Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 44-49

THE RELEASE OF SUPEROXIDE ANION FROM GRANULOCYTES: EFFECT OF INHIBITORS OF ANION PERMEABILITY

Renato Gennaro and Domenico Romeo
Istituto di Chimica Biologica, Università di Trieste, 34127 Trieste, Italy

Received March 26, 1979

SUMMARY: Generation of superoxide anion (O_2^-) by phagocytizing bovine blood granulocytes and extracellular release of the anion were simultaneously assayed by measuring oxygen consumption and O_2^--dependent ferricytochrome c reduction. O_2^- release, but not generation, was markedly inhibited by 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonate and by 1-anilino-8-naphtalene sulfonate, with an I_{50} of about 50 μM. Inhibition of O_2^- release was also observed when a portion of NaCl in the cell suspending medium was replaced by $NaNO_3$ or NaSCN or Na salicylate, with the anions acting in the following order of potency: salicylate > SCN^- > NO_3^-.

Reduction of oxygen to superoxide anion (O_2^-) is markedly increased in both polymorphonuclear and mononuclear phagocytes after exposure to appropriate particulate or soluble stimuli (1-8). While most of the generated O_2^- is likely converted to O_2 and H_2O_2 by the action of endogenous superoxide dismutase (9-11), part of the anion is released into the phagocytic vacuoles and/or into the extracellular space. This highly reactive compound may thus be involved in bacterial killing (1,12) and in tissue damage (13).

The nature of the O_2^- -generating system, its subcellular localization, and the mechanism whereby its activity may be enhanced have partially been clarified (7,8,14-17). Less clear is the mechanism by which O_2^- traverses the cell membrane to reach the surrounding medium and the interior of the phagocytic vacuoles.

Recently, Lynch and Fridovich (18) have shown that O_2^-, generated by xanthine oxidase within vesicles formed from erythrocyte ghosts, escapes into the medium and that this escape is inhibited by stilbene derivatives. Since these compounds are powerful inhibitors of the exchange of anions across the erythrocyte membrane (19-21), Lynch and Fridovich suggested that O_2^- permeates the erythrocyte stroma in the anion channel.

Abbreviations: SITS, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonate; ANS, 1-anilino-8-naphtalene sulfonate; SOD, superoxide dismutase; KRP, Krebs-Ringer phosphate.

0006-291X/79/090044-06$01.00/0

Although to the best of our knowledge the presence of anion channels has never been described in leukocytes, we thought that a system of this type may also be utilized by O_2^- to escape from granulocytes. We therefore tested the effect of SITS and ANS, known inhibitors of anion transport in the erythrocyte as well as in other cells (19-22), on the extracellular release of O_2^- from phagocytizing leukocytes. To distinguish between a possible effect of these compounds on "generation" or "release" of O_2^-, we simultaneously monitored the oxygen consumption by granulocytes and the extracellular recovery of O_2^-, in the absence and in the presence of SITS or ANS.

METHODS: Granulocytes were isolated from bovine blood by employing a technique previously described (5,23). The cells were suspended in a medium consisting of 123 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 16 mM sodium/potassium phosphate, pH 7.4 (KRP medium). Heat-killed *Bacillus mycoides* were opsonized at 37° for 20 min with fresh bovine serum, washed twice and suspended in KRP medium.

Stock solutions of SITS (disodium salt, British Drug House) and of ANS (magnesium salt, recrystallized twice from hot water solutions of the Eastman product) in 0.154 M NaCl were prepared and stored under dim light.

