Neutrophils Exposed to Bacterial Lipopolysaccharide Upregulate NADPH Oxidase Assembly

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Abstract

Bacterial LPS is a pluripotent agonist for PMNs. Although it does not activate the NADPH-dependent oxidase directly, LPS renders PMNs more responsive to other stimuli, a phenomenon known as “priming.” Since the mechanism of LPS-dependent priming is incompletely understood, we investigated its effects on assembly and activation of the NADPH oxidase. LPS pretreatment increased superoxide (O$_2^-$) generation nearly 10-fold in response to N-formyl methionyl leucyl phenylalanine (fMLP). In a broken-cell system, activity was increased in plasma membrane–rich fractions and concomitantly decreased in specific granule–rich fractions from LPS-treated cells. Oxidation-reduction spectroscopy and flow cytometry indicated LPS increased plasma membrane association of flavocytochrome b$_{558}$. Immunoblots of plasma membrane vesicles from LPS-treated PMNs demonstrated translocation of p47-phox but not of p67-phox or Rac2. However, PMNs treated sequentially with LPS and fMLP showed a three- to sixfold increase (compared with either agent alone) in plasma membrane–associated p47-phox, p67-phox, and Rac2, and translocation paralleled augmented O$_2^-$ generation by intact PMNs. LPS treatment caused limited phosphorylation of p47-phox, and plasma membrane–enriched fractions from LPS- and/or fMLP-treated cells contained fewer acidic species of p47-phox than did those from cells treated with PMA. Taken together, these studies suggest that redistribution of NADPH oxidase components may underlie LPS priming of the respiratory burst. (J. Clin. Invest. 1998; 101:455–463.) Key words: inflammation • superoxide • endotoxin • respiratory burst • polymorphonuclear leukocytes

Introduction

Human polymorphonuclear leukocytes (neutrophils or PMNs) are mobilized readily to sites of infection and injury where they destroy invading microorganisms and remove damaged tissue and debris (1). PMNs possess a multicomponent NADPH-dependent oxidase which generates superoxide (O$_2^-$) (for a review see reference 2) and other reactive oxygen species (e.g., H$_2$O$_2$, OH·, and HOCl) that contribute greatly to the ability of PMNs to kill invading pathogens (3, 4). In the absence of a functioning oxidase, as is seen in patients with chronic granulomatous disease (CGD) (5), individuals have increased susceptibility to life-threatening bacterial and fungal infections (6).

In resting PMNs, the inactive oxidase is unassembled, with required components segregated into plasma membrane and cytosolic locations (7–9). During activation, required cytosolic components p47-phox (10–13), p67-phox (10, 14), and Rac2 (15, 16) translocate to the plasma membrane to associate with flavocytochrome b$_{558}$ (7, 17, 18), the key membrane-bound component, thereby assembling the active O$_2^-$-generating complex. Rap1A, a low molecular weight GTP-binding protein associated with flavocytochrome b$_{558}$ (19), and p40-phox, another cytosol-derived component (20), appear to have important but as of yet undefined roles in NADPH activation and O$_2^-$-generation.

Flavocytochrome b$_{558}$ appears to contain all of the redox components necessary for the transfer of electrons from NADPH to molecular oxygen, producing O$_2^-$ (21, 22). In resting cells, ∼10% of flavocytochrome b$_{558}$ is contained within the plasma membrane, 5–15% in the secretory vesicles, and the remainder (75–90%) stored in the membrane of specific granules (23, 24). Flavocytochrome b$_{558}$ is recruited to the plasma membrane from cytosolic granules after stimulation, a process which upregulates the oxidase and enables PMNs to direct production of O$_2^-$ to engulfed pathogens (23–25).

