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ORIENTATION OF THE NADPH DEPENDENT SUPEROXIDE GENERATING OXIDOREDUCTASE ON THE OUTER MEMBRANE OF HUMAN PMN'S[1]

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Summary: The ratio of superoxide formation to O_2 consumption in intact cell suspensions and subcellular particulate fractions rich in oxidoreductase activity, and the response seen upon adding NADPH and $NADP^+$ to these fractions, suggest that the oxidoreductase is orientated in a trans configuration on the outer membrane. Its NADPH electron accepting site appears to lie on the cytosolic side of the outer membrane, whereas its superoxide generating site appears to lie on the opposite, extracellular side of the outer membrane.

The increased rate of respiration seen when intact PMN's[2] interact with a variety of soluble or particulate agents has been linked with formation of O_2^-, and is an early event in the complex series of reactions carried out by phagocytes culminating in the act of phagocytosis (1,2). O_2^- formation is catalyzed through activation of a membrane-bound NADPH dependent oxidoreductase (3-5). An important matter to consider is the vectorial positioning of its active sites on the phagocytic membrane. The oxidoreductase must have an electron accepting site, where it receives electrons from NADPH, and an electron donating site, where electrons received by the enzyme are subsequently transferred to O_2. We have examined the orientation of these sites with respect to their location on the outer membrane of human PMN's. The ratio of O_2^- production to O_2 consumption in whole cell suspensions and subcellular

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[2]Abbreviations: Con A, concanavalin A; GRF, granulocyte rich fraction; HBSS, Hank's buffered saline solution; O_2^-, superoxide; PMN'S, polymorphonuclear leukocytes; SOD, superoxide dismutase; STZ, serum treated zymosan.

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fractions, and the response of each to NADPH, suggest that the oxidoreductase is orientated in a trans configuration across the outer membrane. This interpretation is based upon kinetic data demonstrating a marked increase in NADPH dependent oxidoreductase activity in subcellular fractions with breakage of cells and exposure of the inner membrane to NADPH, and upon observations documenting a shift in the stochiometric ratio of O_2^- production to O_2 consumption associated with membrane invagination and containment of O_2^- within the phagosomal vesicle of intact cell suspensions.

Materials and Methods: Con A, zymosan, NADPH, $NADP^+$, xanthine, xanthine oxidase, ferricytochrome c, cytochalasin B, dimethylsulfoxide and SOD were all purchased from Sigma Chemical Co., St. Louis, Mo. Sodium diatrizoate-ficoll was purchased premixed in phosphate buffer, pH 7.4, from Bionetics Lab Products, Kensington, Md. All other chemicals were of reagent grade commonly found in the laboratory.

Whole blood specimens in 10 ml aliquots were collected by syringe from healthy blood donors in heparin coated round-bottom 16 x 125 mm polystyrene tubes. Buffy coat fractions were isolated by centrifugation of the whole blood at 1150 x g for 15 min, resuspended in 8 ml of 1 mM Na_2EDTA made up in 154 mM NaCl and 32 mM sodium potassium phosphate buffer (EPS), pH 7.0, then layered over 3 ml of diatrizoate-ficoll solution (density = 1.077 g/ml) and centrifuged at 1150 x g for 18 min. Pellets were freed of contaminating red cells by 15 second hypotonic lysis in distilled water and recentrifuged in isotonic EPS. Pure cell fractions were resuspended in Hank's buffered saline solution (HBSS), pH 7.40 , and counted on a Coulter Z particle counter. Granulocyte rich subcellular fractions (GRF) were prepared as described by Hohn and Lehrer (4) except the cells were broken by sonication instead of homogenization. The 27,000 x g GRF pellet was routinely resuspended in HBSS, pH 5.5, made up in 0.34 M sucrose at a final concentration of approximately 10^8 cell equivalents/ml.

