PRODUCTION OF SUPEROXIDE ANION BY AN NADPH-OXIDASE FROM RAT PULMONARY MACROPHAGES

Maureane HOFFMAN and Anne P. AUTOR
The Toxicology Center, Department of Pharmacology, The University of Iowa, Iowa City, IA 52242, USA

Received 4 September 1980
Revised version received 14 October 1980

1. Introduction

Superoxide anion (O_2^-) is produced by polymorphonuclear leukocytes coincident to the respiratory burst which occurs in response to certain soluble and particulate stimuli [1,2]. This univalently reduced form of oxygen plays a role directly or indirectly in the microbicidal function of leukocytes [3,4]. The production of O_2^- by pulmonary macrophages following activation of the cells with soluble or particulate stimuli has also been demonstrated [5]. A membrane-bound flavoprotein dormant in resting leukocytes but activated by specific stimuli catalyzes the reduction of molecular oxygen with NADPH as the source of electrons. Enzymes of this type have been demonstrated to reside in the subcellular granules and plasma membrane of activated leukocytes [6–9]. Cytochrome *b* involvement in this process has also been suggested [10].

A superoxide-generating enzyme has also been reported to be present in the endoplasmic reticulum and mitochondrial membrane, but not the plasma membrane, of resting, non-activated pulmonary macrophages [11]. Here, we describe a pyridine nucleotide-dependent superoxide generating activity with a high affinity for NADPH which may be solubilized from a mixed membrane preparation from resting rat pulmonary macrophages and macrophages stimulated with opsonized, killed yeast particles.

2. Materials and methods

Pulmonary macrophages were isolated from excised, minced lung tissue as in [12]. Cells were suspended in 1 mM phosphate-buffered saline (PBS) and activated, as described for leukocytes, by incubation at 37°C with opsonized killed yeast cells for 7 min in the presence of 1 mM NaN_3 [13]. In control experiments, macrophages were incubated with unopsonized killed yeast cells. The mixed membrane, particulate fraction was isolated as described for leukocytes [14]. Solubilization of the enzyme was accomplished by sonication of the mixed membrane particulate fraction at −5°C using three 15 s pulses at 75 W with a 45 s cooling period between pulses in an extraction buffer. The extraction buffer consisted of 0.34 M sucrose, 0.25% deoxycholate, 15% (v/v) DMSO, 0.1 M Tris–HCl at pH 8.0. This suspension was centrifuged at 105 000 × *g* for 30 min. The pellet was resuspended in extraction buffer and both pellet and supernatant assayed for superoxide production. Protein was determined by the Lowry method [15]. Superoxide anion generation was monitored by measuring the rate of superoxide dismutase inhibitable ferricytochrome *c* reduction at 550 nm. A millimolar extinction coefficient of 18.5 was used for ferricytochrome *c* [16]. Assay mixtures contained 75 μM ferricytochrome *c*, 50 μl mixed membrane-particulate fraction extract or resuspended pellet (test sample), 1.8–90 μM NADPH, 1 mM KPO_4 buffer at the stated pH in 1 ml total vol. This was read against an identical mixture which contained 250 units superoxide dismutase (Biotics Research).

3. Results

3.1. *Production of superoxide anion by extract from resting and activated macrophages*

The maximum activity of the solubilized NADPH-oxidase was ~6.8 nmol ferricytochrome *c* reduced . min^{-1} . mg $protein^{-1}$ ± 1.7 for the stimulated enzyme (n = 5) at pH 7.5. The maximum activity for the

enzyme from unstimulated macrophages was 6.9 nmol ferricytochrome *c* reduced . min^{-1} . mg $protein^{-1}$ ± 2.5 (n = 3). There was negligible superoxide-generating activity in the pellet resuspended following extraction. The K_m for NADPH for the stimulated enzyme was 4.98 μM ± 1.45 (n = 5) at pH 7.5. The K_m for NADPH for the unstimulated enzyme was 21.7 μM ± 3.90 (n = 3) at pH 7.5. The quite different K_m values for NADPH with very similar values for maximal velocity of superoxide production for the activities from stimulated and resting macrophages are presented graphically in fig.1. The slope of Hill plots of the reaction catalyzed by the enzyme from stimulated cells ranged from 0.99–1.07 (n = 5). Hill plots of the reaction catalyzed by the enzyme from resting macrophages had slopes ranging from 0.89–1.0 (n = 3). Addition of 250 units superoxide dismutase to the reaction mixture inhibited the reduction of ferricytochrome *c* by 90–100%.

