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Research Article

Chemoattractant-receptor coupling triggers several biologic responses in phagocytic cells including activation of the respiratory burst. Prior evidence in intact cells implied that stimulation of the respiratory burst by chemoattractants was by a mechanism different from other soluble agents suggesting the possibility that different oxidative enzymes were responsible. We now show that the chemoattractants N-formyl-methionyl-leucyl-phenylalanine and a split fragment of the fifth component of complement (C5a) stimulate an NADPH oxidase activity, measured in the 50,000-g particulate fraction from human polymorphonuclear leukocytes (PMN). Levels of oxidase activity stimulated by the chemoattractants were both time and dose dependent and required the presence of cytochalasin B during stimulation. In contrast, activation by two nonchemotactic stimuli, the ionophore A23187 and phorbol myristate acetate (PMA), did not require cytochalasin B. Temporal patterns of oxidase activation suggested that different stimuli follow different transductional pathways. Chemoattractant-mediated activation was immediate (no lag); peaked by 45 s and declined rapidly to approximately 50% of maximal by 2 min. In contrast, activation by A23187 or PMA had a 15-30-s lag and increased more slowly. Stimulation by A23187 peaked at 5 min, then declined. Stimulation by PMA plateaued at 20 min and did not decline by 90 min. Comparison of K_m values for NADPH and NADH obtained by Lineweaver-Burk analysis of the oxidase activity stimulated by N-formyl-methionyl-leucyl-phenylalanine, A23187, and PMA suggested that [...]

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Activation of the Respiratory Burst Enzyme in Human Polymorphonuclear Leukocytes by Chemoattractants and Other Soluble Stimuli

EVIDENCE THAT THE SAME OXIDASE IS ACTIVATED BY DIFFERENT TRANSDUCTIONAL MECHANISMS

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ABSTRACT Chemoattractant-receptor coupling triggers several biologic responses in phagocytic cells including activation of the respiratory burst. Prior evidence in intact cells implied that stimulation of the respiratory burst by chemoattractants was by a mechanism different from other soluble agents suggesting the possibility that different oxidative enzymes were responsible. We now show that the chemoattractants *N*-formyl-methionyl-leucyl-phenylalanine and a split fragment of the fifth component of complement (C5a) stimulate an NADPH oxidase activity, measured in the 50,000-*g* particulate fraction from human polymorphonuclear leukocytes (PMN). Levels of oxidase activity stimulated by the chemoattractants were both time and dose dependent and required the presence of cytochalasin B during stimulation. In contrast, activation by two nonchemotactic stimuli, the ionophore A23187 and phorbol myristate acetate (PMA), did not require cytochalasin B. Temporal patterns of oxidase activation suggested that different stimuli follow different transductional pathways. Chemoattractant-mediated activation was immediate (no lag); peaked by 45 s and declined rapidly to ~50% of maximal by 2 min. In contrast, activation by A23187 or PMA had a 15–30-s lag and increased more slowly. Stimulation by A23187 peaked at 5 min, then declined. Stimulation

by PMA plateaued at 20 min and did not decline by 90 min. Comparison of K_m values for NADPH and NADH obtained by Lineweaver-Burk analysis of the oxidase activity stimulated by *N*-formyl-methionyl-leucyl-phenylalanine, A23187, and PMA suggested that the same enzyme was activated by all stimuli. Thus, chemoattractants and other soluble stimuli appear to activate the same respiratory burst enzyme in PMN but they utilize different transductional mechanisms and are regulated differently.

INTRODUCTION

Chemoattractant-receptor coupling, as well as phagocytosis, triggers a number of biologic responses in phagocytic cells, including an increase in their oxidative metabolism (1, 2). This phenomenon, termed the respiratory burst, is characterized by the conversion of oxygen to several toxic products, including superoxide anion (O_2^-) and hydrogen peroxide, which are important in the microbicidal, tumoricidal, and inflammatory activities of these cells (2, 3). A wide variety of nonchemotactic soluble stimuli can also activate the respiratory burst (2–4). Recent evidence using whole cells has suggested that the mechanism of activation of the respiratory burst is not the same for all stimuli (5–8). Two possibilities are apparent from these studies. One is that different stimuli activate the same respiratory burst enzyme, but use different transductional pathways. The other is that different stimuli activate different respiratory burst enzymes.

The activity previously identified by several labo-

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ratories (9–12) as the respiratory burst enzyme is a membrane-bound NADPH oxidase that can be localized to a postnuclear particulate fraction from homogenized cells. Activation of this enzyme by both phagocytic and several nonchemotactic soluble stimuli, including phorbol myristate acetate (PMA)¹, digitonin, and the calcium ionophore A23187, has been reported (9–19), but activation by chemoattractants had not yet been demonstrated.

