GENERATION OF SUPEROXIDE ANIONS BY LEUCOCYTES TREATED WITH CYTOCHALASIN E

Akira Nakagawara and Shigeki Minakami

Department of Biochemistry
Kyushu University School of Medicine
Fukuoka 812, Japan

Received March 31, 1975

SUMMARY:
Guinea pig polymorphonuclear leucocytes reduced cytochrome c when treated with cytochalasin E. The reduction was completely inhibited by superoxide dismutase and manganese ions, which indicates that superoxide anions are generated and released into the outside medium by the treatment. The reduction was inhibited by glycolytic inhibitors and cyclic AMP but not by cyclic GMP. The pattern is similar to the cyanide-insensitive respiration of leucocytes during phagocytosis. Nitroblue tetrazolium was also reduced by the leucocytes treated with the cytochalasin, which was inhibited by manganese ions, glycolytic inhibitors and cyclic AMP but was only partially inhibited by superoxide dismutase.

Respiration insensitive to respiratory inhibitors has been observed in polymorphonuclear leucocytes during phagocytosis(1). The respiration is accompanied by the generation of superoxide anions which are supposed to be connected with the killing of phagocytized bacteria(2).

In a previous publication(3), we have shown that cytochalasin E, a member of cytochalasins which inhibit cytokinesis and membrane movement, induces cyanide-insensitive respiration and stimulates hexose monophosphate oxidative pathway in guinea pig polymorphonuclear leucocytes. In the present paper, reduction of exogenous cytochrome c by the leucocytes in the presence of cytochalasin E was studied. The reduction was completely inhibited by superoxide dismutase and Mn^{2+} ions.

EXPERIMENTALS:

Leucocytes were obtained from guinea pigs treated with caseinate solution (4). Contaminated red cells were removed by hypotonic hemolysis. More than 90 % of the cells were polymorphonuclear leucocytes.

Reduction of cytochrome c was measured with a double wave-length spectrophotometer(Hitachi 356) at 550-540 nm with a molar absorption coefficient of 15.5×10^3. Reduction of nitroblue tetrazolium(NBT) was measured after extraction with pyridine according to Baehner and Nathan(5) or with the double wave-length spectrophotometer at 560-800 nm with a molar absorption coefficient of 14.0×10^3. Cytochalasin E was dissolved in dimethyl sulfoxide(DMSO). Other experimental conditions were described in figure legends.

Superoxide dismutase was isolated from ox blood essentially according to McCord and Fridovich(6). Cytochalasin E was obtained from Aldrich Chemical Co., NBT from Sigma and nucleotides from Böhringer u. Söhne, Mannheim. Other reagents were of analytical reagent grade.

RESULTS:

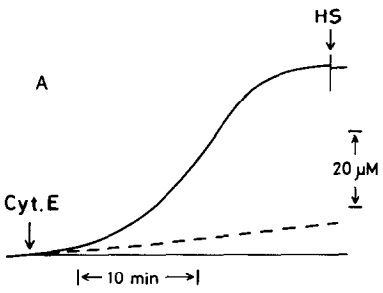
Polymorphonuclear leucocytes treated with cytochalasin E reduced cytochrome c in suspending medium as shown in Fig. 1A. The reduction proceeded gradually with an appreciable lag time and the time course was similar to that of cyanide-insensitive respiration induced by the cytochalasin(Fig. 1 in ref. 3). The rate of the reduction in presence of the cytochalasin was more than ten times of that of the control cells. The lag time was shortened and the reduction rate increased when the cells were incubated with isotonic sucrose solution (Fig. 1B).

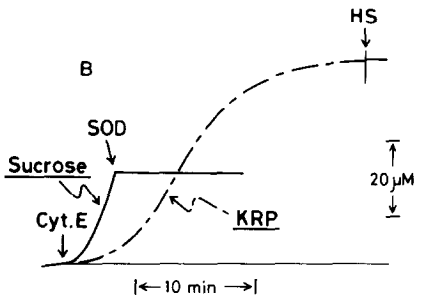
The electron donor is supposed to be superoxide anions(O_2^-), since the reduction was completely inhibited by the addition of superoxide dismutase (Fig. 1B & 1C). The reduction was also inhibited by the addition of Mn^{2+} ions and the reduced cytochrome c was oxidized(Fig. 1D). This effect of Mn^{2+} ions on the reduction of cytochrome c was similar to that by superoxide anions generated by xanthine-xanthine oxidase system.