No reduction of ferricytochrome *c* occurred at any pH >6.0 when either NADPH or test sample was omitted from the assay mixture. However, at pH 5.5 and 6.0 significant reduction of ferricytochrome *c* occurred in the presence of test sample but absence of NADPH. This rate was subtracted from the rate obtained following addition of NADPH for the calculation of NADPH oxidase-catalyzed O_2^- production.

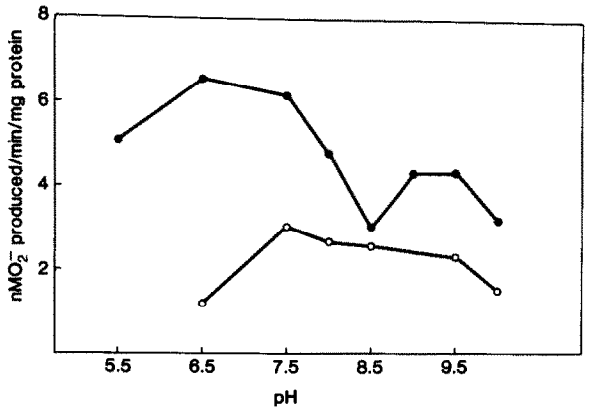

Fig.1. Comparison of double reciprocal plots of superoxide production as a function of NADPH concentration by detergent extracts of unstimulated (○) and stimulated (●) pulmonary macrophages. Macrophages were stimulated with opsonized killed yeast cells. NADPH-oxidase activity was solubilized from macrophage preparations and superoxide production was measured as in section 2. Velocity of the reaction is measured as nanomoles of superoxide produced per minute per mg of protein added to the assay mixture.

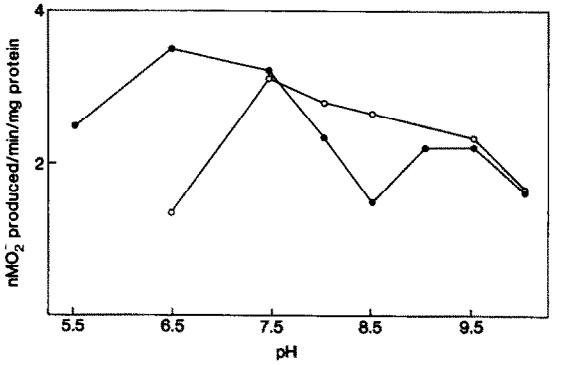

Fig.2. Superoxide production of the solubilized preparation from unstimulated and stimulated pulmonary macrophages as a function of pH. Superoxide production was assayed as in section 2. The buffer was adjusted to the indicated pH by mixing 1 mM solutions of monobasic (pH 4.0) and dibasic (pH 9.0) potassium phosphate.

3.2. *pH-dependence of the NADPH oxidase*

The enzymes from yeast-stimulated and resting pulmonary macrophages maintain activity over a broad pH range (fig.2). A relative maximum occurs at pH 6.5–7.5 for the enzyme from stimulated cells. At higher pH the NADPH-oxidase continues to exhibit significant activity, and a second smaller relative maximum in enzyme activity occurs at pH ~9.0. The solubilized enzyme preparation from resting pulmonary macrophages exhibits maximal activity at pH ~7.5, which declines sharply at lower pH.