Considering the importance of chemoattractants in inflammation, we undertook to examine if these stimuli activate NADPH oxidase activity in human polymorphonuclear leukocytes (PMN) and, if so, to determine if this activity is the same as that previously reported for nonchemotactic agents. Also, we sought to determine by characterizing activation of NADPH oxidase by dissimilar stimuli, if different regulatory mechanisms are involved in triggering the respiratory burst.

METHODS

Isolation of PMN. Human PMN were isolated to >95% purity from heparinized venous blood by dextran sedimentation and Ficoll-Hypaque centrifugation (20). Following hypotonic lysis of erythrocytes with 0.2% NaCl, PMN were resuspended in Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY), containing 10 mM Hepes (Sigma Chemical Co., St. Louis, MO) and 4.2 mM sodium bicarbonate (Mallinckrodt, Inc., Paris, KY), pH 7.4 (HBSS). Cells were counted in a hemocytometer and diluted in HBSS to a final concentration of $\sim 1.5 \times 10^8$ /ml.

Isolation of particulate fractions containing NADPH oxidase activity. Particulate fractions were isolated as previously described (18) with some modifications. PMN at 5×10^7 /ml in HBSS containing 1 mM sodium azide were preincubated at 37°C for 5 min in the presence or absence of either 2×10^{-6} M or 10^{-5} M cytochalasin B (CB) (Sigma Chemical Co.). Two lots of CB were used and the concentration necessary for optimal activation of NADPH oxidase by *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) varied with the lot. CB was stored at -20°C as a 10^{-2} M stock in dimethylsulfoxide (DMSO). After preincubation of cells, the desired stimulus was added and incubation was continued for periods of time ranging from 0 to 90 min. A fivefold excess volume of ice-cold HBSS was added and tubes were immediately placed in a melting ice bath to terminate the reaction. Cells were centrifuged at 200 *g* for 10 min, resuspended in cold 0.34 M sucrose to 2.5×10^7 /ml, and sonicated in an ice-water bath for 25 s at a setting of 35% using an Artek Sonic 300 Dismembrator equipped with a microtip (Artek Systems Corp., Farmingdale, NY). Sonicates were centrifuged at 500 *g* for 10 min; supernates were then centrifuged at 50,000 *g* for 45 min. Final pellets were resuspended in 0.34 M sucrose to yield a protein concentra-

tion of 2–3 mg/ml (21) and were stored at -70°C until time of assay. NADPH oxidase activity was stable for at least 6 mo even with repeated freeze-thaw. Each fraction was assayed immediately after thawing in order to minimize decay at 4°C. No differences in the stability of the enzyme activated by different stimuli were noted.

The following stimuli were kept at -20°C as stock solutions in DMSO: 10^{-2} M fMet-Leu-Phe, 2 mg/ml PMA (3.2 mM), and 10^{-1} M A23187 (all were from Sigma Chemical Co.). A split fragment of the fifth component of human complement (C5a) (generously provided by Dr. D. Chenoweth, UCSD, La Jolla, CA) was purified as described previously (22, 23) and was stored at a concentration of 10^{-5} M at -70°C in HBSS containing 0.5% gelatin (Nutritional Biochemicals Corp., Cleveland, OH). PMA, A23187, and fMet-Leu-Phe were added to the incubation mixture from dilutions made in DMSO to yield a concentration 1,000-fold higher than the final concentration desired. A control set of cells was routinely included, which contained an equivalent amount of DMSO alone, although final concentration of DMSO was never >0.2%. In experiments with C5a, both C5a and fMet-Leu-Phe were added from dilutions made in HBSS containing 0.5% gelatin at a 10 times concentration over the final one desired.

Measurement of NADPH oxidase activity. NADPH oxidase activity was measured as the superoxide dismutase-inhibitable, NADPH-dependent reduction of cytochrome *c* and assays were performed as described previously (5). NADPH was utilized at a concentration of 190 μM in most assays, since NADPH is the likely physiologic substrate for the oxidase (9–19). However, substrate concentration was varied from 9.5 to 190 μM for NADPH, or 0.2 to 1.9 mM for NADH, in assays for Lineweaver-Burk analysis. Assays were performed at ambient temperature using a Cary 219 double-beam spectrophotometer (Varian Associates, Inc., Instrument Group, Palo Alto, CA). Activity was followed continuously and initial slopes after addition of substrate were used for calculations using an extinction coefficient of 21 $\text{mM}^{-1} \text{cm}^{-1}$ for cytochrome *c* (24). Superoxide dismutase (Diagnostic Data, Inc., Mountain View, CA) and cytochrome *c* (type III, Sigma Chemical Co.) were stored as stock solutions at -20°C . NADPH and NADH (Boehringer Mannheim Biochemicals, Indianapolis, IN) were prepared freshly just before use.

