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Reconstitution of Chemotactic Peptide-Induced Nicotinamide Adenine Dinucleotide Phosphate (Reduced) Oxidase Activation in Transgenic COS-phox Cells

Rong He,* Masakatsu Nanamori,* Hairong Sang,* Hong Yin,* Mary C. Dinauer,† and Richard D. Ye2*

A whole-cell-based reconstitution system was developed to study the signaling mechanisms underlying chemoattractant-induced activation of NADPH oxidase. This system takes advantage of the lack of formyl peptide receptor-mediated response in COS-phox cells expressing g91phox, p22phox, p67phox, and p47phox, which respond to phorbol ester and arachidonic acid with O2 production. By exogenous expression of signaling molecules enriched in neutrophils, we have identified several critical components for fMLP-induced NADPH oxidase activation. Expression of PKCα, but not PKCα, -βIII, and -ζ, is necessary for the COS-phox cells to respond to fMLP. A role of PKCα in neutrophil NADPH oxidase was confirmed based on the ability of fMLP to induce PKCα translocation and the sensitivity of fMLP-induced O2 production to rottlerin, a PKCα-selective inhibitor. Optimal reconstitution also requires phospholipase C-β2 and PI3K-γ. We found that formyl peptide receptor could use the endogenous Rac1 as well as exogenous Rac1 and Rac2 for NADPH oxidase activation. Exogenous expression of p40phox potentiated fMLP-induced O2 production and raised the level of O2 in unstimulated cells. Collectively, these results provide first direct evidence for reconstituting fMLP-induced O2 production in a nonhemopoietic cell line, and demonstrate the requirement of multiple signaling components for optimal activation of NADPH oxidase by a chemoattractant. The Journal of Immunology, 2004, 173: 7462–7470.

The phagocyte NADPH oxidase activity is vitally important for host defense against invading microorganisms. In neutrophils, O2 production requires the phagocyte oxidase (phox)3 components g91phox, p22phox, p67phox, and p47phox (reviewed in Ref. 1). Defect in any one of these components compromises the ability of neutrophils to produce O2 and eliminate bacteria, as seen in patients suffering from chronic granulomatous diseases (2, 3). Extensive studies have been conducted to characterize the NADPH oxidase and its activation mechanism. It has been established that the active NADPH oxidase is a complex consisting of the membrane-associated proteins g91phox and p22phox, and the cytosolic proteins p67phox and p47phox, as well as the small GTPase Rac1 and/or Rac2 (reviewed in Ref. 1). Phosphorylation of p47phox and p67phox and activation of Rac, are critical steps preceding membrane translocation of the cytosolic factors and formation of a functional NADPH oxidase complex at the plasma membrane (1). The exact function of another cytosolic protein, p40phox, remains incompletely understood.

Biological processes that trigger the activation of phagocyte NADPH oxidase include phagocytosis of opsonized bacteria and binding of chemoattractants such as fMLP and C5a to their cell surface receptors. These events induce O2 production through activation of FcγRs and G protein-coupled chemoattractant receptors, respectively. In addition, pharmacological agents such as phorbol esters (e.g., PMA) and amphiphilic molecules (e.g., arachidonic acid and SDS) are potent stimuli of the phagocyte NADPH oxidase. These agents are extensively used in characterization of the NADPH oxidase activation mechanisms in both intact cells and cell-free reconstitution assays. The development of the cell-free assay for in vitro reconstitution of NADPH oxidase activity (4–7) has greatly accelerated the characterization of individual phox proteins and their interactions during assembly of a functional NADPH oxidase complex. However, reconstitution of receptor-mediated NADPH oxidase activation remains difficult in the cell-free system, and there are discrepancies in data collected from the cell-free assays and whole-cell-based experiments. For example, the Src homology 3 (SH3) domain located near the C terminus of p67phox is essential for NADPH oxidase activation in intact cells, whereas it is not required in the cell-free assay (8). Furthermore, prenylated Rac1 is sufficient to initiate NADPH oxidase assembly in cell-free assays (9) but unable to mediate fMLP-elicted superoxide generation in neutrophils derived from Rac2 knockout mice (10, 11). These findings imply that NADPH oxidase activation in intact cells requires additional signaling components that are not essential in the cell-free assays.

The chemoattractant fMLP is a potent activator of phagocyte NADPH oxidase. fMLP induces a rapid and transient O2 generation in phagocytes through binding to the Gαi-coupled formyl peptide receptor (FPR) (12, 13). It is presumed that the same NADPH oxidase components are used for both fMLP-elicted and PMA-stimulated response, but additional signaling events must exist for receptor-mediated activation of protein kinase C (PKC) and the
Rac small GTPase, and possibly for phosphorylation and final assembly of the phox proteins. An understanding of the related signaling mechanisms is important for the control of undesirable activation of phagocytes, which contributes to the release of oxidants and tissue damage. Genetic studies involving targeted deletion of phospholipase C-β (PLCβ)2/3 and PI3Kγ have shown that these enzymes are important for chemoattractant-induced NADPH oxidase activation (reviewed in Ref. 14). However it is not clear whether activation of these signaling molecules is sufficient for O2 production, and how they interact with kinases and GTPases that are directly responsible for the phosphorylation and translocation of phox proteins. An area of significant interest is the involvement of different PKC isoforms in fMLP-induced NADPH oxidase activation. Unlike PMA that can activate multiple PKCs, signaling through FPR may only stimulate selected PKC isoforms. Another unresolved issue is the vast disparity among chemoattractant and chemokine receptors in their ability to activate NADPH oxidase. Although all these receptors are capable of mediating leukocyte chemotaxis, only a small fraction of them can stimulate O2 production. These unanswered questions necessitate the development of reconstitution systems in which receptor-mediated NADPH oxidase can be studied in detail.

