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ACTIVATION OF OXYGEN METABOLISM IN POLYMORPHONUCLEAR LEUCOCYTES : ACTIVITY OF SOLUBLE AND MEMBRANE BOUND NADPH AND NADH OXIDASES

ACTIVATION DU MÉTABOLISME DE L'OXYGÈNE DANS LES POLYNUCLÉAIRES : ACTIVITÉ DES NADH- ET NADPH-OXYDASES SOLUBLES ET LIÉES A LA MEMBRANE

P. Rossi *, P. Dri **, G. Berton *, P. Bellavite *

ABSTRACT : The NADH and NADPH oxidase activities of cell-free particles and supernatants obtained from resting and phorbol myristate acetate (PMA)-activated guinea-pig polymorphonuclear leucocytes (PMN) were compared. The supernatants obtained from resting PMN contained a very low NADH and NADPH oxidase activities which were not significantly modified in supernatants obtained from PMA-activated PMN. The NADH and NADPH-oxidase activities of the supernatants were insensitive to mitochondrial inhibitors. Sulfhydryl reagents inhibited the NADPH oxidase activity and stimulated the NADH-oxidase activity of the supernatants. The cell-free particles obtained from resting granulocytes also had a very low NADH and NADPH oxidase activities. On the contrary, the oxidase activity of cell-free particles obtained from PMA-activated cells was found markedly increased with both NADH and NADPH as substrates. A correlation was found between the degree of activation of the oxidase of cell-free particles and the degree of activation of the respiratory burst of intact PMN. The response of the particulate NADH and NADPH-oxidase to mitochondrial inhibitors and sulfhydryl reagents was comparable to that of intact stimulated PMN. The K_m of the oxidase for NADPH was lower than the concentration of NADPH in intact PMN whereas the K_m of the oxidase for NADH was higher than the concentration of NADH in intact PMN. It is concluded that only the NADH and NADPH oxidase activities of cell-free particles may play a role in the activation of the respiratory burst and that NADPH is the physiological substrate in intact cells.

NADPH oxidase ; NADH oxidase ; polymorphonuclear leucocytes ; respiratory burst ; superoxide anion.

One of the properties shared by granulocytes and macrophages is that of undergoing a dramatic increase in respiration during phagocytosis or following interaction with certain soluble stimuli. The enzymatic basis of this respiratory burst is controversial. Table I shows the enzymes that in the past have been proposed as likely candidates in the activation of the respiratory burst. Evidence obtained in our and other laboratories have shown that the key enzyme of the

* Istituto di Patologia Generale dell'Università di Padova, sede di Verona, Strada Le Grazie, 37134 Verona (Italy).

** Istituto di Patologia Generale dell'Università di Trieste, Italy.

respiratory burst is a membrane bound NADPH oxidase [2, 8, 10, 11, 15, 17, 18]. In a paper which appeared last year, a renewed emphasis had been given to a soluble NADH-oxidase, as a possible key enzyme of the respiratory burst, on the basis of the isolation and characterization of a NADH-oxidase from the supernatant of homogenates of guinea pig granulocytes in isotonic alkaline KCl [3].

In the present paper, the NADH and NADPH oxidase activities of the supernatants and of the cell-free particles obtained from guinea-pig polymorphonuclear leucocytes are compared, in order to establish their relative role in the respiratory burst.

METHODS

Materials

Cytochrome c (type VI), superoxide dismutase (type I), phorbol 12-myristate 13-acetate (PMA), NADPH (type III), NADH (grade II), N-ethylmaleimide (NEM), p-chloromercuribenzoic acid (pCMB) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were obtained from Sigma Chemical Co., St. Louis, Mo. Other reagents were of the best grade commercially available.

Table I. — Enzymes or systems proposed as primary oxidase

Myeloperoxidase	[17]
« Soluble » NADH oxidase	[3, 4]
NADH-NBT reductase	[20]
NAD(P)H dehydrogenase-cytochrome b	[21]
NADPH oxidase	[2, 8, 10, 12, 15, 16, 18]
Amino acid oxidase	[5]
Ascorbate oxidase	[7]

Preparation of the cells

Guinea-pig peritoneal polymorphonuclear leucocytes were obtained from peritoneal inflammatory exudates induced by sodium caseinate as previously described [9]. After centrifugation of the exudates, the packed cells were freed of contaminating erythrocytes by a brief hypotonic treatment. The cells were finally resuspended in Krebs-Ringer phosphate buffer (KRP) pH 7.4 containing 5mM glucose and 0.5mM $CaCl_2$.