The rate of O_2 consumption of resting and Con A or STZ (4) activated PMN's was measured on a Gilson K-IIC Oxygraph equipped with a water-jacketed Clark electrode. Routine assays, except where stated elsewise in the text, were carried out with 2 to 5 x 10^6 PMN's in HBSS, pH 7.40, and cytochalasin B (4.6 ug/ml) in the presence of Con A (104 ug/ml) or STZ (83 ug/ml). The final concentration of NADPH or $NADP^+$ in these and subcellular assays was 1mM. Routine subcellular assays were carried out in HBSS adjusted to pH 5.5 and in the presence of 0.34 M sucrose, 1mM $MnCl_2$ and 0.5 mM NaN_3. The reaction volume was 1.5 ml.
O_2^- formation and SOD activity were quantitated by a modification of the SOD inhibitable cytochrome c assay of McCord et al. (6). The total assay volume in assaying O_2^- formation was 1.2 ml. Paired assays (3.6 x 105 PMN's/ml) were stopped by the addition of 4 ml ice cold water and, following sedimentation of the residual cell suspensions, the absorbance of control (+SOD) and experimental (-SOD) test mixtures was read on Gilford 300 or Varian Cary 219 spectrophotometer. The molar absorbtivity coefficient for ferrocytochrome c at 550 nm was taken as 21.1 x 10^3 (7).

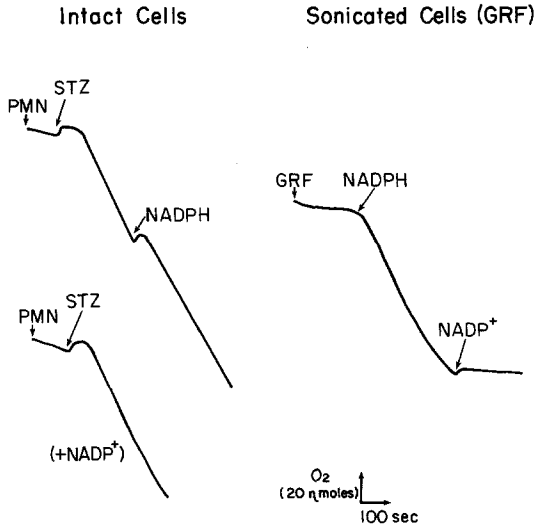

Fig. 1. Effect of exogenous additions of NADPH and $NADP^+$ on the activity of intact (left) and sonicated (right) cell suspensions following STZ activation of resting cells.

RESULTS

When intact PMN's were assayed for O_2^- generating activity following activation by STZ, no difference in respiratory burst activity was observed whether NADPH or $NADP^+$ was included in assay mixture compared to control assays carried out in the absence of these cofactors. Similar results were also seen when cells were monitored for bust activity by O_2 consumption. Following sonication of these cells and isolation of GRF, NADPH dependent O_2^- generating and O_2 uptake activity was demonstrable, and subject to inhibition by addition of $NADP^+$ (Fig. 1). The GRF from STZ fed cells was approximately 17-fold richer in oxidoreductase activity than fractions isolated from unfed nonphagocytizing cells. Moreover, our results are consistent with those of Hohn and Lehrer (4). We also observed that the NADPH dependent oxidoreductase required Mn^{+2} for full activity, and was optimally active at pH 5.5 (Table I).

The ratio of O_2^- generated to O_2 consumed in intact cell isolates with soluble Con A as stimulant was 0.95, or approximately 1:1. This decreased

Table I. NADPH Oxidoreductase Activity in GRF from Fed and Unfed PMN's.

Experiment[a]	O_2 Uptake Rate (nmoles/min)
Complete assay[b], pH 5.5	
+ GRF (fed cells)	2.07
- GRF	0.13
- NADPH	0.17
- Mn^{+2}	0.03
+ GRF (unfed cells)	0.25
Complete assay, pH 7.4	
+ GRF (fed cells)	0.12

[a]GRF assays expressed per 10^6 cell equivalents.