In some preliminary experiments, 0.042% deoxycholate (Sigma Chemical Co.) was included in the assay mixture (25), which resulted in a three- to fourfold enhancement of activity measured in fractions from cells stimulated with either fMet-Leu-Phe or PMA. The overall pattern of activity seen with these stimuli did not change in the presence of detergent, nor was activity observed using detergent in fractions from cells stimulated by chemoattractants in the absence of CB. Deoxycholate was not included in subsequent assays.

RESULTS

Activation of NADPH oxidase by chemoattractants and nonchemotactic agents. Exposure of PMN to the chemoattractant fMet-Leu-Phe activated an NADPH-dependent oxidase activity that was measurable in a 50,000-*g* particulate fraction. As shown by the tracing in Fig. 1, little cytochrome *c* reduction was obtained before the addition of NADPH and activity was still very low after NADPH was added if the source of enzyme was a particulate fraction from unstimulated

¹ Abbreviations used in this paper: C5a, a split fragment of the fifth component of complement; CB, cytochalasin B; DMSO, dimethylsulfoxide; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution containing 10 mM Hepes and 4.2 mM sodium bicarbonate, pH 7.4; PMA, phorbol myristate acetate.

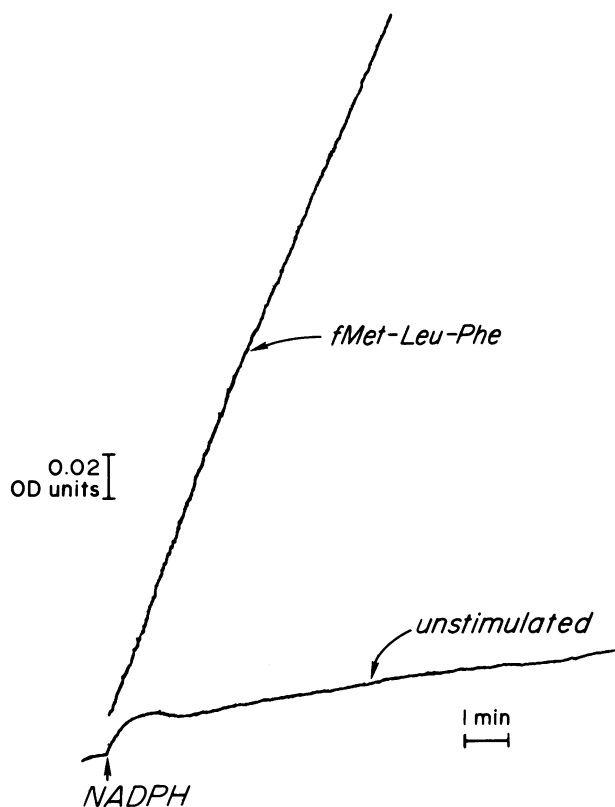


FIGURE 1 Representative tracings of NADPH-dependent cytochrome *c* reduction (NADPH oxidase activity) by particulate fractions from unstimulated or fMet-Leu-Phe-stimulated PMN. Particulate fractions were isolated from PMN exposed to either DMSO or 10^{-6} M fMet-Leu-Phe for 1 min in the presence of CB and assays were performed as described in Methods. Concentration of each fraction in the assay was 0.8 mg/ml. Specific activity was 0.1 and 3.0 nmol O_2^- /min per mg for unstimulated and fMet-Leu-Phe fractions, respectively.

cells. However, if a particulate fraction from cells stimulated with 10^{-6} M fMet-Leu-Phe for 1 min was used, a substantial rate of reduction was seen after addition of NADPH.

Summarized data of the NADPH oxidase activity in fractions from cells stimulated by either chemoattractants or two nonchemotactic soluble agents are given in Table I. Stimulation of PMN by 10^{-6} M fMet-Leu-Phe resulted in a 25-fold increase in NADPH oxidase activity over levels from unstimulated cells. Activation by another chemoattractant, purified C5a at 10^{-7} M, gave a 10-fold increase in oxidase activity. The lower level of activity with C5a as compared with fMet-Leu-Phe is most likely because of the different doses used. Higher amounts of C5a were not tested because quantities of the purified compound were limited. The ionophore A23187 stimulated NADPH oxi-

dase activity 20-fold and the most effective stimulus was PMA (300-fold enhancement over resting).