Cell activation

Cell suspensions (10^7 leucocytes/ml) were prewarmed at 37 °C and then incubated with PMA (0.1 µg/ml) for 90 s under continuous stirring. Resting cells were incubated under identical conditions in the presence of dimethylsulfoxide instead of PMA. At the end of the incubation, the cell suspensions were diluted five fold with ice-cold KRP and centrifuged at 250 *g* for 7 min. The pellet was then resuspended and washed in 0.154M KCl containing 3.2mM $KHCO_3$ (alkaline isotonic KCl).

Cell homogenization and preparation of the subcellular fractions

Packed cells were suspended at 15 % concentration (v/v) with alkaline isotonic KCl and homogenized for 5 min in a Potter-type homogenizer equipped with a teflon pestle driven by a motor. The homogenate was then centrifuged 10 min at 250 *g* and the supernatant fluid (S_1) was collected. The pellet was resuspended in alkaline isotonic KCl and was

subjected to an identical homogenization procedure. The homogenate was centrifuged 10 min at 250 *g* and the supernatant fluid (S_2) was collected. The pellet, containing nuclei, cell debris and a few unbroken cells, was discarded. The supernatants S_1 and S_2 were combined and centrifuged at 40.000 *g* for 15 min. The clear supernatant fluid obtained after this centrifugation was designated as « supernatant » and the pellet was resuspended in alkaline isotonic KCl and designated « cell-free particles ». The protein content of these fractions was determined by the method of LOWRY [13].

Assay of NADH and NADPH oxidase activities

The O_2^- forming activity of the supernatant fraction and of cell-free particles in the presence of NADPH or NADH was measured. The method is based on the spectrophotometric determination of the superoxide-inhibitable reduction of cytochrome c at 550 nm [2]. The assays were carried out in a double beam Perkin Elmer 576 spectrophotometer at 37 °C. Both the reference and the sample cuvette contained 50 mM Hepes pH 7.0, 0.15mM ferricytochrome c and NADH or NADPH in a final volume of 1 ml. The reference cuvette contained in addition 30 µg of superoxide dismutase. The reaction was initiated by the addition of a suitable amount of supernatant fraction or cell-free particles. The amount of cytochrome c reduced by O_2^- was calculated by using an extinction coefficient of $21.1 mM^{-1} \cdot cm^{-1}$ for cytochrome c (reduced minus oxidized) [24].

RESULTS

Table II shows the NADH and NADPH oxidase activities of the supernatants and of the cell-free particles obtained from resting and PMA-treated guinea pig granulocytes. The data show that : 1) the supernatants of resting PMN contain a very low O_2^- forming activity in the presence of either NADH or NADPH ; 2) the K_m for NADPH is one order of magnitude lower than that for NADH ; 3) both the activities and the K_m do not vary significantly in the supernatants obtained from PMA-treated granulocytes ; 4) the cell-free particles from resting PMN have also a very low oxidase activity with both NADH and NADPH as substrates. The values are around the lower limit of sensitivity of the assay method. For this reason and owing to the wide scatter of the experimental data, the range of the values are given in the table and the K_m was not measured in resting preparations ; 5) the rate of oxidation of both NADH or NADPH is markedly higher in stimulated cells than in resting cells. The K_m for NADPH is one order of magnitude lower than that for NADH. The Vmax with NADH or NADPH are similar.

Figure 1 shows the dose-response curves of the oxygen consumption by intact cells and of the activation of NADPH oxidase of cell-free particles as a function of increasing concentration of PMA. The stimulation of respiration by intact cells increases by increasing PMA concentration up to 10 ng/ml ; this behaviour is paralleled by the activation of the NADPH oxidase activity of cell-free particles. Similar results were obtained with NADH.

Table III shows the effect of several metabolic inhibitors on the NADH and NADPH oxidase activities of the supernatants and the cell-free particles obtained from stimulated guinea-pig granulocytes. Both activities are insensitive to cyanide, azide and rotenone in analogy with the respiratory burst of intact cells [6, 18, 19]. ATP inhibits the NADH oxidase activity of the supernatants and of the cell-free particles, while the NADPH oxidase activity is only slightly affected. The sulfhydryl reagents NEM and pCMB inhibit the NADPH oxidase activity of the supernatants and of the cell-free particles and the NADH oxidase

Table II. — Activity and kinetic properties of the NAD(P)H-dependent $O_2^{\cdot-}$ production by cell-free particles and supernatants from resting and PMA-activated guinea-pig PMN

