volume of cuvette 0.1 ml). In the case of the chemiluminescence method, the ratio of amplitude of signal in the presence of neutrophils to amplitude of signal in their absence was 3, and it was observed with cells in a concentration of 40,000/ml (volume of cuvette 1 ml), i.e., in a concentration 10 times smaller, but with the same abeolute number of cells as in the method using NR. However, if it is considered that chemiluminescence of activated neutrophils in the presence of luminol is inhibited by SOD by 50%, and NR formation is reduced by 90%, this means that the sugge5ted method is more specific in relation to $O_2^{\cdot-}$.

When the formylated tripeptide (formly-methionyl-leucylphenylalanine) was used as activator, similar results were obtained. In the case of activation of neutrophils by opsonized zymosan, however, a marked increase was observed in the velocity of self-oxidation of HA, and this reduced the sensitivity of the method.

LITERATURE CITED

1. L. S. Vartanyan and S. M. Gurevich, Biokhimiya, **51**, No. 5 (1989).
2. Yu. E. Rashba, V. A. Chernikov, L. M. Baider, and L. S. Vartanyan, Biol. Membr., **3**, No. 8, 838 (1986).
3. Yu. E. Rashba, L. S. Vartanyan, L. M. Baider, and L. A. Krinitskaya, Biofizika, **34**, No. 1, 57 (1989).
4. A. Boyum, Scand. J. Clin. Lab. Invest., Suppl. 97, 77 (1968).
5. F. E. Patton, G. M. Rosen, and E. J. Rauckman, Molec. Pharmacol., **18**, 588 (1980).
6. G. M. Rosen, E. Finkelstein, and E. J. Rauckman, Arch. Biochem., **215**, 3G7 (1982).