Differences in the levels of NADPH oxidase activity recovered in particulate fractions could be due to differences in localization of the enzyme. The distribution of NADPH oxidase activity and protein in subcellular fractions from PMN stimulated by fMet-Leu-Phe, C5a, A23187, or PMA was examined and no differences were found with the various stimuli under the same conditions as described in Table I. The data were combined and are given in Table II. The amount of NADPH oxidase activity recovered in the particulate fraction ranged from 65 to 88% with only 3% remaining in the supernatant. The activity recovered in the 500-g pellet is similar to the amount of protein recovered and probably represents unbroken cells or large membrane aggregates. Thus, the particulate fraction appears to be valid to use for measurements of NADPH oxidase activity triggered by different stimuli.

The presence of CB during stimulation was necessary for obtaining activity from cells stimulated by chemoattractants. Cytochalasin D (10^{-5} M) and dihydrocytochalasin B (10^{-5} M) were also effective (data not shown). CB was not required during either the isolation procedure or the assay and had little effect on the activity from cells stimulated by A23187 or PMA.

Activation of NADPH oxidase by chemoattractants was dependent on the concentration of the stimulus added to the cells (Fig. 2, top panels). Similar dose dependencies were seen with A23187 and PMA (Fig. 2, bottom panels). The ED_{50} calculated from these

TABLE I
NADPH Oxidase Activity in Particulate Fractions from PMN Stimulated by Chemoattractants or Other Soluble Agents

Stimulus*	NADPH oxidase activity†	
	CB present during stimulation	CB absent during stimulation
	nmol O_2^- /min/mg	
None (DMSO)	0.07±0.01 (16)	0.06±0.03 (3)
fMet-Leu-Phe, 10^{-6} M	1.80±0.22 (21)	0.07±0.01 (4)
C5a, 10^{-7} M	0.72±0.26 (5)	0.07±0.01 (2)
A23187, 5×10^{-6} M	1.67±0.21 (12)	1.47±0.13 (5)
PMA, 1.6×10^{-6} M	23.1±2.1 (16)	34.2±1.7 (4)

* Neutrophils were treated with either DMSO or the indicated stimulus for various lengths of time in the presence or absence of CB. The times of stimulation were 1 min for fMet-Leu-Phe, 30 s for C5a, 5 min for A23187, and 15 min for PMA.

† Isolated particulate fractions were assayed for NADPH-dependent O_2^- production using 190 μ M NADPH. Values given are the mean±SEM with the number of experiments shown in parentheses.

TABLE II
Distribution of NADPH Oxidase Activity and Protein in Subcellular Fractions from Stimulated PMN

Fraction*	NADPH oxidase activity recovered†	Protein recovered†
	%	
500-g pellet	10.9±1.8 (8.5-19.5)	12.0±1.4 (8.7-18.1)
50,000-g pellet (particulate fraction)	77.3±3.1 (64.7-88.1)	41.3±2.0 (34.4-48.8)
50,000-g supernatant	3.2±1.0 (0-7.5)	43.9±1.0 (40.3-47.6)
Total recovery	91.4±2.8 (84.2-104.1)	97.2±1.4 (93.1-103.4)

* Fractions were isolated from cells stimulated with 10^{-6} M fMet-Leu-Phe for 1 min; 10^{-7} M C5a for 30 s; 5×10^{-6} M A23187 for 5 min; or 1.6×10^{-6} M PMA for 15 min in the presence of CB.

† Distribution is expressed as the percentage of the total activity or protein in the unfractionated sonicate that was present in each fraction. Results are given as the mean±SEM of six experiments with the range shown in parentheses. Of the six experiments, two were performed with fMet-Leu-Phe as stimulus, two with PMA, and one each with A23187 and C5a.

curves for fMet-Leu-Phe and PMA are approximately one log higher than the effective dose, 50% (ED_{50}) reported for O_2^- release using intact cells (6, 26, 27). If we assume that the peak response with C5a has not been reached at 10^{-7} M (the highest dose tested), then

the ED_{50} for C5a may also be 10-fold higher than that reported in whole cells (28). Tauber et al. (29) have shown that the ED_{50} for PMA depends on the cell density used. Because of technical reasons, stimulation of cells for activation and isolation of NADPH oxidase