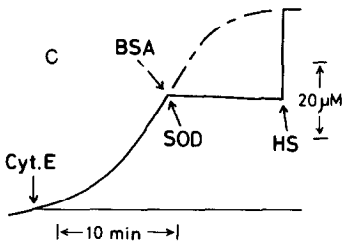
Cytochrome c was also reduced by polymorphonuclear leucocytes during phagocytosis of heat-killed E. Coli(Fig. 1E). The rate was only about two to three fold compared with the control, though the cyanide-insensitive respiration during phagocytosis was more than that of the cytochalasin-treated cells. The reduction was inhibited by superoxide dismutase as shown by Curnutte and Babior(2). No stimulation of the reduction was observed when Ehrlich ascites tumor cells were incubated either with cytochalasin E or with E. Coli.

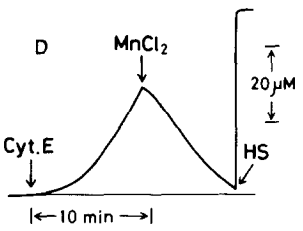
The reduction of cytochrome c was inhibited by glycolytic inhibitors and SH-inhibitors(Fig. 1F & 1G), but not by respiratory inhibitors(Fig. 1H). Cyanide slightly stimulated the reduction, probably due to the inhibition of cytosol superoxide dismutase(7).

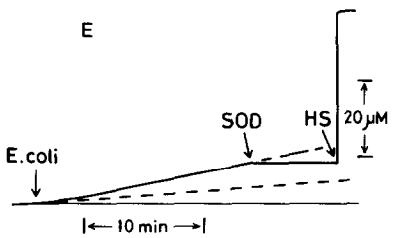
We have previously shown that the cyanide-insensitive respiration induced

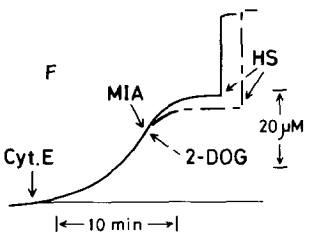

A
HS
20 μM
Cyt.E
|← 10 min →|

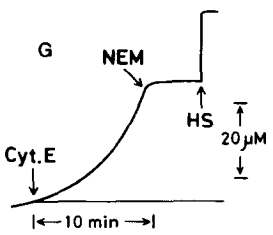

B
HS
SOD
Sucrose
20 μM
Cyt.E
KRP
|← 10 min →|

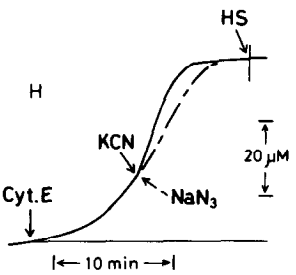

C
BSA
20 μM
SOD
HS
Cyt.E
|← 10 min →|


D
MnCl2
20 μM
Cyt.E
HS
|← 10 min →|


E
SOD
HS
20 μM
E.coli
|← 10 min →|


F
HS
MIA
20 μM
Cyt.E
2-DOG
|← 10 min →|


G
NEM
HS
20 μM
Cyt.E
|← 10 min →|


H
HS
KCN
20 μM
Cyt.E
NaN3
|← 10 min →|

by phagocytosis is inhibited by cyclic AMP but not by cyclic GMP, while that induced by trypsin-treated microsomes is inhibited by both nucleotides and that induced by methylene blue by neither of them(8). The reduction of cytochrome c induced by the cytochalasin was inhibited by cyclic AMP(Fig. 2A) and theophylline(Fig. 2B), but not by cyclic GMP(Fig. 2C). A membrane permeable cyclic AMP-analog, dibutyryl cyclic AMP(Bu_2-cAMP), strongly inhibited the reduction(Fig. 2D). The reduction was slightly stimulated by the addition of 2 mM ATP but not by the addition of AMP, NADH and NADPH in the same concentration The mechanism of the stimulation is not clear but it might be related to the ecto-ATPase described by DePierre and Karnovsky(9).