Simultaneous assays of O_2 consumption by and O_2^- release from granulocytes (8) were carried out as follows. $2x10^7$ cells in 2 ml of KRP medium, containing 5 mM glucose, were incubated at 37° in the vessel for O_2 consumption measurement, described elsewhere (24). After recording of basal respiration, ferricytochrome c (150 μM, Sigma, Type III) was injected into the cell suspension, immediately followed by an addition of *B. mycoides* (100 bacteria per granulocyte). After 3 min of continuous recording of O_2 consumption, 10 μl of a SOD (Truett Laboratories) solution (3 mg/ml) were added to the vessel with a microsyringe to stop O_2^- -dependent reduction of cytochrome c. The cell suspension was then rapidly transferred to a conical tube and centrifuged in an Eppendorf 3200 centrifuge for 2 min at max speed. After a 1:1 dilution with KRP medium, the absorbance of reduced cytochrome c in the cell-free supernates was read at 550 nm in a split-beam spectrophotometer. The reference cuvette contained all the reagents at the same concentration of sample cuvette, including cytochrome c in the oxidized form. The absorbance units were converted into nmoles of cytochrome c reduced by using an absorbance coefficient of 21.1 mM^{-1} cm^{-1} (reduced minus oxidized) (25). Specificity of ferricytochrome c reduction by O_2^-, and thus the amount of O_2^- released from granulocytes, was evaluated by including SOD in the cell suspending medium before addition of cytochrome c and bacteria. The recovery of cytochrome c was checked by reduction of the hemoprotein in the sample cuvette with Na dithionite.

The effect of SITS and ANS on O_2 consumption and extracellular release of O_2^- was determined by adding either reagent to the cell suspension 2 min before cytochrome c and bacteria.

Oxidation of xanthine (200 μM) by xanthine oxidase (10 mU/ml, Boehringer) was assayed in KRP medium, containing 5 mM glucose and 0.1% bovine serum albumin, by recording the consumption of oxygen (24). Reduction of ferricytochrome c by O_2^-, generated by the xanthine/xanthine oxidase system, was measured at 37°

by following at 550 nm the increase in absorbance during a reaction time of 3 min. These controls showed that the inhibition of O_2^- -dependent cytochrome c reduction by 100 μM SITS (8%) and by 100 μM ANS (13%) were due to a comparable inhibition of the xanthine oxidase activity. Inhibition of O_2^- -dependent cytochrome c reduction by 10 mM Na salicylate (54%) or NaSCN (6%) could also be fully ascribed to inhibition of xanthine oxidase activity.

RESULTS: Fig. 1 shows that in the presence of either SITS or ANS the amount of O_2^- recovered in the medium surrounding phagocytizing granulocytes is markedly reduced. The I_{50} of both reagents is approximately 50 μM, and maximal inhibition is about 80%.

SITS and ANS affect neither cytochrome c reduction (see "Methods") nor cytochrome c recovery, as determined by total reduction of the hemoprotein with Na dithionite. Furthermore, the generation of O_2^-, as measured by the consumption of O_2, is not modified by ANS and only minimally decreased by SITS (16% inhibition at 200 μM SITS) (Fig. 1).

When a portion of NaCl (10 μmoles/ml) in the KRP medium is replaced by an equivalent amount of either $NaNO_3$ or NaSCN or Na salicylate, the extracellular recovery of O_2^- is inhibited by 8%, 19% and 64%, respectively. The simultaneously determined consumption of O_2, which monitors O_2^- generation, is not modified by NO_3^- and SCN^-, being decreased to 86% of control by salicylate (these values are means of 3 determinations).

DISCUSSION: The experiments presented here have shown that SITS and ANS, known inhibitors of the exchange of anions across the plasma membrane of various cell types (19-22), inhibit the extracellular release of O_2^- from phagocytizing granulocytes, thereby indicating that O_2^- can exit from the granulocyte through an anion channel. Grounds in support of this suggestion are offered by the observation that i) the I_{50} of SITS and ANS for anion transport in erythrocytes and for O_2^- release from granulocytes is comparable (19,22); ii) partial replacement of Cl^- with either NO_3^- or SCN^- or salicylate also causes inhibition of O_2^- release, the order of potency of these anions being the same as that of inhibition of Cl^- efflux from erythrocytes (26); iii) the recovery of O_2^- from concanavalin A-stimulated granulocytes is inhibited by p-diazobenzene sulfonate† (27), another inhibitor of anion transport in the erythrocyte (20).