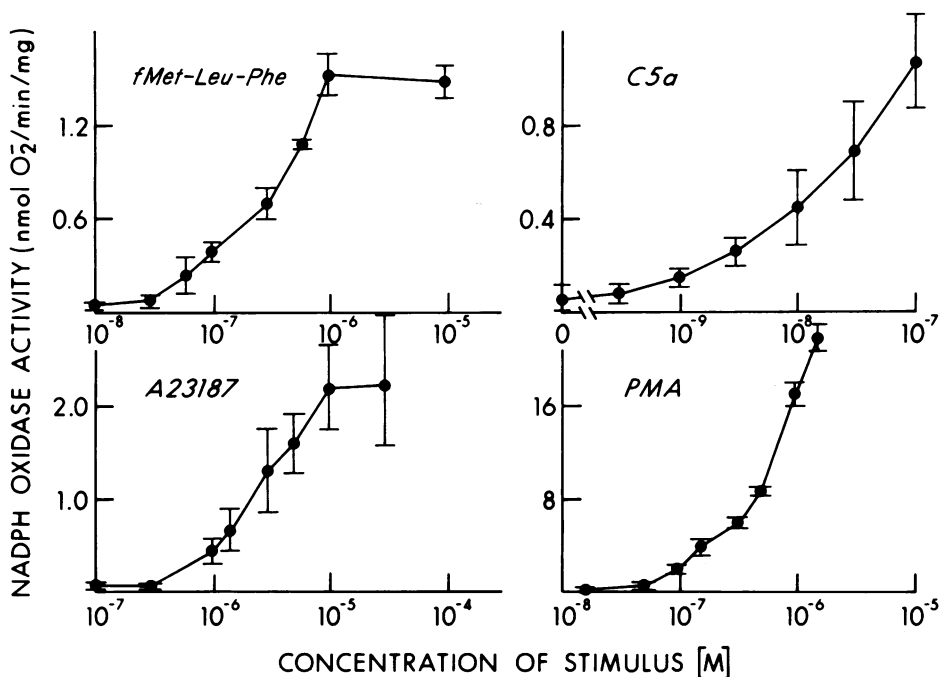


FIGURE 2 NADPH oxidase activity as a function of stimulus concentration. PMN were exposed to various concentrations of either fMet-Leu-Phe for 1 min, C5a for 30 s, A23187 for 5 min, or PMA for 15 min in the presence of CB. Particulate fractions were then isolated and assayed. Data are expressed as the mean±SEM of two to five experiments with each dose of stimulus.

is conducted at a much higher cell density than stimulation for O_2^- release by intact cells. In an experiment not shown, we found that the ED_{50} of fMet-Leu-Phe for O_2^- release by intact cells was shifted from 2×10^{-8} M to 2×10^{-7} M if the cell density was increased from 2×10^6 cells/ml to 5×10^7 cells/ml. Thus, the shift to a higher ED_{50} that we observe for oxidase stimulation appears to be caused by the higher cell density used during stimulation.

Temporal patterns of NADPH oxidase activation by different stimuli. NADPH oxidase activity varied dramatically with the time of exposure of the cells to the stimulus (Fig. 3). Activation by either fMet-Leu-Phe or C5a was initiated immediately with no detectable lag and peaked between 30 and 45 s. Levels of activity fell rapidly, decreasing to 30–40% of maximal by 10 min. In contrast, activation by A23187 or PMA had a 15–30-s lag before activity above resting levels was observed. Oxidase activity stimulated by A23187 peaked at 5 min and then declined slowly. Activity stimulated by PMA reached a plateau by 20 min and did not decline for up to 90 min.

The decline in oxidase activity seen with chemoattractants as stimuli did not appear to be caused by

inactivation of the ligand. Stimulation was conducted in the presence of 1 mM azide, which has been shown to prevent autoinactivation of chemotactic factors in human neutrophils (30). In experiments not shown, 10^{-6} M fMet-Leu-Phe after exposure to 5×10^7 cells/ml for 2 min was tested for its ability to either activate NADPH oxidase or to stimulate intact cell O_2^- release in fresh cells. Results showed that the ligand was 75% effective compared with buffer-treated fMet-Leu-Phe, and that the ED_{50} for O_2^- release was unchanged. Thus, the level of oxidase activity at 2 min (<40% of maximal) was much lower than the capability of the ligand to stimulate fresh cells. Also, if fresh fMet-Leu-Phe (10^{-6} M) were added to cells at 5 min after the initial dose, no restimulation of oxidase activity was observed (data not shown). These results suggest that oxidase inactivation is a regulatory process of the stimulated cells and not a by-product of ligand destruction.

Determination of K_m values. To examine if the same oxidase was activated by chemoattractants and other soluble stimuli, the apparent K_m values for NADPH and NADH of the activity obtained with each stimulus were determined by Lineweaver-Burk analysis and compared by analysis of variance (F test).