4. Discussion

A superoxide-generating NADPH oxidase activity can be solubilized from a mixed membrane particulate fraction of pulmonary macrophages. The activity is present in both resting cells and those stimulated by opsonized killed yeast cells. The K_m of the NADPH-oxidase from resting macrophages for NADPH, however, is 4-fold greater than that from stimulated macrophages. The low K_m for NADPH (4.98 μM) of the enzyme from stimulated macrophages is in marked contrast to K_m values ~8-fold higher which have been reported for the NADPH-oxidase from leukocytes [9,17], as well as the K_m of 8.3 mM for NADPH reported for the enzyme in ER and mitochondrial membranes from resting pulmonary macrophages [11]. This suggests that the enzyme activity described here which is solubilized from a preparation containing a

mixture of cellular membranes from pulmonary macrophages is unlike the enzyme activity isolated and described by others.

The equality of the maximal rate of superoxide production from resting and stimulated macrophages may indicate that the same enzyme or enzymes in the same amount are present both before and after activation. However, the much lower K_m and the apparent shift in pH dependence of the enzyme after stimulation would indicate that a change in the enzyme has occurred during stimulation which is stable enough to survive the fractionation and solubilization procedures. It has been suggested that transformation of leukocyte NADPH-oxidase from allosteric to normal hyperbolic kinetics occurs following stimulation of leukocytes, thus effectively lowering the K_m [8]. Allosteric kinetics were not a characteristic of the solubilized NADPH-oxidase obtained in this study from resting pulmonary macrophages as indicated by the slope of the Hill plot and the linearity of the double reciprocal plot. However, it is possible that the pulmonary macrophages may be partially stimulated by the cell isolation procedure or may reside in the lung in a partially activated state because of their constant contact with the external environment. Partial activation of the macrophages by any means would obscure a change in the kinetics of the NADPH from allosteric to hyperbolic.

It can be concluded from these data that:

(1) Pulmonary macrophages possess an NADPH-oxidase which in the presence of substrate generates superoxide anion;

(2) The apparent K_m of this enzyme for NADPH decreases upon stimulation of the cells, which would have the effect of increasing enzyme activity under conditions of unchanged substrate concentration.

Acknowledgements

M. H. was supported by NIH grants GM 12675 and the Medical Scientists Training Program GM 07337. A. P. P. is recipient of a Research Career Development Award from the National Institutes of Child Health and Human Development HD 0068.

References

[1] Babior, B. M. (1978) New Engl. J. Med. 298, 659–668.
[2] DeChatelet, L. R. (1978) J. Reticulo. Soc. 24, 73–91.
[3] Babior, B. M., Kipnes, R. S. and Curnette, J. T. (1973) J. Clin. Invest. 52, 741–744.
[4] Baehner, R. L., Murrmann, S. K., Davis, J. and Johnston, R. B. (1975) J. Clin. Invest. 56, 571–576.
[5] Holian, A. and Danielle, R. P. (1979) FEBS Lett. 108, 47–50.
[6] Curnette, J. T., Kipnes, R. S. and Babior, B. M. (1975) New Engl. J. Med. 243, 628–632.
[7] Briggs, R. T., Karnovsky, M. L. and Karnovsky, M. J. (1977) J. Clin. Invest. 59, 1088–1098.
[8] DeChatelet, L. R., Shirley, P. S., McPhail, L. C., Iverson, D. B. and Doellgast, G. J. (1978) Infect. Immun. 20, 398–405.
[9] Gabig, T. G. and Babior, B. M. (1979) J. Biol. Chem. 254, 9070–9074.
[10] Segal, A. W. and Jones, O. T. G. (1978) Nature 276, 515.
[11] Andrew, P. W., Lowrie, D. W., Jackett, P. S. and Peters, T. J. (1980) Biochim. Biophys. Acta 611, 61–71.
[12] Stevens, J. B. and Autor, A. P. (1977) Lab. Invest. 37, 470–478.
[13] Babior, B. M. and Kipnes, R. S. (1977) Blood 50, 517–524.
[14] Gabig, T. G., Kipnes, R. S. and Babior, B. M. (1978) J. Biol. Chem. 253, 6663–6665.
[15] Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[16] Margoliash, E. and Frohivirt, N. (1959) Biochem. J. 71, 570–572.
[17] Tauber, A. I. and Goetzl, E. J. (1979) Biochem. 18, 5576–5584.