[b]Complete assay mixture was 1 mM $MnCl_2$, 0.5 mM NaN_3 and 0.34 M sucrose made up in HBSS. The final concentration of NADPH was 1 mM.

to 0.20 in STZ fed cells. In the GRF the ratio became 0.03 (Table II). Thus, there was approximately a 3-fold loss of NADPH oxidoreductase activity in preparing subcellular GRF as measured by O_2 uptake analysis, but a 33-fold loss as measured by O_2^- trapping analysis.

Table II. Comparison of Intact Cell and Subcellular NADPH Dependent Oxidoreductase Activity.[a]

		nmoles/min/10^6 cells ($^+$SD)		
SOURCE	STIMULANT	O_2 Uptake	O_2^- Trapped	Ratio (O_2^- Trapped/ O_2 Uptake)
Intact cells[b]	Con A	4.3 $\pm$ 0.9	4.1 $\pm$ 0.7	0.95
	STZ	4.0 $\pm$ 0.6	0.78 $\pm$ 0.2	0.20
Subcellular[c] GRF	STZ	1.3 $\pm$ 0.6	0.04 $\pm$ 0.02	0.03

[a]Values presented are averages of five assays carried out on separate lots of cells.

[b]Assayed at 37°.

[c]Assayed at 20°.

DISCUSSION

The spatial arrangement of the NADPH dependent oxidoreductase's electron accepting and donating sites on the outer membrane can be viewed in terms of a cis or trans configuration as shown in Fig. 2. With phagocytosis the outer membrane invaginates to form an intact phagosomal vesicle (1,8,9). If the electron donating site is positioned on the outer membrane it will become positioned on the inner membrane of the phagosomal vesicle with concomitant closure of the vesicle wall. (cf., Fig. 2). In theory a loss in apparent O_2^- generating capacity accompanying formation of the phagosomal vesicle

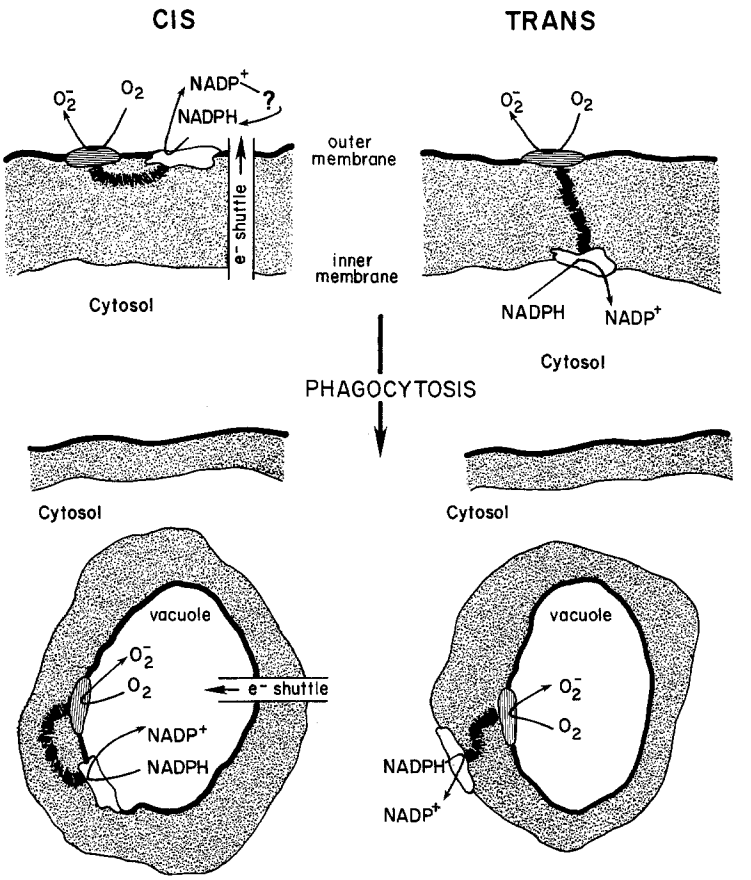

Fig.2. Theoretical model illustrating the cis (left) and trans (right) configurations of the O_2^- generating NADPH Oxidoreductase's active sites on the outer PMN membrane. Phagocytosis is associated with invagination of the membrane, causing the O_2^- generating site to be carried to the inner side of newly-formed phagosomal vesicles.

should occur because with closure of the vesicle there is no longer direct access to O_2^- by O_2^- trapping agents suspended in the extracellular medium.

