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SIMULTANEOUS DEMONSTRATION OF PHAGOCYTOSIS-CONNECTED OXYGEN CONSUMPTION AND CORRESPONDING NAD(P)H OXIDASE ACTIVITY: DIRECT EVIDENCE FOR NADPH AS THE PREDOMINANT ELECTRON DONOR TO OXYGEN IN PHAGOCYTIZING HUMAN NEUTROPHILS[†]

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SUMMARY: Phagocytosis-connected oxygen consumption by human neutrophils and corresponding NAD(P)H oxidase were measured by an oxygen electrode with sequential additions of opsonized zymosan, Renex 30 (0.067%), and NAD(P)H. At a concentration of 0.15 mM substrate, NADPH oxidase activity of stimulated neutrophils was twice that required to account for accompanying oxygen consumption, and was about 20 times higher than that activity obtained from resting cells. NADH oxidase activity of phagocytizing cells, however, was negligible at the same concentration of substrate. With high recovery of oxidase activity, these results strongly suggest that NADPH is the dominant electron donor to oxygen in phagocytizing human neutrophils.

Oxygen is activated by phagocytizing leukocytes to biologically active oxygen species ($O_2^{-}\cdot$, H_2O_2, $OH\cdot$, $^1O_2$) which are subsequently used for bacterial killing (1). For some time, this process was observed as cyanide-insensitive respiration (2,3). Many reports have been published concerning the enzyme or enzyme complexes responsible for this cyanide-insensitive respiration, but some controversy still revolves around the physiologically significant electron donor to oxygen (4-6, for review). Karnovsky and his coworkers have reported that NADH oxidase activity can explain phagocytosis-connected oxygen activation in animal (7) and human granulocytes (8). Other groups (1,3,9) have reported that the so-called NADPH oxidase is the dominant enzyme responsible for the oxygen activation function in human neutrophils. Because no direct comparison between oxygen consumption by phagocytizing cells and corresponding oxidase activity exists, and partially because of the failure to demonstrate that a single oxidase could account for the full oxygen burst of phagocytes (5), the question of the true electron donor to oxy-

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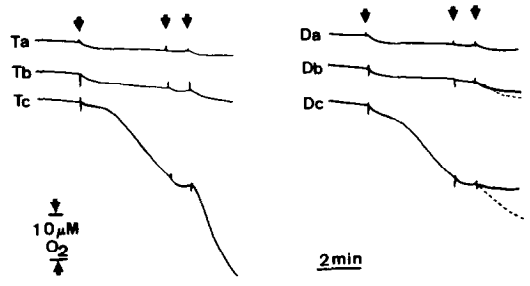

Fig. 1. Phagocytosis-connected oxygen consumption by intact neutrophils and corresponding NADPH oxidase (T) or NADH oxidase (D). a-Opsonized zymosan (1.2mg/ml), Renex 30 (0.067%), and NAD(P)H (0.15 mM) were added in this order to Hepes-saline buffer (pH 7.4) containing 1 mM NaCN; b-Unopsonized zymosan (1.2mg/ml), Renex 30 (0.067%), and NAD(P)H (0.15 mM for solid lines, and 1.5 mM for dashed lines) were added in this order to a neutrophil suspension (1.0×10^6 cells/ml) in the presence of 1 mM NaCN; c-Same as b, but opsonized zymosan was added instead of unopsonized zymosan.

gen in phagocytizing leukocytes has remained. In this communication, a simple technique is presented with evidence supporting NADPH as the predominant electron donor to oxygen in phagocytizing human neutrophils.

MATERIALS AND METHODS

Human neutrophils were separated from heparinized normal blood by dextran sedimentation, followed by Conray-Ficoll techniques (10). Contaminating red blood cells were hypotonically eliminated. Purity of polymorphonuclear leukocytes was more than 98%. Zymosan, opsonized or unopsonized, was suspended in Hepes-saline buffer to 60 mg/ml and dispersed by sonication before use. Xanthine and xanthine oxidase were purchased from Boehringer-Mannheim, Ltd. Superoxide dismutase, catalase and NADPH were obtained from Sigma Chemical Co. NADH and Renex 30 (polyoxyethylene (12) tridecyl ether) were purchased from PL Biochemicals, Inc., and ICI Americas, Inc., respectively.