Lipopolysaccharide (LPS or endotoxin) elicits a variety of PMN responses, including receptor upregulation (26, 27), actin assembly (28), and adherence (29), and primes the cell for enhanced release of superoxide (O$_2^-$) in response to other stimuli such as N-formylated bacterial peptides (26, 30–34). Thus, LPS released from invading pathogens at sites of infection may, in a sense, sensitize local phagocytic cells to be more responsive and thereby amplify the inflammatory response. This amplified response may in turn rapidly eliminate the pathogens and/or result in untoward sequelae, such as manifestations of the systemic inflammatory response syndrome. However, LPS itself does not elicit significant O$_2^-$ generation, and the molecular basis of PMN priming by LPS is unknown.

To define further the mechanisms of priming for an enhanced respiratory burst, we investigated the effects of LPS on...
the NADPH oxidase. Our data indicate that LPS treatment (a) upregulated plasma membrane association of flavocytochrome b55 and (b) significantly enhanced assembly of the cytosolic oxidase factors with the plasma membrane–bound NADPH oxidase after stimulation with N-formyl methionyl leucyl phenylalanine (fMLP). The accentuated assembly of the oxidase at the plasma membrane correlated directly with the enhanced respiratory burst observed in intact cells. Our results suggest that increased plasma membrane association of NADPH oxidase components after LPS exposure is the basis for the enhanced respiratory burst observed in LPS-treated PMNs.

Methods

Materials. Endotoxin-free dextran was purchased from USB Biologicals (Cleveland, OH). GTP-γ-S and superoxide dismutase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). RNase-free Percoll was purchased from Pharmacia Biotech (Piscataway, NJ) and was assayed for endotoxin content. Endotoxin-free Hypaque (50% diatrizoate sodium injection grade, USP) was obtained from Nycomed (New York, NY). Endotoxin-free 0.9% NaCl and dH2O (both injection grade, USP) were obtained from Baxter Healthcare Corp. (Deerfield, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified.

Endotoxin preparation. LPS was purified from Salmonella minnesota as described previously (35). Stock solutions were prepared in Dulbecco’s PBS without calcium or magnesium (DPBS) or H2O and were sonicated 15–30 min at 25°C in a water bath sonicator (model 1200; Branson Ultrasonics Corp., Danbury, CT) before each use.

Neutrophil isolation. Heparinized, venous blood was obtained from healthy individuals (or from a patient with X-linked CGD) in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, and PMNs were isolated as described previously using Hypaque-Ficoll density-gradient separation after dextran sedimentation (36). Purified PMNs (>96% of the cells in the preparation) were resuspended in DPBS (or in phosphate-free loading buffer for 3P labeling) and kept on ice until use. All reagents and materials used in the preparation of PMNs (e.g., DPBS, Hypaque-Ficoll, dextran/NaCl, dH2O, and saline solutions) were essentially endotoxin-free (i.e., <10 pg/ml) as determined by Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Inc., Walkersville, MD).

Neutrophil priming. PMNs (2–10 × 10⁶ cells/ml) in DPBS (or loading buffer) were mixed gently at 37°C for the indicated times with LPS (15 or 100 ng/ml, as specified) and then centrifuged at 500 g for 10 min at 4°C. Treated PMNs were resuspended in an appropriate assay buffer (either DPBS plus glucose [DPBS + 1%] for superoxide assays, DPBS for flow cytometry, or relaxation buffer [23] for nitrogen cavitation) at specified cell densities (see below). Untreated PMNs were incubated in a manner identical to that for the LPS-treated cells but without LPS. For fMLP-activated PMNs, cells were incubated with or without LPS, as above, and then with or without 1 µM fMLP at 37°C for the indicated time(s). PMN treatments were terminated by placing cells immediately on ice, and all subsequent procedures were carried out at 0–4°C.

Intact PMN superoxide assays. PMNs were adjusted to 10⁶ cells/ml and treated with or without 100 ng/ml LPS for 60 min at 37°C. After incubation, LPS-treated or untreated PMNs were incubated with or without 1 µM fMLP at 37°C for an additional 10 min, and O₂⁻ generation was measured by the superoxide dismutase–inhibitable reduction of ferricytochrome c at 550 nm as described previously (37).