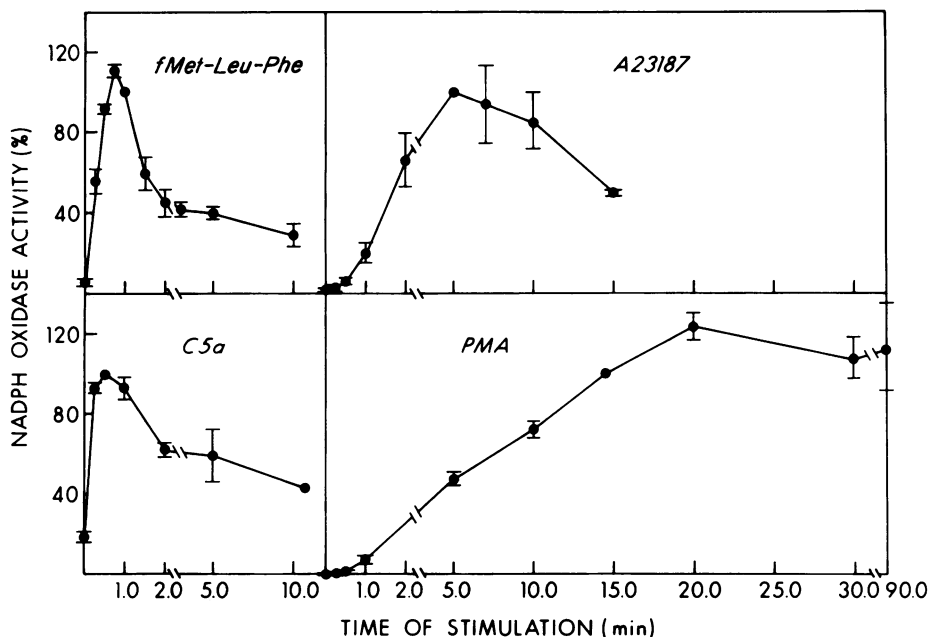


FIGURE 3 Temporal pattern of activation of NADPH oxidase by various stimuli. PMN were exposed for the indicated times to either 10^{-6} M fMet-Leu-Phe, 10^{-7} M C5a, 5×10^{-6} M A23187, or 1.6×10^{-6} M PMA in the presence of CB. Particulate fractions were then isolated and assayed. Data are expressed as the mean \pm SEM of two to eight experiments at each time point with each stimulus, except for only one experiment at 10 min with C5a. Specific activity (nanomoles O_2^- per minute per milligram) at 100% for each stimulus (expressed as mean \pm SEM with number of experiments in parentheses) was as follows: fMet-Leu-Phe, 1 min— 1.6 ± 0.4 (7); C5a, 30 s— 0.6 ± 0.3 (3); A23187, 5 min— 2.3 ± 0.4 (5); PMA, 15 min— 23.9 ± 3.3 (8).

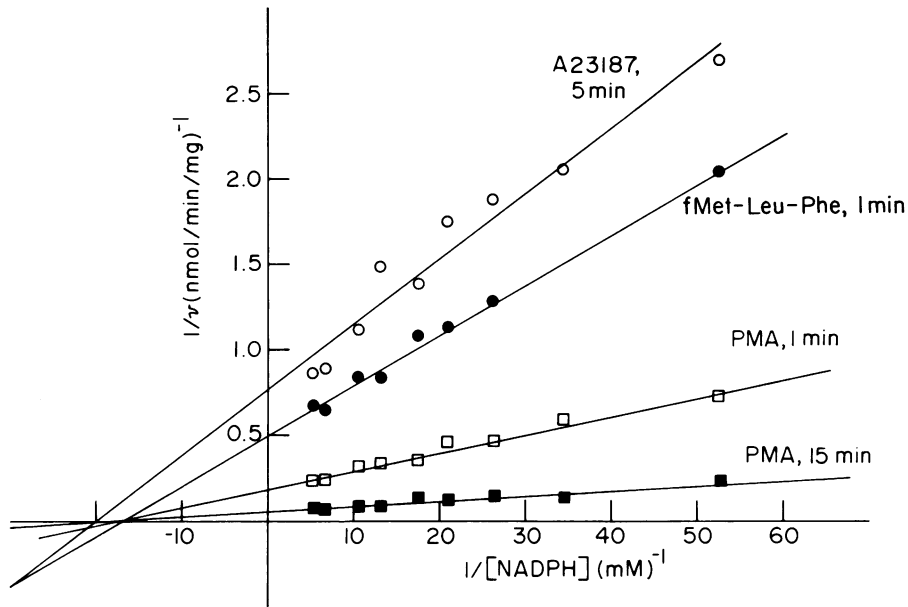


FIGURE 4 Lineweaver-Burk plots of NADPH oxidase activity in PMN stimulated by various agents. PMN were exposed to either 5×10^{-6} M A23187 for 5 min, 10^{-6} M fMet-Leu-Phe for 1 min, 1.6×10^{-6} M PMA for 1 min, or 1.6×10^{-6} M PMA for 15 min in the presence of CB. Particulate fractions were then isolated and assayed at NADPH concentrations varying from 9.5–190 μ M. Lines were drawn by linear regression analysis, with r values of 0.978 for A23187, 0.997 for fMet-Leu-Phe, 0.987 for PMA at 1 min, and 0.965 for PMA at 15 min. Plots shown are representative of three to four experiments with each stimulus.