Phagocytosis-connected oxygen consumption and NAD(P)H oxidase were simultaneously assayed at 25°C on a polarographic apparatus described previously (11). Oxygen concentration was calibrated by using NADH and the electron transport particles from beef heart mitochondria (12). The net volume of the assay well after fitting a Clark-type electrode was 0.15 ml. Cell viability was checked on a light microscope by the trypan blue dye exclusion technique within 20 sec after addition of 0.067% Renex 30. Lactate dehydrogenase (EC 1.1.1.27) released from neutrophils into the medium was separated by centrifugation (Beckman Microfuge B) 30 sec after the addition of Renex 30. The separation procedure was completed within 1.5 min. The enzyme activity was assayed by the method of Kornberg (13).

RESULTS

Resting or phagocytosis-connected oxygen consumption by human peripheral neutrophils and corresponding NAD(P)H oxidase activity were measured at 25°C by an oxygen electrode in the presence of 1 mM NaCN (Fig. 1). Oxidase activity was assayed by adding NAD(P)H 50-60 sec after the addition of Renex 30

Table I. Phagocytosis-connected oxygen consumption rate of neutrophils and corresponding NADPH oxidase or NADH oxidase activity.

Electron donor	Phagocytosis-connected O_2 Consumption Rate	Oxidase		Ratio*
		Resting	Stimulated	
	(Δ nmol O_2/min/10^6 neutrophils)			
NADPH (0.15 mM)	5.6 ± 0.6	0.6 ± 0.3	11.5 ± 1.0**	2.0
NADH (0.15 mM)	4.7 ± 0.4		0.2 ± 0.3	
NADH (1.5 mM)	5.5 ± 0.2	1.5 ± 0.9	4.3 ± 0.4***	0.5

Each value is the mean ± S.D. obtained from 3 assays.
*Ratio = (oxidase activity of stimulated cells minus oxidase activity of resting cells)/phagocytosis-connected O_2 consumption rate.
**$p<0.001$, compared to resting activity
***$p<0.05$, compared to resting activity

for elimination of oxygen consumption by intact neutrophils. At a concentration of 0.067%, the detergent killed the cells completely (Table II), eliminating stimulated oxygen consumption activity of intact neutrophils within 20 sec, and releasing all lactate dehydrogenase from the cytoplasm to the medium. Detergent-treated cells, of course, failed to respond to the challenge by opsonized zymosan (data not shown). Significant increases in oxidase activity were observed only in two assays as shown in Fig. 1 (Tc and dashed line of Dc). One was NADPH oxidase (Tc) of Renex 30-treated cells, previously stimulated by opsonized zymosan at a concentration of 0.15 mM substrate, and the other was NADH oxidase (Dc) observed in the identical cells, but at a substrate concentration of 1.5 mM. These results are summarized in Table I.

NADPH oxidase activity was twice the oxygen consumption rate of phagocytizing neutrophils at a concentration of 0.15 mM substrate (Table I). NADH oxidase was negligible at 0.15 mM substrate but became significant at a concentration of 1.5 mM NADH. The net increase in the NADH oxidase activity obtained by phagocytic stimulation at the latter substrate concentration represented half of the oxygen consumption by phagocytizing neutrophils. NADPH

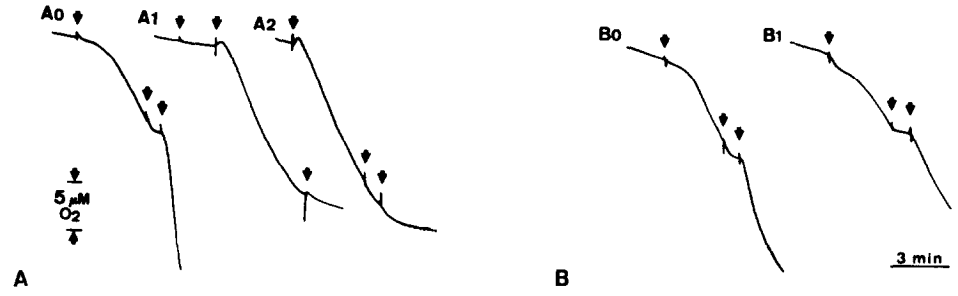

Fig. 2A. Effects of the exposure of neutrophils to exogenous active oxygen species on NADPH-dependent oxygen consumption by Renex 30-treated cells. AO-Same as Fig. 1, Tc, but the final concentration of NADPH was adjusted to 1 mM; A1-Renex 30 (0.067%), xanthine oxidase (67 μg/ml), and NADPH (1 mM) were added in this order to a neutrophil suspension (1.0 x 10^6 cells/ml) containing about 35 μM xanthine; A2-Xanthine oxidase, Renex 30, and NADPH were added in this order to cell suspension specified in A1. A dashed line indicates the time course without added NADPH.