With soluble, nonphagocytizeable Con A as stimulant, the ratio of O_2^- produced to O_2 consumed was 0.95, but dropped to 0.20 upon feeding intact cells particulate, phagocytizeable STZ. This apparent loss in O_2^- generating activity was not caused by "quenching" or dismutation of O_2^- by STZ. Aliquots of STZ did not inhibit xanthine oxidase generated O_2^- production. The disparity between O_2^- generated and O_2 consumed is most apparent in the GRF assays (cf., Table II). Based upon the morphology of the phagosomal vesicle and its relationship to the PMN's original outer membrane, these observations are consistent with the concept that the electron donating (O_2^- generating) site is located on the extracellular side of the outer membrane, and subsequently gets carried inside the vesicle during phagocytosis.

Based upon the results illustrated in Fig. 1, the NADPH electron accepting site must lie on the inner, cytoplasmic side of the membrane. Intact cell suspensions were unaffected by exogenous addition of NADPH or $NADP^+$, indicating that the NADPH site is not accessible to experimental manipulation from the extracellular side of the membrane. The NADPH binding site was readily accessible to experimental manipulation when cells were sonicated to allow exposure of the cytoplasmic side of the PMN's membrane to these cofactors. NADPH dependent burst activity subject to inhibition by $NADP^+$ was demonstrable (cf., Table I and Fig. 1). Thus the oxidoreductase appears to be positioned in the *trans* configuaration with its electron accepting (NADPH) and donating (O_2^- generating) sites spanning the cell membrane as shown in Fig. 2.

Babior *et al.* (3,10) reported O_2^- generating activity associated with the GRF of approximately the same level as in this study. Recently, Tauber and Goetzl (11) presented data on the partial purification of the oxidoreductase in which they obtained slightly higher activity. Neither group measured the concomitant O_2 uptake rate associated with O_2^- generating

activity in their GRF. However, Hohn and Lehrer (4) assayed oxidoreductase activity in a similarly prepared GRF obtained from human PMN's by O_2 uptake analysis and observed much higher enzyme activity on the order of 10 nmoles/min per 10^6 cell equivalents. The apparent discrepancy between O_2^- generating activity and O_2 uptake activity in these GRF fractions can be explained, as shown in this study, as an artifact of the method used to assess O_2^- formation caused by the vectorial arrangement of the NADPH dependent O_2^- generating enzyme on the outer membrane of intact PMN's. The reason this discrepancy is less apparent in assaying whole cell suspensions may be because a major portion of O_2^- can still be trapped through entry of trapping agents into the partially formed vesicles of whole cells whereas this is not possible following isolation of vesicular GRF fractions.

An alternate explanation is that there are two, or possibly more, NADPH oxidoreductases activated with stimulation of PMN suspensions, and that differences seen in subcellular fractions are due to selective lability of one or more of the oxidoreductases during isolation of these fractions. It is also possible that the oxidoreductase seen in intact cell suspensions is somehow modified upon isolation with a change in its catalytic specificity away from univalent reduction of O_2. However, the morphology of membrane folding, and our preliminary vectorial analysis suggests a trans configuration for the oxidoreductase on the outer membrane is most probable.

Singer (12) has noted the importance of asymmetry in biomembranes for a number of biologically important chemical reactions including the positioning of synapses and gap junctions on the outer membranes of cells, the formation of plaques on halobacterial membranes, and the juxtapositioning of spectrin and anchorin on the cytoplasmic wall of erythrocytes. The asymmetric positioning of the NADPH dependent O_2^- generating oxidoreductase on the PMN's membrane may be important in assuring that O_2^- generation is released to the inner side of the phagosomal vesicle where bacterial killing must occur.

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