Neutrophil fractionation. PMNs (5 × 10⁶ cells/ml in DPBS) treated with or without LPS for 70 min, or with or without LPS for 60 min with or without 1 µM fMLP at 37°C for 10 additional min, were treated with 2–4 mM diisopropyl fluorophosphate for 15 min on ice, and then PMN subcellular fractions (plasma membrane– and specific granule–enriched fractions and a cytosol-derived fraction) were isolated using Percoll step gradients as described by Borregaard et al. (23). Plasma membrane– or specific granule–enriched fractions were resuspended in broken-cell assay buffer (38) to 10⁷ cell equivalents (CE)/ml for broken-cell superoxide assays or in relaxation buffer (23) for analysis by SDS-PAGE. Cavities used for quantitative comparison were obtained from an equal number of PMNs.

Broken-cell NADPH oxidase reconstitution assay. For broken-cell assays using SDS as the activating agent, 3 × 10⁶ CE neutrophil cytosol and 6 × 10⁶ CE neutrophil membranes were combined with 100 µM ferricytochrome c, 10 µM FAD, 10 µM GTP-γ-S, 100 µM SDS, and buffer (38) to 600 µl final volume. After a 3-min incubation with SDS, NADPH was added to a final concentration of 200 µM, and superoxide dismutase–inhibitable O₂⁻ production was monitored continuously at 550 nm on a single beam spectrophotometer (model DU 640; Beckman Instruments, Inc., Fullerton, CA) using an Auto 6 sampler (Beckman Instruments, Inc.) for 8–10 min. The concentrations of cytosol and membrane fractions used in these assays were adjusted so that O₂⁻ generation was not saturating. All samples used for comparison were analyzed simultaneously using the Auto 6 sampler.

Broken-cell assays activated with phosphatidic acid (PA) and diacylglycerol (DiC8) were done essentially as described by McPhail and colleagues (39). The contents of the PA and DiC8 assays were identical to the SDS assays except that 40 µg PA and 10 µg DiC8 replaced SDS, and each assay was incubated at room temperature for 30 min before addition of NADPH.

Spectral analysis of flavocytochrome b55. Plasma membrane– and specific granule–enriched fractions were isolated from untreated and LPS-treated PMNs as described above. The λ-peak at 427 nm in oxidized minus dithionite-reduced difference spectra was used to calculate the amount of flavocytochrome b55 in the plasma membrane– or specific granule–enriched fractions as described previously using an extinction coefficient of 106 mM⁻¹ cm⁻¹ (40). The recovery of flavocytochrome b55 from the gradients was 72.2–91.6% as determined by spectrophotometry and 87.2–101.7% by SDS-PAGE and subsequent densitometry.

Flow-cytometric analysis of plasma membrane–associated flavocytochrome b55. Plasma membrane association of flavocytochrome b55 was determined using an mAb (7D5) which recognizes an extracellular epitope of flavocytochrome b55 (41, 42). PMNs (2 × 10⁶ cells/ml in DPBS) treated with or without LPS (100 ng/ml for 5, 10, 20, 40, 60, and 90 min at 37°C) and with or without fMLP (1 µM added to identical untreated and LPS-treated PMNs after incubation for 60 min and further incubated for 1, 2, 5, 10, and 30 min at 37°C) were resuspended in FACScan® buffer (DPBS/2% normal goat serum) to a final concentration of 10⁷ cells/ml. Plasma membrane association of flavocytochrome b55 was detected using 7D5 and a fluorescein-conjugated goat anti-mouse IgG antibody (Organon Teknika Corp., Durham, NC) by the method of Jones et al. (43). After staining, samples were analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) at the University of Iowa Core Flow Cytometry facility. Unfixed PMNs were used for all flow-cytometric analyses, and a single live gate was used to eliminate debris and any contaminating cells.