NADPH concentration was varied from 9.5 to 190 μ M and representative Lineweaver-Burk plots are shown in Fig. 4. Cells were stimulated with either A23187 for 5 min, fMet-Leu-Phe for 1 min, PMA for 1 min, or PMA for 15 min. Activity at both an early

time and a maximal activation time for PMA was examined because it was conceivable that different enzymes were activated at different times. As can be seen, all of the lines intersect the x axis between -17 and -20 , which corresponds to K_m values of 50–60 μ M. Summarized data of the K_m and V_{max} values with NADPH as substrate are given in Table III. Mean K_m values varied from 60 μ M with A23187 as stimulus to 44 μ M with PMA for 15 min as stimulus, and are not significantly different ($F_{3,10} = 2.573$). V_{max} values varied with both the stimulus used and with the time of stimulation (using PMA as stimulus). NADH was also an effective substrate for the enzyme (data not shown); however, the K_m for NADH of the activity stimulated by fMet-Leu-Phe was ~ 10 -fold higher than that for NADPH, while the V_{max} was the same ($K_m = 450 \pm 20$ μ M, $V_{max} = 2.9 \pm 0.8$ nmol O_2^- /min per mg; mean \pm SEM, $n = 2$).

TABLE III
 K_m and V_{max} Values for NADPH Oxidase Activity Triggered by Various Stimuli

Stimulus*	K_m † μ M	V_{max} † nmol O_2^- /min/mg
fMet-Leu-Phe	59 \pm 4 (4)	3.3 \pm 0.8 (4)
A23187	60 \pm 6 (3)	2.2 \pm 0.7 (3)
PMA, 1 min	54 \pm 4 (3)	4.6 \pm 0.7 (3)
PMA, 15 min	44 \pm 6 (3)	22.7 \pm 4.8 (3)

* Neutrophils were treated with the indicated stimulus for various times in the presence of CB and particulate fractions were isolated. Conditions of stimulation were 10^{-6} M fMet-Leu-Phe for 1 min; 5×10^{-6} M A23187 for 5 min; 1.6×10^{-6} M PMA for 1 min; or 1.6×10^{-6} M PMA for 15 min.

† K_m and V_{max} were determined in each individual experiment by Lineweaver-Burk analysis of data obtained at 9–10 substrate concentrations. These values were combined for the mean \pm SEM shown. Total number of experiments performed with each stimulus is given in parentheses.

DISCUSSION

Although chemoattractants had been shown to trigger the respiratory burst in whole cells (6, 26–28, 31–33), activation of NADPH oxidase has not been previously demonstrated (5, 25). Recent evidence using whole cells suggested that the mechanism of activation of the respiratory burst by chemoattractants was different

from other stimuli. Superoxide production stimulated by chemotactic factors but not PMA was enhanced by increasing the concentration of extracellular calcium or by the presence of CB (6, 26–28). Also, pretreatment of PMN with a nonpenetrating protein-denaturing agent inhibited activation of the respiratory burst by fMet-Leu-Phe or concanavalin A (34), but not by PMA or A23187, and had no effect on lysosomal enzyme release triggered by any of the stimuli (5). These results had two possible explanations. Chemoattractants either activate the same respiratory burst enzyme by different transductional pathways or they activate a different respiratory burst enzyme. Our evidence supports the first possibility.

The data demonstrate that the chemoattractants, fMet-Leu-Phe and C5a, activate an NADPH oxidase in human PMN. Activation of the oxidase by chemoattractants is both time and dose dependent and, unlike other soluble stimuli, requires the presence of CB during the stimulation phase. The precise mode of action here of CB is not clear, but cytochalasin D and dihydrocytochalasin B, which inhibit microfilament function, but not glucose transport (35), were also effective in initiating expression of NADPH oxidase activity by chemotactic factors. This suggests that the state of actin filament assembly may regulate NADPH oxidase activation induced by chemoattractant receptors, but not by A23187 or PMA. However, a non-CB-mediated mechanism of microfilament disruption has been reported in macrophages (36), and it is conceivable that activation by PMA and A23187 may be influenced by this or another unknown process.