	Resting cells		PMA-activated	
	supernatant	cell-free particles *	supernatant	cell-free particles
0.15mM NADH	0.77 ± 0.22	0-1.47	1.31 ± 0.17	18.79 ± 6.4
1.0mM NADH	1.25 ± 0.45	0-1.55	1.88 ± 0.13	53.40 ± 16.3
0.15mM NADPH	0.66 ± 0.29	0-0.45	0.74 ± 0.12	45.70 ± 8.4
1.0mM NADPH	0.74 ± 0.36	0-0.69	0.92 ± 0.23	65.25 ± 15.6
Vmax NADH	1.46 ± 0.28	—	1.57 ± 0.57	62.5 ± 18.7
Vmax NADPH	0.73 ± 0.38	—	1.32 ± 0.6	54.0 ± 18.0
K_m NADH	0.178 ± 0.07mM	—	0.116 ± 0.03mM	0.64 ± 0.11mM
K_m NADPH	0.016 ± 0.016mM	—	0.017 ± 0.004mM	0.057 ± 0.01mM

Values of NADPH oxidase activity are given as nmoles $O_2^{\cdot-} \cdot min^{-1} \cdot mg^{-1}$ protein. The data are means ± SD of five experiments. * Due to the high variability, the data are expressed as range of activity.

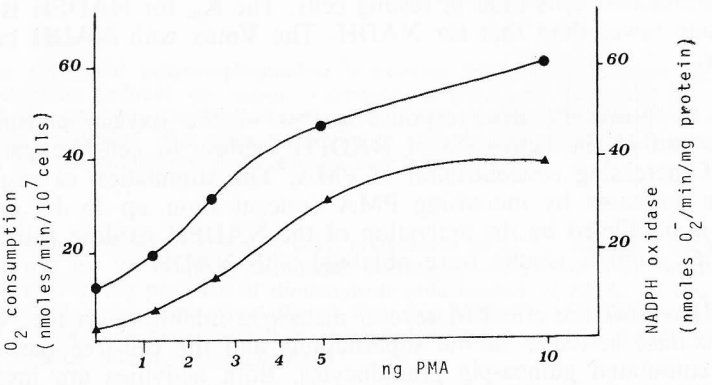

Fig. 1. — Relationship between the activation of O_2 consumption (●) by intact guinea-pig PMN and the activation of the NADPH oxidase activity (▲). O_2 consumption was measured in the presence of different amounts of PMA and NADPH oxidase measured as $O_2^{\cdot-}$ forming activity of cell free particles obtained by centrifugation for 20 min at 100,000 *g* of cells withdrawn from the electrode chamber and sonicated at 4 °C with two 5-s pulses using a MSE sonicator at the maximal intensity (150 W).

Table III. — Effect of inhibitors on the NAD(P)-H dependent O_2^- production by cell-free particles and supernatants of PMA-activated guinea-pig PMN

Addition		Supernatant		Cell-free particles	
		NADH	NADPH	NADH	NADPH
None		100	100	100	100
NaN_3	2.0mM	80	93	100	104
KCN	0.5mM	85	91	90	87
Rotenone	0.025mM	88	111	96	100
ATP	0.5mM	36	81	23	85
NEM	0.1mM	188	71	—	—
NEM	1.0mM	179	36	17	23
pCMB	0.1mM	151	33	3	3.5
pCMB	1.0mM	77	0	0	0

The results are expressed as percentage of O_2^- production in the control mixture which contained no inhibitor. NADH 1mM, NADPH 0.15mM.

activity of the cell-free particles. On the contrary, the compounds stimulate the NADH oxidase activity of the supernatant. It is worthy recalling that NEM and pCMB inhibit the respiratory burst of intact cells [6, 14, 23].

Table IV shows the NADPH and NADH concentration in guinea-pig granulocytes measured by three different groups of authors. It is evident that the concentration of NADPH is higher than the K_m of the oxidase for NADPH, whereas the concentration of NADH is lower than the K_m of the enzyme for NADH (table II).

Table IV. — NADPH and NADH concentration in guinea-pig PMN

	NADPH (mM)	NADH (mM)
SBARRA's group [22]	0.136	0.172
ROSSI's group [15]	0.273	0.076
FREI's group * [1]	0.100	0.160

* Values calculated by BADWEY and KARNOVSKY [3].

DISCUSSION

This paper reports the results of a comparative study of the NADH and NADPH oxidase activities of supernatant and cell-free particles obtained from resting and PMA activated guinea-pig peritoneal polymorphonuclear leucocytes. As far as we know, such a comparison has never been done. Since a particulate NADPH oxidase or a soluble NADH oxidase [3, 4] have each been proposed as the key enzyme of the respiratory burst, we believed the present study to be necessary in order to establish more clearly the substrate requirement (NADH or NADPH) and the soluble or particulate nature of the oxidase responsible for the respiratory burst.