Fig. 2B. Effect of pretreatments of lysed neutrophils and NADPH with superoxide dismutase and catalase on NADPH oxidase. Bo-Same as Fig. 1, Tc, but the final concentration of NADPH was adjusted to 0.3 mM. Cyanide was not present in the assay mixture. B1-Same as Bo, but the neutrophil suspension contained superoxide dismutase (45 μg/ml) and catalase (90 μg/ml). NADPH added was previously treated with these two enzymes at room temperature for 20 min at the following final concentrations in Hepes-saline buffer, pH 7.4: NADPH, 15 mM; superoxide dismutase, 330 μg/ml; catalase, 660 μg/ml.

oxidase and NADH oxidase activities were significantly lower in resting (challenged by unopsonized zymosan) and Renex 30-treated cells (approximately 1/20 and 1/3 of activities obtained from stimulated, lysed cells, respectively).

As the NADPH oxidase activity in stimulated and detergent-treated neutrophils was dramatically higher than that reported previously (see Discussion), the contributions of non-specific oxidation processes were carefully examined. In order to evaluate the possible contribution of free radical-initiated chain reactions produced during phagocytosis (14) and oxidase activity of myeloperoxidase (15,16) to NADPH-dependent oxygen consumption by stimulated and Renex 30-treated human neutrophils, the following two experiments were performed. In one experiment (Fig. 2, A1, A2), neutrophils were exposed to active oxygen species produced by the xanthine-xanthine oxidase system. The amount and the rate of oxygen consumption by the system were comparable to those of phagocytizing neutrophils (Fig. 2, Ao). These cells failed to respond to added NADPH whether they were exposed to active oxygen species before (A2) or

after (A1) treatment with Renex 30, or when NADPH was added during the production of active oxygen (Fig. 2, A2). These results eliminated any contribution of free radicals (labile or stable) to observed NADPH oxidase, but did not eliminate the possible contribution of myeloperoxidase, assuming that the oxidase activity of myeloperoxidase could be activated specifically in phagocytizing neutrophils.

In the second experiment (Fig. 2, B1), neutrophils were challenged by opsonized zymosan in the presence of superoxide dismutase and catalase and in the absence of cyanide. Phagocytizing neutrophils were killed by the detergent, followed by the addition of NADPH which had been preincubated with superoxide dismutase and catalase at room temperature for 20 min. (The oxidase activity of myeloperoxidase, dependent upon $O_2^{-\cdot}$ (15) or H_2O_2 (17), was inhibited by the presence of superoxide dismutase and catalase, respectively.) NADPH-dependent oxygen consumption in stimulated and Renex 30-treated cells increased significantly, but the extent of the increase was about half of that observed in the control experiment (Fig. 2, Bo). These results suggest that oxygen molecules are recovered by catalase to a significant extent from hydrogen peroxide which is produced by the dismutation reaction of superoxide anions, but not that the decreased activity is attributable to myeloperoxidase oxidase activity.

DISCUSSION

In spite of several outstanding reports (1,4,5, for review), the NAD(P)H oxidase specific to the oxygen burst of phagocytes has been considered a difficult enzyme to be examined precisely because of the limitation in the amount obtainable for experiments and because of its instability. The technique presented in this paper has overcome many of the difficulties formerly encountered in assessing this respiratory burst-specific oxidase activity.

Among the detergents we examined, Renex 30 and Renex 31 were valuable for the simultaneous assay of oxygen consumption and corresponding NAD(P)H oxidase

activity. Other detergents, for example, Triton X-100 and sodium deoxycholate, occasionally gave significant NADPH oxidase activity, but it was much lower than that obtained by Renex 30 treatment and was not so reproducible. Renex 30 produced slightly higher oxidase activity than Renex 31.

