Vol. 91, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1979 Pages 222-226

SUPEROXIDE PRODUCTION BY PHAGOCYTES

Another look at the effect of cytochrome c on oxygen uptake by stimulated neutrophils

Bernard M. Babior

Blood Research Laboratory and Department of Medicine, Tufts-New England Medical Center, Boston, Mass. 02111

Received September 27, 1979

Summary

The widely held view that stimulated phagocytes liberate O_2^- into the extracellular medium is supported by the alterations in oxygen uptake which occur when ferricytochrome c is added to a suspension of zymosan-treated neutrophils. An explanation consistent with this view is provided for some previously reported results (FEBS Lett. 100, 27) which initially appeared to conflict with the notion that O_2^- is released by phagocytes.

The exposure of neutrophils to suitable stimuli results in the activation of an enzyme which catalyzes the following reaction: $2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP$ (1). This reaction can be demonstrated by measuring either oxygen uptake or O_2^- production. According to the reaction stoichiometry, oxygen uptake and O_2^- production should occur on a mole for mole basis, but equivalence is rarely observed in practice (2,3). This is because not all the O_2^- produced by the cell will be detected in the assay, and also because some of the oxygen taken up by the neutrophil will be returned during the course of secondary reactions involving O_2^-.

An example of such a secondary reaction is the reduction of cytochrome c by O_2^-: ferricytochrome c + O_2^- → ferrocytochrome c + O_2. In this reduction, one molecule of oxygen is liberated for every molecule of O_2^- which reacts with the cytochrome. Because of this reaction, the addition of ferricytochrome c to a neutrophil suspension in which oxygen is being consumed to form O_2^-

0006-291X/79/210222-05$01.00/0

should lead to a diminution in net oxygen uptake, because some of the oxygen which has been converted to O_2^- will be regenerated by the reaction between the O_2^- and the cytochrome.

Recently, a report has appeared from Segal's laboratory suggesting that O_2^- is in fact not released from stimulated neutrophils (4). This suggestion was based on the observation that the addition of ferricytochrome c to a suspension of activated neutrophils did not lead to the expected fall in oxygen uptake. Because this suggestion is at variance with the conclusions drawn by many other workers concerning the production of O_2^- by neutrophils (5,6), I decided to restudy this question in the hope of reconciling the differences.

Materials and methods

Neutrophils (7) and opsonized zymosan (8) were prepared as previously described and suspended at appropriate concentrations in Hanks buffered saline solution containing 1.5 mM HEPES buffer (pH 7.4) (Hanks/HEPES). Ferricytochrome c (Sigma type VI) and bovine erythrocyte superoxide dismutase (Sigma) were dissolved in Hanks/HEPES.

Oxygen uptake and O_2^- production were both measured at 37° in a Gilson oxygen uptake chamber (effective volume 1.2 ml) fitted with a Yellow Springs oxygen electrode. To measure oxygen uptake, neutrophils plus enzymes as indicated, in a total volume of 1.3 ml, were placed in the chamber of the oxygen electrode and incubated for 5 minutes with vigorous stirring to permit temperature equilibration. Opsonized zymosan (2.5 mg in 0.1 ml, prewarmed to 37°) was then added, and oxygen uptake was followed for the next 5 minutes. To measure O_2^- production, neutrophils and cytochrome c (total volume 1.3 ml) were placed in the chamber of the oxygen electrode and brought to 37° as described above. Opsonized zymosan (2.5 mg in 0.1 ml, prewarmed to 37°) was then added. O_2^--dependent cytochrome c reduction was terminated by the addition of 10 µg superoxide dismutase either immediately (reference) or after 2 minutes (sample). Two and a half minutes after the addition of the zymosan, the reaction mixture was transferred from the oxygen electrode into a test tube placed in melting ice, the cells and zymosan were removed by centrifugation at 0°, and O_2^--dependent cytochrome c reduction was determined by difference spectroscopy (sample vs reference) as described elsewhere (9).

Results and discussion

Experiments were done at two cell concentrations: 2×10^7 cells/ml, the concentration used in Segal's experiments, and 4×10^6 cells/ml. Oxygen uptake by zymosan-stimulated neutrophils at these two concentrations is shown in Fig.1. Uptake at the higher cell concentration is similar to that reported by Segal.

The effect of superoxide dismutase and ferricytochrome c on oxygen uptake by neutrophils was determined. As reported by Segal, the presence of 0.25 mM

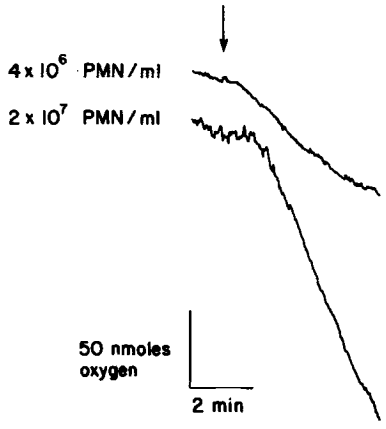

Fig. 1. Oxygen uptake by zymosan-stimulated neutrophils. Reaction mixtures contained neutrophils as the concentrations indicated, together with the opsonized zymosan. The zymosan was added at the point indicated by the arrow. Oxygen uptake was determined as described in the text.