Translocation of NADPH oxidase components and immunoblotting. Plasma membrane–enriched fractions were prepared as described above using the method of Borregaard (23). 1–2 × 10⁶ CE plasma membrane vesicles obtained from PMNs treated with or without LPS (100 ng LPS/ml for 5, 10, 20, 40, 60, or 70 min as indicated at 37°C) and/or with or without fMLP (1 µM for an additional 10 min at 37°C) were separated using 10% SDS-PAGE and then transferred to nitrocellulose as described previously (see p. 10.2.1 in reference 44). Immunoblots were processed using polyclonal antibody to p47-phox, p67-phox, and Rac2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by detection using enhanced chemiluminescence (Super Signal substrate; Pierce Chemical Co., Rockford, IL). Plasma membrane association of proteins was quantitated using a scanning laser densitometer (model CS-9000U; Shimadzu Corp., Kyoto, Japan).
Phosphorylation and immunoprecipitation of p47-phox. 1.2 × 10⁶ PMNs were resuspended in 1 ml loading buffer (10 mM Na-Hepes, 138 mM NaCl, 2.7 mM KCl, and 7.5 mM D-glucose, pH 7.5), prepared with endotoxin- and RNase-free reagents: contaminating LPS was removed by filtration through a 1-ml Detoxi-Gel column (Pierce Chemical Co., Rockford, IL) with 0.5 mM [³²P]orthophosphate and incubated at room temperature for 60 min. After loading, 2 × 10⁷ PMNs were treated with or without LPS (100 ng/ml for 60 min at 37°C) and then with or without fMLP (1 μM for 10 min at 37°C) and iced immediately. After diisopropyl fluorophosphate treatment as above, cells were resuspended in 100 μl lysis buffer (1% Triton X-100, 0.5 mg/ml leupeptin and pepstatin A, 1 mM PMSF, 1 mM sodium orthovanadate, and 0.5% cetyl trimethyl ammonium bromide in Tris-buffered saline, pH 7.5) for 20 min on ice. Insoluble material was removed by centrifugation (14,000 g for 30 s at room temperature), and the supernatants were diluted to 1 ml with 50 mM Tris-HCl, 190 mM NaCl containing 2.5% Triton X-100, and 1 mM sodium orthovanadate. p47-phox was immunoprecipitated using polyclonal antibody to p47-phox and *Staphylococcus* protein A (Pansorbin cells; Calbiochem Corp., La Jolla, CA) using a previously described method (45). *Staphylococcus* protein A–immune complexes were boiled in SDS-sample buffer without reducing agent and resolved with 10% SDS-PAGE. Dried gels were subjected to autoradiography using XAR films (Eastman Kodak Co., Rochester, NY), and the [³²P]orthophosphate incorporated into p47-phox was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Since the amount of p47-phox immunoprecipitated from cells treated with or without LPS and/or fMLP and PMA was compared, we verified that the rabbit antiserum against p47-phox recognized both native and phosphorylated p47-phox with equal efficiency (data not shown). Briefly, 10⁷ PMNs were warmed to 37°C and stimulated with buffer alone (control) or 2 μg/ml PMA for 10 min. Cells were lysed and processed as described above for immunoprecipitation. Immunoprecipitates were resolved by 10% SDS-PAGE, and the amount of p47-phox was quantitated. The same amount of p47-phox was recovered from control or PMA-stimulated cells.

Analysis of p47-phox phosphorylation by nonequilibrium pH-gradient electrophoresis (NEPHGE) and SDS-PAGE was performed as described previously (46). Briefly, PMNs treated with or without LPS (100 ng/ml for 60 min at 37°C) and then with or without fMLP (1 μM for 10 min at 37°C) were disrupted by sonication, and the plasma membrane–enriched fraction was isolated as described previously (8). After pH resolution by NEPHGE, gels were subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblots were processed using polyclonal antibody to p47-phox and developed using enhanced chemiluminescence (Super Signal substrate).