Because NADPH oxidase activity was recovered almost exclusively from the precipitate fraction (Table II) and the pyridine nucleotide binding site of the oxidase seemed to face the intracellular space[1], significant preference for NADPH rather than NADH as an electron donor to oxygen may have been due to the selective transport of NADPH into stimulated and Renex 30-treated cells. This possibility, however, was ruled out by complete release of lactate dehydrogenase from the cells whether the cells had been stimulated or not. Oxidase activities observed in our assay system were different from NAD(P)H-dependent oxygen consumption observed in guinea pig granulocytes suspended in hypotonic medium (18). More than 95% of the latter cells excluded trypan blue, and the release of lactate dehydrogenase into the medium was negligible.

In addition to the total dependence of NADPH oxidase on phagocytic stimulation, NADPH oxidase activity completely accounted for the stimulated oxygen consumption by intact neutrophils (Fig. 1, Table I) at the physiological concentration of substrate (19,20). This is the first report directly demonstrating that NADPH oxidase activity is primarily responsible for whole cell oxygen consumption in phagocytizing neutrophils. NADH oxidase activity failed to fully account for the net increase in oxygen consumption by neutrophils (Fig. 1, Dc), even at a concentration of 1.5 mM NADH, which may be 5 times higher than the concentration estimated for the intracellular space of human neutrophils (19,20). These results strongly suggest that NADPH is the predominant electron donor to oxygen in phagocytizing human neutrophils. Our preliminary experiments also suggest NADPH as the predominant electron donor

[1]Unpublished data and reference 24. Green, et al. (24) addressed the orientation of NADPH oxidase on cell surface, but the activity was measured at pH 5.5 in the presence of 1 mM $MnCl_2$.

Table II. Effects of neutrophil treatment with Renex 30 on cell viability and enzyme release.

Cells	Viability (%)	Enzyme Release (%)	
		NADPH Oxidase*	Lactate Dehydrogenase**
Resting	0	-	105 ± 5
Stimulated	0	9	95 ± 10

*Activity was assayed 6 min after Renex 30 addition. Recovery of the activity in the particulate fraction was 110% (= Δ 3.6 nmols O_2/min/10^6 cells, n=2).
**Total activity was Δ 5.9 ± 0.4 nmols NADH/min/10^6 cells (n=3).

during the respiratory burst of other types of phagocytes, including human monocytes, granulocytes and alveolar and peritoneal macrophages of guinea pigs.

There have been many documented, but controversial, reports concerning the electron donor for oxygen activation in human neutrophils during phagocytosis. Baehner, et al. (8,21) suggested NADH as the candidate for electron donor with evidence that NADH oxidase activity in neutrophils from patients with chronic granulomatous disease is low. Because of the poor recovery of the oxidase activity, and the absence of demonstrated significant increases in the activity of stimulated neutrophils, the role of NADH should be re-evaluated. Although Briggs, et al. (22) histochemically demonstrated NADH oxidase at the cell surface of human neutrophils, exaggerated cerium deposits dependent on exogenous NADH did not seem compatible with the slight NADH-dependent increase in oxygen consumption by phagocytizing cells (for further discussion, see ref. 1,4). Other authors (1,4, for review, 9) have recently reported that NADPH was the major electron donor to oxygen in human neutrophils. Although oxidase activity, recovered in subcellular fractions used for the assays, was low, and the specific activities (nmol $O_2^{\cdot-}$/min/mg protein) were disappointingly lower than those reported in this manuscript (about 0.3-6.3%), dominance of NADPH was consistent and phagocytic stimulation was essential to obtain high NADPH oxidase ($O_2^{\cdot-}$-forming enzyme) activity. These findings are compatible with our present results.

The net increase in our oxidase activity was estimated to be approximately 210 nmols O_2 consumed/min/mg protein with the assumption that 1 mg protein corresponded to 1.5 x 10^7 neutrophils (8). We have also measured $O_2^{-}\cdot$ production, H_2O_2 formation and NADPH oxidation under various conditions[2], confirming our high recovery of O_2 reduction activities in these neutrophils. The highest activity of the oxidase ($O_2^{-}\cdot$-forming enzyme) so far reported in human neutrophils is about 70 nmols $O_2^{-}\cdot$/min/mg protein of partially purified enzyme (V_{max} calculated from Fig. 4 of ref. (23)). Since about 80% of the oxygen consumed by the stimulated and Renex 30-treated neutrophils was converted to superoxide anion[2], the NADPH oxidase activity in our assay system may exceed 4 times that obtained with partially purified enzyme. The high recovery of NADPH oxidase activity and the significant increase in the activity during phagocytosis have permitted us to succeed in demonstrating this activity in whole blood preparations (manuscript in preparation).

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