Table 1. The effect of cytochrome c and superoxide dismutase on oxygen uptake by zymosan-stimulated neutrophils. Oxygen uptake was determined as described in the text. The concentrations of selected constituents were as follows: neutrophils, 4 x 10^6/ml; cytochrome c, 0.25 mM; superoxide dismutase, 10 µg/ml. Oxygen uptake is expressed as nmoles consumed during the designated time intervals. The results represent the mean ± SE of 4 experiments.

Additions	Oxygen uptake (nmoles)	
	2 min	5 min
None	35.2 ± 1.7	80.4 ± 5.0
Cytochrome c	23.2 ± 2.5	57.1 ± 5.7
Dismutase	31.2 ± 2.1	65.3 ± 3.2
Cytochrome c plus dismutase	35.5 ± 4.8	72.0 ± 1.7

ferricytochrome c had little effect on oxygen consumption in experiments carried out with neutrophils at a concentration of 2 x 10^7 cells/ml (results not shown). When a lower concentration of neutrophils was treated with the same concentration of cytochrome c, however, a decrease in net oxygen uptake of over 30% was observed. The further addition of superoxide dismutase returned oxygen uptake toward control levels. Cytochrome c was found to have only a slight effect on oxygen uptake in the presence of superoxide dismutase. These results, shown in Table 1, are consistent with the widely held notion that stimulated neutrophils release O_2^- into the medium.

What accounts for the differences between the results at the two cell concentrations? One possibility has to do with the efficiency with which the O_2^- released by the neutrophil was trapped by the cytochrome. When the cell concentration is high, it might be expected that only a small fraction of the liberated O_2^- will react with the cytochrome, the remainder undergoing rapid dismutation and other secondary reactions. Under such circumstances, the return of oxygen due to the reaction of O_2^- with cytochrome c might be too small relative to the total oxygen uptake to be detected in the assay. Conversely, at a low cell concentration, most of the liberated O_2^- is likely to react with the cytochrome, with the return of a substantial portion of the oxygen originally consumed in the O_2^--forming reaction.

The results in Table 2 show that the foregoing explanation appears to account for the present observations. In assays of O_2^--dependent cytochrome c reduction carried out at a neutrophil concentration of 4×10^6/ml, the amount of cytochrome reduced at a cytochrome c concentration of 0.1 mM was the same as that reduced when the cytochrome concentration was increased by a factor of 2.5. This finding indicates that all the O_2^- released by the neutrophils into the extracellular medium was trapped by the cytochrome even at the lower of the two cytochrome c concentrations. In contrast are the results obtained at 2×10^7 cells/ml. At this cell concentration, half again as much O_2^- was captured by 0.25 mM cytochrome c as was trapped by the lower concentration of the cytochrome. Furthermore, even at the highest cytochrome concentration, the amount of cytochrome c reduced by O_2^- was far less than the amount expected on the basis of the results obtained at 4×10^6 cells/ml,

Table 2. O_2^--dependent cytochrome c reduction by zymosan-stimulated neutrophils. O_2^--dependent cytochrome c reduction was determined as described in the text. The concentrations of neutrophils and cytochrome c are shown in the table. The results represent the mean ± SE of 5 experiments.

Cell concentration	O_2^--dependent cytochrome c reduction (nmoles/2 min)	
	0.1 mM cyt c	0.25 mM cyt c
4×10^6 PMN/ml	22.4 ± 4.2	21.6 ± 2.7
2×10^7 PMN/ml	35.8 ± 3.2	53.6 ± 8.6

suggesting that substantial quantities of O_2^- still escaped detection. It appears probable from these results that most of the O_2^- released by the stimulated neutrophils under the conditions employed in Segal's experiments failed to react with the cytochrome, and it is for this reason that the expected drop in oxygen uptake was not observed in those experiments.

The present observations provide additional evidence that stimulated neutrophils liberate O_2^- into the medium. They also illustrate some of the problems associated with the determination of substances as short-lived as the O_2^- radical.

Acknowledgement. I thank Janet Mitchell for excellent technical assistance. Supported in part by Grant AI 11827 from the National Institute of Allergy and Infectious Diseases.

References

1. Babior, B.M., Curnutte, J.T., McMurrich, B.J. (1976) J. Clin. Invest. 58, 989-996.
2. Root, R.K., Metcalf, J.A. (1977) J. Clin. Invest. 60, 1266-1279.
3. Weening, R.S., Wever, R., Roos, D. (1975) J. Lab. Clin. Med. 85, 245-252.
4. Segal, A.W., Meshulam, T. (1979) FEBS Lett. 100, 27-32.
5. Babior, B.M. (1978) N. Engl. J. Med. 298, 659-665.
6. Klebanoff, S.J., Clark, R.A. (1978) The neutrophil: function and clinical disorders, North-Holland, New York, p. 294-295.
7. Babior, B.M., Kipnes, R.S., Curnutte, J.T. (1973) J. Clin. Invest. 52, 741-744.
8. Hohn, D.C., Lehrer, R.I. (1975) J. Clin. Invest. 55, 707-713.
9. Curnutte, J.T., Whitten, D.M., Babior, B.M. (1974) N. Engl. J. Med. 290, 593-597.