Cytochalasin E also stimulated the reduction of nitroblue tetrazolium by leucocytes. The time course was similar to that of the cytochrome reduction induced by the cytochalasin and reduction rates of both dyes were comparable (Fig. 3A). Although superoxide dismutase inhibited the reduction only to the extent of 30 to 40 %(Fig. 3B), other properties of the NBT reduction were similar to those of the cytochrome reduction as shown in Table I: the reduction was inhibited by Mn^{2+}, 2-deoxyglucose and SH-inhibitors but not by respiratory inhibitors. Bu_2-cAMP and theophylline inhibited the reduction.

Figure 1. Reduction of cytochrome c by leucocytes. The cells($1.7\text{-}2.3 \times 10^7$) were suspended in 1.5 ml of a modified Krebs Ringer Phosphate solution($CaCl_2$ reduced to 0.5 mM) pH 7.4 containing 50 μM cytochrome c at 37°C. Five microliter of cytochalasin E(Cyt.E) solution(1 mg/ml DMSO) was added as indicated. The reduction of cytochrome c was measured at 550-540 nm and the complete reduction was shown by the addition of sodium hydrosulfite(HS). (A). Time course of the reduction. The control experiment was with 5 μl DMSO. (B). The reduction with leucocytes suspended in 0.25 M sucrose solution buffered with 15 mM Tris-HCl pH 7.4. The control experiment was with the modified Krebs Ringer Phosphate(KRP) solution. Superoxide dismutase(SOD. 20 μg) was added at the time indicated. (C). Effect of superoxide dismutase on the reduction. The control experiment was with bovine serum albumin solution(BSA) of the same protein concentration. (D). Effect of 1 mM $MnCl_2$ on the reduction. (E). The reduction induced by the addition of heat-killed E. Coli(dry weight 0.8 mg). One thirtieth volume of guinea pig blood serum was added to KRP solution. The control experiment was without E. Coli. (F). Effect of 2 mM monoiodoacetate (MIA) or 25 mM 2-deoxyglucose(2-DOG) on the reduction. (G). Effect of 1 mM N-ethylmaleimide(NEM) on the reduction. (H). Effect of 1 mM KCN or 10 mM NaN_3 on the reduction.