**Results and Discussion**

**Priming of intact PMNs by LPS.** To determine the effect of LPS on PMN O₂⁻ generation, cells were incubated for 60 min at 37°C with or without LPS, and then subsequently stimulated with 1 μM fMLP for an additional 10 min (Fig. 1). PMNs treated with LPS alone generated very little O₂⁻ (2.5±0.4 nmole O₂⁻/10⁶ PMNs vs. 1.4±0.9 for resting cells) (Fig. 1, bar 2 vs. bar 1). However, PMNs treated with LPS produced nearly 10-fold more O₂⁻ when stimulated subsequently with fMLP (24±1.8 nmole O₂⁻/10⁶ PMNs) than cells not treated with LPS before fMLP exposure (3.2±0.3) (Fig. 1, bar 4 vs. bar 3). Optimal priming effects in the absence of serum, as a source of LPS-binding protein (47), required pretreatment of PMNs with 100 ng LPS/ml for ~60 min as reported previously (30, 34), or as little as 1 ng LPS/ml in the presence of 100 ng LBP/ml (data not shown). Pretreating PMNs with LPS increased greatly the amplitude of the respiratory burst induced by fMLP but did not alter the duration of NADPH oxidase activity (data not shown). The synergy between LPS and fMLP as agonist for PMN O₂⁻ generation is consistent with previous reports (30, 32, 34, 48).

**Enhanced O₂⁻ production by plasma membrane–rich fractions of LPS-primed PMNs.** Because the NADPH oxidase catalyzes the production of O₂⁻, we used a broken-cell NADPH oxidase reconstitution system to test the hypothesis that LPS directly affects NADPH oxidase components. PMNs treated with or without LPS were fractionated, and the O₂⁻-generating activities of the plasma membrane– and specific granule–enriched fractions were compared. As shown in Fig. 2, incubation of cytosol and plasma membrane–rich fractions from LPS-treated PMNs produced approximately two times more O₂⁻ (1.2±0.24 nmole O₂⁻/min/10⁶ CE; bar 3) than analogous fractions from untreated cells (0.65±0.28; bar 1) in the SDS-activated broken-cell assay. Similar results were obtained in a broken-cell system activated by PA and Dic8, agonists which might better represent signaling pathways triggered by fMLP (39, 49). Again, cytosol and plasma membrane–rich fractions from LPS-treated PMNs elicited nearly twofold greater O₂⁻-generating activity (0.49±0.24 nmole O₂⁻/min/10⁶ CE) compared with cytosol and plasma membrane–rich fractions from untreated cells (0.26±0.14) (Fig. 2, bar 6 vs. bar 4). A similar enhancement of broken-cell O₂⁻-generating activity was observed when plasma membrane–rich fractions (from LPS-pretreated PMNs) were combined with cytosol obtained from either untreated or LPS-treated cells (compare bars 2 and 3 and bars 5 and 6 in Fig. 2). Thus, most of the enhanced

![Figure 1](image-url)  
*Figure 1.* O₂⁻ generation from PMNs after exposure to bacterial LPS. 5 × 10⁷ PMNs were incubated for 60 min at 37°C with or without LPS (100 ng/ml) and then with or without fMLP (1 μM) for 10 min at 37°C as indicated. O₂⁻ generation was determined as described in Methods, and results are expressed as the mean±SD of four separate experiments.
broken-cell $O_2^-$-generating activity reflected changes in the properties of the plasma membrane–rich fractions derived from LPS-treated PMNs.