†Goldstein et al. (27) have attributed this effect to inhibition of O_2^- "generation" and used this result as evidence that a leukocyte ectoenzyme is responsible for O_2^- production. They did not measure, however, the effect of this reagent on O_2 consumption, this assay providing the only direct evidence for inhibition of O_2^- synthetase.

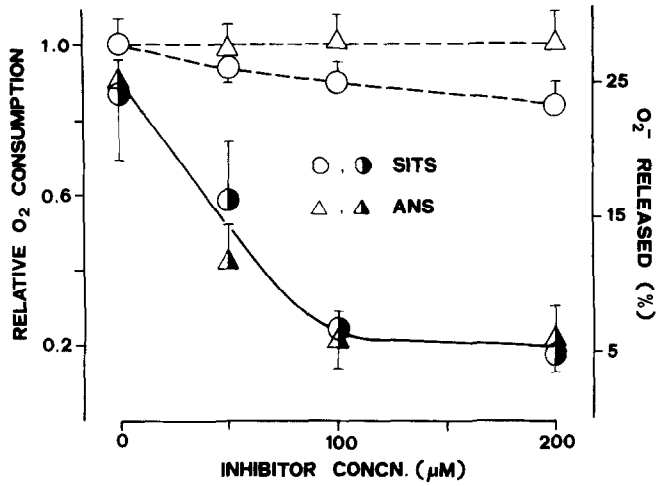

Fig. 1 Effect of SITS and ANS on oxygen consumption and O_2^- release by phagocytizing granulocytes. $2x10^7$ cells were suspended at 37° in KRP medium, containing 5 mM glucose. Oxygen consumption and O_2^- release were measured 3 min after granulocyte exposure to *B. mycoides* (100 bacteria/cell) in the presence of 150 μM ferricytochrome c. Values of oxygen consumption (○, △) are relative to the respiration measured in the absence of either SITS or ANS (77.1 ± 8.8 nmoles O_2/3 min). Values of O_2^- release (◑, ▲) are per cent of O_2^- recovered extracellularly with respect to O_2 consumed. Data are means ± SEM for five (SITS) or four (ANS) experiments.

As for the physiological relevance of a mechanism for anion transport in the granulocyte plasma membrane, apart from its involvement in O_2^- release from the stimulated cell, it is possible that it may be used for exchange of iodide (28-30), an important cofactor for the myeloperoxidase-mediated antimicrobial system (28,30).

Furthermore, it is attractive to speculate that a similar system for O_2^- transport may also be present on the surface of the mononuclear phagocytes. These cells produce and release O_2^- when exposed to either soluble or phagocytosable stimuli (2,6), and the extent of O_2^- release depends on the state of activation of the phagocytes (6). Johnston et al. (6) have suggested that the enhanced O_2^- release from chemically elicited or immunologically activated macrophages might reflect a quantitative increase in the triggering of O_2^- synthetase and/or an alteration in the intracellular concentration or in the intrinsic activity of the enzyme. Were an O_2^- transport system also present on the surface of macrophages, a third possibility would be that the alteration

brought about by the chemical or immunological activation is associated with an enhanced efficiency of this system.

Studies aimed at the unravelling of the mechanism of anion transport in erythrocytes have led to the identification of a membrane protein with a specific anion binding site directly involved in transport (20,21). A better elucidation of the mechanism of O_2^- transport across the plasma membrane of phagocytic cells should derive from the identification of a similar anion-binding protein. Investigations along this line are in progress in our laboratory.

ACKNOWLEDGEMENTS: This research was aided by grants from the italian Consiglio Nazionale delle Ricerche (CT 77.01477.04) and from the University of Trieste.