The supernatants of resting guinea-pig granulocytes were found to contain a very low NADH and NADPH oxidase activities. These oxidase activities were not significantly modified in supernatants obtained from PMA-activated granulocytes neither quantitatively nor as far as the kinetic properties (K_m and Vmax) were concerned. Therefore, it is unlikely that the NADH and NADPH oxidase activities of the supernatants of granulocytes are involved in the activation of the oxidative metabolism of these cells.

The cell-free particles obtained from resting granulocytes had also a very low NADH and NADPH oxidase activities, but these activities were markedly increased in particles obtained from PMA-activated granulocytes. In addition, the degree of activation of both NADH and NADPH-oxidase of cell-free particles paralleled the degree of activation of the respiratory metabolism of intact cells (fig. 1). These data indicate that both the NADH and NADPH oxidase activities of cell-free particles might be involved in the activation of the respiratory burst. This conclusion is further strenghtened by the studies on the effect of inhibitors on the NADH and NADPH oxidase activities of the cell-free particles and of the supernatants. In fact, while the oxidase of cell-free particles in the presence of either NADH or NADPH as substrates responded to inhibitors in the same way as intact stimulated cells do, the NADH oxidase of the supernatant was stimulated by sulfhydryl reagents which, instead, inhibit the respiratory burst of intact cells.

Since the oxidation of both NADH and NADPH is increased in particles obtained from stimulated cells, the question now arises as to whether NADPH or NADH is the physiological substrate in intact cells. The data of the concentrations of NADPH and NADH in intact cells (table IV) show that the concentration of NADPH is higher than the K_m of the oxidase for NADPH (table II), whereas the concentration of NADH is lower than the K_m of the enzyme for NADH (table II). Thus, if one measures the O_2^- forming activity in the presence of a concentration of the substrates similar to that present within the cell, the activity with NADPH is much greater than that with NADH. This suggests that in intact cells the physiological substrate is NADPH since its oxidation is favoured compared with that of NADH.