**LPS induces mobilization of flavocytochrome $b_{558}$ to the plasma membrane.** It should be noted that whereas plasma membrane–rich fractions from LPS-treated cells showed increased NADPH oxidase activity when incubated with cytosol plus SDS or PA plus DiC8, these membrane fractions in the absence of added cytosol expressed very little activity (0.25±0.03 nmole $O_2^-$/min/10$^7$ CE for assays without added cytosol vs. 1.2±0.24 for assays containing added cytosol); $O_2^-$-generating activity of plasma membrane–rich fractions (without added cytosol) from resting cells is 0.13±0.02 nmole $O_2^-$/min/10$^7$ CE. These findings seem most compatible with a mobilization of flavocytochrome $b_{558}$–containing specific granules to the plasma membrane during PMN treatment with LPS rather than preassembly of the active $O_2^-$-generating complex. To test more directly the effect of LPS treatment on the subcellular localization of flavocytochrome $b_{558}$, we quantitated flavocytochrome $b_{558}$ in plasma membrane–rich fractions and in specific granules isolated from control and LPS-treated PMNs using oxidation-reduction difference spectroscopy. Spectral analysis revealed an increase in flavocytochrome $b_{558}$ in plasma membrane–rich fractions and a corresponding decrease in flavocytochrome $b_{558}$ from the specific granule–rich fractions after LPS treatment (Table I), consistent with an induction of specific granule exocytosis by LPS. The changes in flavocytochrome $b_{558}$ content of plasma membrane–rich fractions and specific granules paralleled changes in broken-cell NADPH oxidase activity expressed by these fractions in concert with cytosol. Thus, whereas the activity of plasma membrane–rich fractions (Fig. 2, bars 1–6) was increased by LPS treatment, the activity of the specific granules was decreased (Fig. 2, bars 7 and 8).

The data shown in Table I reproduce the subcellular distribution of flavocytochrome $b_{558}$ in control PMNs reported previously (23, 24), and suggest that upregulation of plasma membrane–associated flavocytochrome $b_{558}$ by LPS is the result of granule–plasma membrane fusion. However, one limitation of these data is that secretory vesicles copurify with plasma membrane vesicles using Percoll gradients (25), and, therefore, flavocytochrome $b_{558}$ recovered in plasma membrane–rich fractions of resting cells is derived from both plasma membrane and secretory vesicles. However, it is likely LPS elicits secretory vesicle mobilization before that of specific granules, in accordance with a reported hierarchy of granule exocytosis (25,50).

To monitor more directly the mobilization of flavocytochrome $b_{558}$ to the plasma membrane during LPS treatment, PMNs were analyzed by flow cytometry using an mAb (7D5) which recognizes an extracytoplasmic epitope of flavocytochrome $b_{558}$. FACScan analysis indicated 7D5 bound PMNs from healthy individuals, but did not bind to PMNs from a patient with X-linked CGD, indicating the specificity of 7D5 for PMNs incubated with or without LPS were fractionated, and flavocytochrome $b_{558}$ in plasma membrane– and specific granule–enriched fractions was quantitated by oxidation-reduction spectroscopy. Flavocytochrome $b_{558}$ concentration (from 10$^7$ CE) is expressed as the mean±SD of four separate experiments. Statistical analyses were performed using the paired Student’s $t$ test. *$P < 0.007$ vs. untreated plasma membranes. **$P < 0.01$.