REFERENCES

1. Babior, B.M., Kipnes, R.S., and Curnutte, J.T. (1973) J. Clin. Invest. 52, 741-744.
2. Drath, D.B., and Karnovsky, M.L. (1975) J. Exp. Med. 141, 257-262.
3. Goldstein, I.M., Roos, D., Kaplan, H.B., and Weissmann, G. (1975) J. Clin. Invest. 56, 1155-1163.
4. Weening, R.S., Weaver, R., and Roos, D. (1975) J. Lab. Clin. Med. 85, 245-252.
5. Gennaro, R., Schneider, C., de Nicola, G., Cian, F., and Romeo, D. (1978) Proc. Soc. Exp. Biol. Med. 157, 342-347.
6. Johnston, R.B., Godzik, C.A., and Cohn, Z.A. (1978) J. Exp. Med. 148, 115-127.
7. Kakinuma, K., and Minakami, S. (1978) Biochim. Biophys. Acta 538, 50-59.
8. Dri, P., Bellavite, P., Berton, G., and Rossi, F. (1979) Mol. Cell. Biochem. 23, in press.
9. DeChatelet, L.R., McCall, C.E., McPhail, L.C., and Johnston, R.B. (1974) J. Clin. Invest. 53, 1197-1201.
10. Patriarca, P., Dri, P., and Rossi, F. (1974) FEBS Lett. 43, 247-251.
11. Salin, M.L., and McCord, J.M. (1974) J. Clin. Invest. 54, 1005-1009.
12. Johnston, R.B., Keele, B.B., Misra, H.P., Lehmeyer, J.E., Webb, L.S., Baehner, R.L., and Rajagopalan, K.V. (1975) J. Clin. Invest. 55, 1357-1372.
13. McCord, J.M. (1974) Science 185, 529-531.
14. Babior, B.M., Curnutte, J.T., and McMurrich, B.J. (1976) J. Clin. Invest. 58, 989-996.
15. Romeo, D., Zabucchi, G., and Rossi, F. (1977), in Movement, Metabolism and Bactericidal Mechanisms of Phagocytes (Rossi, F., Patriarca, P., and Romeo, D., eds.) pp. 153-165, Piccin Medical Books, Padova.
16. Patriarca, P., Cramer, R., and Dri, P., Ibidem, pp. 167-174.
17. DeChatelet, L.R., Shirley, P.S., McPhail, L.C., Iverson, D.B., and Doellgast, G.J. (1978) Infect. Immun. 20, 398-405.
18. Lynch, R.E., and Fridovich, I. (1978) J. Biol. Chem. 253, 4697-4699.
19. Cabantchik, Z.I., and Rothstein, A. (1972) J. Membr. Biol. 10, 311-330.
20. Rothstein, A., Cabantchik, Z.I., and Knauf, P. (1976) Fed. Proc. 35, 3-10.

21. Cabantchik, Z.I., Knauf, P.A., and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239-302.
22. Fortes, P.A., and Hoffman, J.F. (1974) J. Membr. Biol. 16, 79-100.
23. Carlson, G.P., and Kaneko, J.J. (1973) Proc. Soc. Exp. Biol. Med. 142, 853-856.
24. Zabucchi, G., and Romeo, D. (1976) Biochem. J. 156, 209-213.
25. VanGelder, B.F., and Slater, E.C. (1962) Biochim. Biphys. Acta 58,593-595.
26. Dalmark, M., and Wieth, J.O. (1972) J. Physiol. (Lond.) 224, 583-610.
27. Goldstein, I.M., Cerqueira, M., Lind, S., and Kaplan, H.B. (1977) J. Clin. Invest. 59, 249-254.
28. Klebanoff, S.J. (1967) J. Exp. Med. 126, 1063-1078.
29. Stolc, V. (1971) Biochem. Biophys. Res. Commun. 45, 159-166.
30. Root, R.K., and Stossel, T.P. (1974) J. Clin. Invest. 53, 1207-1215.