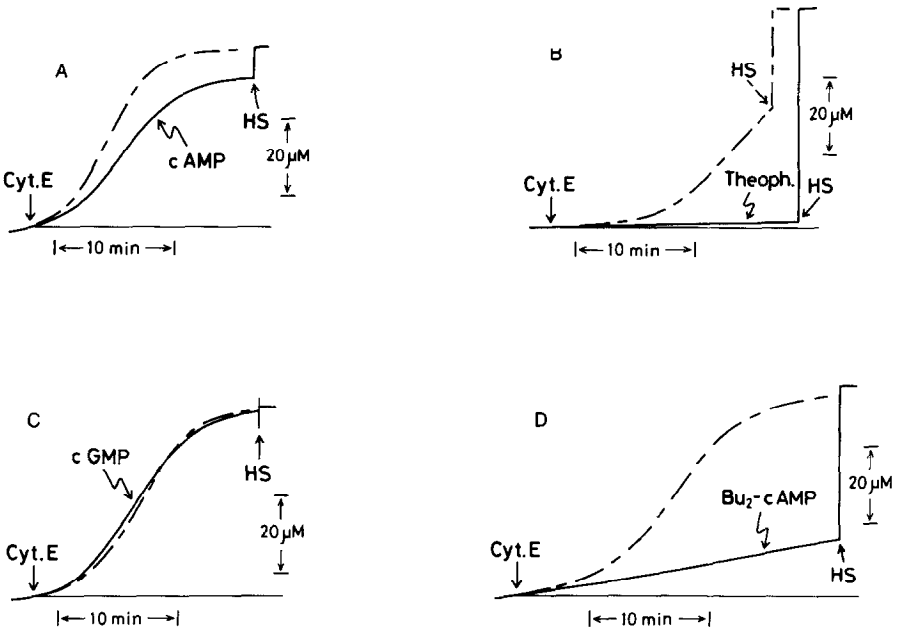


Figure 2. Effect of cyclic nucleotides on the cytochrome reduction. Experimental conditions were as in Fig. 1. (A). Effect of cyclic AMP(cAMP). The cells were preincubated with 5 mM cAMP in KRP solution at 37°C for 30 min and centrifuged. The cells were resuspended in fresh medium to measure the cytochrome reduction. The reduction rate was slower than the control(— - —) which was treated in the same way but without cAMP. The reduction stopped before the complete reduction of cytochrome c. (B). Effect of 4.4 mM theophylline(Theoph.). (C). Effect of cyclic GMP(cGMP). The cells were treated with 3 mM cGMP as in (A). (D). Effect of dibutyryl cyclic AMP(Bu_2-cAMP). The cells were suspended in KRP solution containing 3 mM Bu_2-cAMP and kept at 37°C for 10 min before the addition of cytochalasin E.

DISCUSSION:

Curnutte and Babior(2) have observed the reduction of cytochrome c by superoxide anions released from leucocytes during phagocytosis and contribution of the anions in intracellular killing of phagocytized bacteria has been discussed(10-12).

In the present study, cytochrome c was similarly reduced by leucocytes treated with cytochalasin E. The cytochalasin stimulates cyanide-insensitive respiration and hexose monophosphate oxidative pathway as reported in a previous communication(3). The reduction was completely inhibited by superoxide

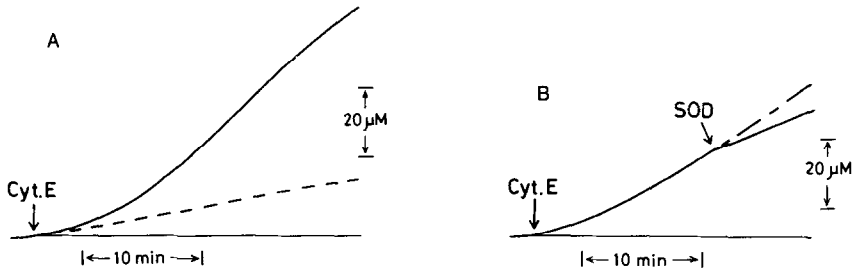


Figure 3. Reduction of nitroblue tetrazolium by leucocytes. The cells(2×10^7) were suspended in 1.5 ml glucose-free Hanks balanced salt solution containing 0.05 % NBT at 37°C. The reduction was measured at 560-800 nm. Five microliter of cytochalasin E solution(1 mg/ml DMSO) was added at the time indicated. (A). Time course of the reduction. The control experiment was with 5 μl of DMSO. (B). Effect of superoxide dismutase(20 μg). The control experiment (— - —) was without the addition of the enzyme.

dismutase, which indicates that the electron donor is superoxide anions. The cytochalasin induced O_2^--formation had characteristics similar to the metabolic changes associated with phagocytosis: 1) it was resistant to cyanide and azide, 2) sensitive to glycolytic inhibitors and SH-inhibitors, and 3) inhibited by intracellular elevation of cyclic AMP. It is suggested that the cytochalasin react with the cellular membrane and stimulate oxidation of NADH or NADPH to generate superoxide anions through mediation of cyclic nucleotide levels.

Although the reduction of cytochrome c by the cytochalasin-treated cells was qualitatively similar to that by the cells phagocytizing bacteria, stoichiometry of cyanide-resistant O_2-uptake and the cytochrome reduction was different. In the cytochalasin-treated cells, the amount of cytochrome c reduced was roughly comparable to that of the oxygen consumed, while in the phagocytizing cells the reduction of cytochrome c was less than one tenth of the oxygen consumed. Superoxide anions are probably dismutated or utilized for oxidation when the bacteria are ingested, while the anions are released to the medium when the cells are treated with the cytochalasin.