### Table I. Subcellular Distribution of Flavocytochrome $b_{558}$ after LPS Exposure

<table>
<thead>
<tr>
<th>PMN treatment</th>
<th>Plasma membrane</th>
<th>Specific granule membrane</th>
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<tr>
<td></td>
<td>conc. (pmol)</td>
<td>% total</td>
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<tr>
<td>Untreated PMNs</td>
<td>4.33±0.85</td>
<td>(25.9%)</td>
</tr>
<tr>
<td>LPS-treated PMNs</td>
<td>6.42±0.66</td>
<td>(40.6%)‡</td>
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PMNs incubated with or without LPS were fractionated, and flavocytochrome $b_{558}$ in plasma membrane– and specific granule–enriched fractions was quantitated by oxidation-reduction spectroscopy. Flavocytochrome $b_{558}$ concentration (from 10$^7$ CE) is expressed as the mean±SD of four separate experiments. Statistical analyses were performed using the paired Student’s $t$ test. *$P < 0.01$ vs. untreated plasma membranes. **$P < 0.007$ vs. untreated plasma membranes. †$P < 0.05$ vs. untreated specific granule membranes.
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Furthermore, PMNs treated with or without LPS (100 ng/ml for up to 90 min at 37°C) and subsequently with or without fMLP (1 μM) for an additional 10 min and then probed with 7D5, an mAb which recognizes an extracytoplasmic epitope of flavocytochrome b₅₅₈. PMNs stained with a control IgG1 mAb of the same isotype as 7D5 and untreated PMNs from an individual with CGD were included for comparison (A). Results are from one experiment representative of five (A, healthy cells) or two (B, CGD cells).

Figure 3. Plasma membrane association of flavocytochrome b₅₅₈ after LPS treatment. PMNs from healthy subjects (A) or from an individual with X-linked CGD (B) were treated with or without LPS (60 min at 37°C with 100 ng LPS/ml) and subsequently with or without fMLP (1 μM) for an additional 10 min and then probed with 7D5, an mAb which recognizes an extracytoplasmic epitope of flavocytochrome b₅₅₈. PMNs stained with a control IgG1 mAb of the same isotype as 7D5 and untreated PMNs from an individual with CGD were included for comparison (A). Results are from one experiment representative of five (A, healthy cells) or two (B, CGD cells).

Figure 4. Time-dependent mobilization of flavocytochrome b₅₅₈ to the plasma membrane after LPS treatment. PMNs from healthy subjects were treated with (○) or without (●, ▲) 100 ng LPS/ml for the indicated times at 37°C and probed with 7D5, or were stimulated subsequently with fMLP (1 μM) (△, ▲) for the additional indicated times and then probed with 7D5. Results are expressed as the mean fluorescence ± SD of three to five separate experiments.
that translocation had similar kinetics as the LPS-induced up-regulation of flavocytochrome b and that of LPS priming in general (30, 33, 34), providing additional evidence that LPS priming for an augmented respiratory burst is the direct result of redistribution of NADPH oxidase components (Fig. 5, C and D). Analogous fractions from PMNs treated with fMLP alone showed a similarly small increase in all three cytosolic oxidase components (Fig. 5, A and B). However, when fMLP was added to LPS-pretreated PMNs, translocation of all three cytosolic protein species was enhanced dramatically; the levels of p47-phox, p67-phox, and Rac2 in the plasma membrane–enriched fractions were \( \approx 10 \)-fold above normal resting levels (Fig. 5, A and B). The combined effect of LPS and fMLP on the translocation of p47-phox, p67-phox, and Rac2 is much greater than additive, and correlates well with the relative levels of \( O_{2}\text{--} \) -generating activity expressed by intact PMNs under these different conditions.