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BIBLIOGRAPHY

1. Aellig (A.), Maillard (M.), Phavorin (A.), Frei (J.). — The energy metabolism of the leukocyte. *Enzyme,* 1977, **22**, 207-212.
2. Babior (B.M.), Curnutte (J.T.), McMurrich (B.J.). — The particulate superoxide forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. *J. clin. Invest.,* 1976, **58**, 989-996.
3. Badwey (J.A.), Karnovsky (M.L.). — Production of superoxide and hydrogen peroxide by an NADH-oxidase in guinea pig polymorphonuclear leukocytes. Modulation by nucleotides and divalent cations. *J. biol. Chem.,* 1979, **254**, 11530-11537.
4. Baehner (R.L.), Gilman (N.), Karnovsky (M.L.). — Respiration and glucose oxidation in human and guinea pig leucocytes : comparative studies. *J. clin. Invest.,* 1970, **49**, 692-700.
5. Cline (M.J.), Lehrer (R.I.). — D-amino acid oxidase in leukocytes : a possible D-amino acid-linked antimicrobial system. *Proc. nat. Acad. Sci., Wash.,* 1969, **62**, 756-763.
6. Curnutte (J.T.), Babior (B.M.). — Effects of anaerobiosis and inhibitors on O_2^- production by human granulocytes. *Blood,* 1975, **45**, 851-861.
7. De Chatelet (L.R.), Cooper (M.R.), McCall (C.E.). — Stimulation of the hexose monophosphate shunt in human neutrophils by ascorbic acid : mechanism of action. *Antimicrobial. Agents Chemother.,* 1972, **1**, 12-16.
8. De Chatelet (L.R.), McPhail (L.C.), Mullikin (D.), McCall (C.E.). — An isotopic assay for NADPH oxidase activity and some characteristics of the enzyme from human polymorphonuclear leukocytes. *J. clin. Invest.,* 1975, **55**, 714-721.
9. Dri (P.), Bellavite (P.), Berton (G.), Rossi (F.). — Interrelationship between oxygen consumption, superoxide anion and hydrogen peroxide formation in phagocytosing guinea pig polymorphonuclear leucocytes. *Molec. cell. Biochem.,* 1979, **23**, 109-112.
10. Hohn (D.C.), Lehrer (R.I.). — NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J. clin. Invest.,* 1975, **55**, 707-713.
11. Iyer (G.J.N.), Islam (M.F.), Quastel (J.H.). — Biochemical aspects of phagocytosis. *Nature,* 1961, **192**, 535-541.
12. Kakinuma (K.), Kaneda (M.). — Kinetic studies on the H_2O_2 (O_2^-)-forming enzyme in guinea pig leukocytes. *FEBS Letters,* 1980, **111**, 90-94.
13. Lowry (O.H.), Rosenbrough (N.J.), Farr (A.L.), Randall (R.J.). — Protein measurement with Folin reagent. *J. biol. Chem.,* 1951, **193**, 265-275.
14. Mandell (G.). — Functional and metabolic derangements in human neutrophils induced by a glutathione antagonist. *J. Reticuloendothel. Soc.,* 1972, **11**, 129-137.
15. Patriarca (P.), Cramer (R.), Moncalvo (S.), Rossi (F.), Romeo (D.). — Enzymatic basis of metabolic stimulation in leucocytes during phagocytosis : the role of activated NADPH oxidase. *Arch. Biochem. Biophys.,* 1971, **145**, 255-262.
16. Paul (B.B.), Strauss (R.R.), Jacobs (A.A.), Sbarra (A.J.). — Direct involvement of NADPH oxidase with the stimulated respiratory and hexose phosphate shunt activity in phagocytizing leukocytes. *Exp. Cell Res.,* 1972, **73**, 456-462.
17. Roberts (J.), Quastel (J.H.). — Oxidation of reduced triphosphopyridine nucleotide by guinea pig polymorphonuclear leucocytes. *Nature, Lond.,* 1964, **202**, 85-86.
18. Rossi (F.), Zatti (M.). — Changes in the metabolic pattern of polymorphonuclear leucocytes during phagocytosis. *Brit. J. exp. Path.,* 1964, **45**, 548-559.
19. Sbarra (A.J.), Karnovsky (M.L.). — The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. biol. Chem.,* 1959, **234**, 1355-1362.
20. Segal (A.W.), Peters (T.J.). — Characterization of the enzyme defect in chronic granulomatous disease. *Lancet,* 1976, **1**, 1363-1365.
21. Segal (A.W.), Webster (D.), Jones (O.T.G.), Allison (A.G.). — Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. *Lancet,* 1978, **2**, 446-449.
22. Selvaraj (R.J.), Sbarra (A.J.). — The role of the phagocyte in host-parasite interactions. VII. Di- and triphophopyridine nucleotide kinetics during phagocytosis. *Biochim. Biophys. Acta,* 1967, **141**, 243-249.
23. Takanaka (K.), O'Brien (P.J.). — Mechanisms of hydrogen peroxide formation by leukocytes. Evidence for a plasma membrane location. *Arch. Biochem. Biophys.,* 1975, **169**, 428-535.
24. Van Gelder (B.F.), Slater (E.C.). — The extinction coefficient of cytochrome c. *Biochim. Biophys. Acta,* 1962, **58**, 593-595.

RÉSUMÉ : Les activités NADH- et NADPH-oxydase des particules acellulaires et des surnageants, obtenus à partir de leucocytes polynucléaires (PMN) de cobaye au repos et activés par le phorbol myristate acétate (PMA), ont été comparées. Les activités NADH- et NADPH-oxydase des surnageants provenant des PMN au repos sont très faibles ; elles ne sont pas modifiées de façon significative dans les surnageants provenant des PMN activés par PMA. Les activités NADH- et NADPH-oxydase des surnageants sont insensibles aux inhibiteurs mitochondriaux. Les réactifs sulfhydryle inhibent l'activité NADPH-oxydase et stimulent l'activité NADH-oxydase des surnageants. Les particules acellulaires provenant des granulocytes au repos ont également des activités NADH- et NADPH-oxydase très faibles. Au contraire, l'activité oxydase des particules acellulaires provenant de cellules activées par PMA est nettement accrue avec NADH et NADPH comme substrats. Il existe une corrélation entre le degré d'activation de l'oxydase des particules acellulaires et le degré d'activation de la respiration des PMN intacts. La réponse des NADH- et NADPH-oxydase particulaires aux inhibiteurs mitochondriaux et aux réactifs sulfhydryle est comparable à celle des PMN stimulés intacts. Le K_m de la NADPH-oxydase est plus faible que la concentration de NADPH dans les PMN intacts, alors que le K_m de la NADH-oxydase est plus élevé que la concentration de NADH dans les PMN intacts. On peut en conclure que seules les activités NADH- et NADPH-oxydase des particules acellulaires peuvent jouer un rôle dans l'activation de la respiration et que vraisemblablement NADPH est le substrat physiologique dans les cellules intactes.