It may be necessary to mention the effect of Mn^{2+} ions on the cytochrome reduction. It has been shown that Mn^{2+} ions are strong inhibitor of NADPH-

TABLE I

Effect of inhibitors on NBT reduction induced by cytochalasin E

	% of control
control	100
+ 1 mM $MnCl_2$	4
+ 20 mM 2-deoxyglucose	1
+ 1 mM monoiodoacetate	0
+ 1 mM N-ethylmaleimide	1
+ 3 mM dibutyryl cyclic AMP	46
+ 4 mM theophylline	1

Experimental conditions were essentially according to Baehner and Nathan(5), except that latex beads were replaced by cytochalasin E. The cells were incubated at 37°C in 1.0 ml medium containing 0.4 ml Hanks balanced salt solution, 0.4 ml 0.1 % NBT in saline, 5 μl 0.2 M KCN and 10 μl of cytochalasin E solution (0.2 mg/ml DMSO). After 15 min incubation, the reaction was stopped by the addition of 10 ml 0.5 N HCl and the mixture was centrifuged(1,000 x g for 15 min at 4°C). The precipitate was extracted with pyridine and the formazan was measured at 515 nm. For 2-deoxyglucose experiment, glucose in the medium was omitted. For experiment with dibutyryl cyclic AMP, the cells were preincubated with the nucleotide for 20 min at 37°C.

---

dependent oxidation of microsomal lipid(13) and that the oxidation of formate by leucocyte preparation is dependent on Mn^{2+} ions(14). The observation that the reduction of cytochrome c by superoxide anions was inhibited and the reduced cytochrome was oxidized by the addition of Mn^{2+} ions, may be due to the stimulation of superoxide dismutation to form hydrogen peroxide.

Reduction of NBT by leucocytes during phagocytosis has been used for diagnosis of chronic granulomatosis, a hereditary disorder lacking NAD(P)H oxidizing system. The NBT reduction by the cytochalasin-treated cells had almost identical with the cytochrome reduction, though the former was only partially inhibited by superoxide dismutase. Although the contribution of superoxide anions in the NBT reduction was questioned due to the failure of dismutase to inhibit the NBT reduction(15), this might be explained by the molecular weight difference of NBT and cytochrome c. It is probable that NBT reacts with superoxide anions on the cellular membrane before superoxide dismutase attacks the anions, while cytochrome c interact with the anions

after they are released from the cellular surface, so that the reduction of cytochrome c could be completely inhibited by the dismutase.

REFERENCES:

1. Sbarra, A.J., and Karnovsky, M.L.(1959) J.Biol.Chem. 234, 1355-1362.
2. Curnutte, J.T., and Babior, B.M.(1974) J.Clin.Invest. 53, 1662-1672.
3. Nakagawara, A., Takeshige,K., and Minakami, S.(1974) Exptl.Cell.Res. 87, 392-394.
4. Takeshige,K., Nakagawara,A., Hatae,T., and Minakami,S.(1975) J.Biochem.(Tokyo) 77, 559-566.
5. Baehner, R.I., and Nathan, D.G.(1968) New Eng.J.Med. 278, 971-976.
6. McCord, J.M., and Fridovich, I.(1969) J.Biol.Chem. 244, 6049-6055.
7. Salin, M., and McCord, J.M.(1974) J.Clin.Invest. 54, 1005-1009.
8. Nakagawara, A., Takeshige, K., and Minakami, S.(1975) J.Biochem.(Tokyo) 77, 567-573.
9. DePierre, J.W., and Karnovsky, M.L.(1974) J.Biol.Chem. 249, 7111-7120.
10. Klebanoff, S.J.(1974) J.Biol.Chem. 249, 3724-3728.
11. Allen, R.C., Yevich, S.J., Orth,, R.W., and Steele, R.H.(1974) Biochem.Biophys.Res.Commun. 60, 909-917.
12. Yost,F.J., and Fridovich, I.(1974) Arch.Biochem.Biophys. 161, 395-401.
13. May, H.E., and McCay, P.B.(1968) J.Biol.Chem. 243, 2296-2305.
14. Roberts, J., and Quastel, J.H.(1964) Nature 202, 85-86.
15. Briggs, R.T., Drath,D.B., Karnovsky, M.J., and Karnovsky, M.L.(1974) J.Cell Biol. 63, 36a.