We did not observe any oxidase activation by chemoattractants in the absence of CB in agreement with earlier reports (5, 25). Low levels of O_2^- generation without CB have been measured during chemoattractant stimulation of whole cells (5, 6, 26–28). There are at least two possibilities to explain this discrepancy. First, the activity seen in whole cells without CB may be caused by activation of an enzyme different than NADPH oxidase. The presence of other superoxide-generating enzymes in phagocytic cells has been reported (37). Alternatively, the amount of enzyme activated in the absence of CB may be below the level of detection in our isolation and assay system. At this time we cannot distinguish between these two possibilities, although the quantitative explanation seems more plausible.

Comparison of kinetic parameters obtained by Lineweaver-Burk analysis of NADPH oxidase activity stimulated by fMet-Leu-Phe, A23187, or PMA shows that the enzyme activated by all three stimuli has the same K_m (Table II, Fig. 4, text). The average K_m values for NADPH ($54 \pm 3 \mu M$, mean \pm SEM, $n = 13$) and for NADH (fMet-Leu-Phe-activated enzyme) agree well

with previously reported values for NADPH oxidase activity in particulate fractions or phagocytic vesicles from cells stimulated by either digitonin (13), PMA (14, 15), or phagocytosis (12, 15–17). Prior determinations of K_m were made using fractions from cells stimulated in the absence of CB. Our data suggest that the same enzyme is activated both in the presence and absence of CB. The variation in V_{max} values implies that different quantities of the enzyme are activated by different stimuli and by the same stimulus (PMA) at different times. These data support the concept that the same respiratory burst enzyme is activated by all stimuli. However, final proof will require more direct evidence, such as purification of the enzyme activated by different stimuli, that is beyond the scope of this report.

Examination of the temporal pattern of activation of NADPH oxidase by different stimuli yielded several interesting results. First, activation of NADPH oxidase by chemoattractants appears to occur via a different transductional mechanism than other soluble stimuli. The most pronounced difference is the lack of a detectable lag with fMet-Leu-Phe and C5a-mediated activation, while a 15–30-s lag is apparent with A23187 and PMA. A lag period of ~ 1 min has also been found for activation of NADPH oxidase by digitonin in guinea pig granulocytes (13). Similar time differences between onset of stimulation by chemoattractants, PMA, and A23187 have been reported in whole cell studies of membrane potential changes (38), O_2^- production (39), degranulation (39), aggregation (40), cyclic AMP levels (41), and chemiluminescence (42). Although the exact lag periods have varied depending on the assay utilized, chemoattractants invariably show a shorter lag period than A23187 or PMA. Our data are the first evidence that differences in time courses of O_2^- production or chemiluminescence observed in whole cells are linked directly to activation of the respiratory burst enzyme and are not caused by a secondary phenomenon such as the diffusion rate of O_2^- or the nature of the light-producing reactions. The basis for the lag observed with A23187 or PMA is presently unknown and could be related to either membrane diffusion limitations or requirement for the formation of an intermediate. In any case, we can conclude that the mechanism of interaction of PMA and A23187 with PMN to activate the respiratory burst enzyme is quite different from that of chemoattractants.

The kinetic patterns of activation of the respiratory burst enzyme by different stimuli also suggest that different regulatory mechanisms are triggered with each stimulus. Chemoattractant-mediated activity is rapidly suppressed, while PMA-mediated activity is not. Previous studies with whole cells had shown that

O₂⁻ production stimulated by chemoattractants ceased after 1–2 min (6, 26–28, 43). Our results clearly demonstrate the triggering by chemoattractants of a deactivation mechanism that directly affects the level of the respiratory burst enzyme. This deactivation mechanism differs from those previously reported for NADPH oxidase activity (16, 44, 45), which were prevented by the presence of azide and CB during stimulation (phagocytosis), or were observed after addition of a competitive inhibitor to displace the stimulus from its receptor (concanavalin A). The “shut off” mechanism triggered by chemoattractants occurs in the presence of CB and azide, occurs without external displacement of the stimulus, and cannot be explained by destruction of the ligand. This rapid suppression of oxidase activity during activation by chemoattractants may serve a protective role in vivo.

We conclude that chemoattractants appear to activate the same respiratory burst enzyme in human PMN as do other stimuli, but that the transductional mechanism is different. Further, the activity of the respiratory burst enzyme is finely regulated by a balance between the rate of an activation mechanism(s) and the rate of deactivation process(es), which may be unique for different stimuli. Elucidation of the nature of these regulatory mechanisms will be important in understanding and modulating the bactericidal, tumoricidal, and inflammatory functions of phagocytic cells.

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