**Phosphorylation of p47-phox induced by LPS and/or fMLP.** Previous studies have shown that phosphorylation and translocation of p47-phox correlate with activation of the respiratory burst in PMNs (46, 53, 54). To investigate further the possible role of p47-phox phosphorylation in LPS priming, we analyzed the effects of LPS pretreatment on the phosphorylation of p47-phox in fMLP-stimulated PMNs. LPS pretreatment of PMNs caused a detectable, albeit slight increase in the phosphorylation of p47-phox versus control cells (Fig. 6A). Phosphorylation of p47-phox was increased roughly fivefold when PMNs were treated with fMLP alone but was enhanced only slightly further in cells pretreated with LPS before addition of fMLP (Fig. 6A). Thus, whereas translocation of p47-phox correlated with augmented \( O_{2}\text{--} \) -generating activity in cells treated sequentially with LPS and fMLP (compare Figs. 1 and 5), phosphorylation of p47-phox after fMLP stimulation did not appear similarly enhanced by LPS pretreatment (compare Figs. 1 and 5 with Fig. 6). Because the results shown in Fig. 6A include combined membrane and cytosolic pools of p47-phox, it could be argued that differences in the plasma membrane–bound phosphorylated p47-phox might be masked by additional phosphorylated cytosolic species. Plasma membrane–rich fractions from PMNs pretreated with LPS and then
treated with fMLP do not show a substantially greater accumulation of more acidic (i.e., phosphorylated) p47-phox species than do cells at rest or after treatment with LPS or fMLP alone (Fig. 6 B). The limited effects of LPS and/or fMLP on phosphorylation of p47-phox are particularly striking when juxtaposed with the effects of PMA, both quantitatively and qualitatively. Incorporation of 32P into p47-phox was nearly sevenfold greater after PMA treatment than after LPS alone (Fig. 6 A), whereas incorporation of 32P into p47-phox after a sequential treatment of LPS and fMLP was similar in magnitude to that of PMA (Fig. 6 A). By contrast, plasma membrane–rich fractions from PMNs treated with PMA contained at least eight acidic derivatives of p47-phox, including several species more acidic than the most anodal species accumulating after treatment with LPS and/or fMLP. Analogous fractions obtained from PMNs treated with LPS, fMLP, or LPS and fMLP sequentially contained only one or two more acidic species of p47-phox than did untreated PMNs (Fig. 6 B). These findings suggest that even limited phosphorylation of p47-phox induced by LPS and/or fMLP may play an important role in p47-phox translocation and respiratory burst activation (46, 53–55). Since there are few anodal plasma membrane–associated species of p47-phox in any of the LPS/fMLP combinations (Fig. 6 B), the effects of LPS and fMLP may only transiently involve phosphorylation of p47-phox or be limited to one or two important phosphorylation sites affecting a small percentage of total p47-phox. However, we cannot exclude the possible existence of an alternative phosphorylation-independent pathway of NADPH oxidase activation as suggested previously by Robinson et al. (56), or a possible lack of correlation between phosphorylation and translocation with certain agonists as reported previously (51). Studies are currently under way to determine the location and kinetics of these limited LPS- and/or fMLP-induced phosphorylation events.

In summary, our data suggest that LPS priming for enhanced \( \cdot \mathrm{O}_2^- \) generation by fMLP-stimulated PMNs reflects redistribution of NADPH oxidase components, thereby facilitating increased assembly of the NADPH oxidase. The effects of LPS and fMLP on this assembly process appear to be distinct: LPS pretreatment induces mainly translocation of flavocytochrome \( \mathrm{b}_{558} \) from specific granules to the plasma membrane, with limited translocation of p47-phox but little concomitant translocation of p67-phox or Rac2 from the cytosol. In contrast, subsequent treatment with fMLP rapidly induces translocation of all three cytosolic components to the plasma membrane, with little additional recruitment of flavocytochrome \( \mathrm{b}_{558} \). Previous attempts (30, 32) anticipated the more detailed characterization of cytosolic components and precise definition of the subcellular distribution of the flavocytochrome \( \mathrm{b} \) available today. Specifically, the method used by Forehand et al. to isolate plasma membrane vesicles also coisolated specific granules (32). Therefore, LPS-augmented, plasma membrane–associated \( \cdot \mathrm{O}_2^- \)-generating activity resulting from flavocytochrome \( \mathrm{b} \) redistribution would have gone unnoticed as reported (32).

The events reported here appear to be directly responsible for the enhanced respiratory burst observed in LPS-primed PMNs, since translocation of cytosolic oxidase components is closely associated with \( \cdot \mathrm{O}_2^- \) generation (8, 9). However, it is apparent that other more proximal events result in upregulation of the NADPH oxidase after LPS exposure and involve a variety of signaling pathways (28, 32, 33, 52). Recently, El Benna...


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