To accomplish this goal, we explored several possibilities for reconstituting FPR-mediated NADPH oxidase activation in intact cells. A previous study demonstrates that expression of p49\(\text{phox}\), p67\(\text{phox}\), and p47\(\text{phox}\) in the erythroleukemia cell line K562 renders the cells responsive to PMA in O2 production assay (15). Reconstitution of NADPH oxidase activity was also achieved by expression of p47\(\text{phox}\) in EBV-transformed B cells that lack this cytotoxic factor (16). In both cases, the reconstituted cells produced a relatively small amount of O2 compared with neutrophils. More recently, one of our laboratories generated a stable COS-7 line expressing p99\(\text{phox}\), p22\(\text{phox}\), p67\(\text{phox}\), and p47\(\text{phox}\) (the COS-phox cell line) (17). Stimulation of these cells with PMA and arachidonic acid led to potent production of O2, suggesting the possibility of using these genetically amenable cells to identify signaling molecules downstream of the activated FPR. Whereas K562 is a cell line of hemopoietic lineage and may already have the necessary signaling components for FPR signaling, COS-7 is an epithelial cell line that lacks the hemopoietic specific proteins required for FPR signaling (18). We exploited this property of the COS-phox cells to examine the roles of selected signaling molecules in FPR-mediated NADPH oxidase activation by taking a gain-of-function approach. In this study, we report reconstitution of fMLP-induced NADPH oxidase activity in the transgenic COS-phox cells. Our results indicate that the novel PKC isoform, PKCα, plays an important role in fMLP-induced O2 production in both the transfected COS-phox cells and human neutrophils. Our results also suggest a role of p40\(\text{phox}\) in positive regulation of fMLP-induced NADPH oxidase activation.

Materials and Methods

Reagents

The N-formyl peptide fMLP, PMA, and isoluminol were purchased from Sigma-Aldrich (St. Louis, MO). HRP and superoxide dismutase (SOD) were purchased from Roche (Indianapolis, IN). Pertussis toxin was obtained from List Laboratories (Campbell, CA). Cytochalasin D and rottlerin were obtained from Calbiochem (San Diego, CA). Polyclonal rabbit serum against Go2 was prepared against a synthetic peptide with the sequence KACNKLKDCLGF. The following Abs were purchased from the indicated sources (in parentheses): mouse Abs against Myc-epitope tag and HA-epitope tag (Covance, Richmond, CA), rabbit polyclonal Abs against PLCβ2 and PKCβII (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse mAb against p40\(\text{phox}\) (Upstate, Lake Placid, NY), a mouse mAb to β-actin (Santa Cruz), a mouse mAb against Rac1 (BD Pharmingen, San Diego, CA), and rabbit polyclonal Abs against nonphosphorylated and phospho-PKCα (Th505) (Cell Signaling Technologies, Beverly, MA).

Expression vectors

A full-length cDNA for human FPR was subcloned into the prey vector (BD Pharmingen). Plasmids containing cDNA inserts for wild-type Goi proteins were gifts from Drs. C. Knall and G. Johnson (National Jewish Center, Denver, CO). The PLCβ2 expression vector was a gift from Dr. D. Wu (University of Connecticut Health Center, Farmington, CT). Preparation and characterization of the HA-tagged PKCs, PKCδ, and PKCɛ expression constructs were described in a previous publication (19). The GFP expression vector EGFP-N1 was from Clontech (Palo Alto, CA). A full-length cDNA for human Rac1 (Guthrie Research Institute, Sayre, PA) was subcloned into the prey vector expression vector (BD Pharmingen). A Myc-tagged Rac1 expression construct was provided by Dr. U. Knaus (Scirpiss Research Institute, La Jolla, CA). Myc-tagged p101 and p101 constructs were provided by Dr. A. Sinaraki (University of Rochester, Rochester, NY). The p40\(\text{phox}\) expression vector was a gift from Dr. S. Chanock (National Cancer Institute, National Institutes of Health, Bethesda, MD).

Preparation of human neutrophils

Peripheral blood was drawn from healthy donors, using a protocol approved by the Institutional Review Board at the University of Illinois (Chicago, IL). Neutrophils were prepared using Percoll gradient centrifugation based on the method of Umler and Flad (20), as detailed in a previous publication (21). The prepared cells contained ~97% neutrophils with viability ≥98%. Neutrophils were resuspended in serum-free RPMI 1640 medium at a density of 2 × 10^6 cells/ml before use. Blood cells from different donors (n ≥ 3) were used in experiments.

Cell culture and transient transfection

The transgenic Cos-phox cells were generated as described previously (17). The stable cell line was maintained at 37°C with 5% CO2 in DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were grown by limiting dilution with the presence of 0.6 mg/ml hygromycin (Sigma-Aldrich), 0.8 mg/ml neomycin (Invitrogen Life Technologies, Carlsbad, CA), and 1 μg/ml puromycin (Calbiochem). LipofectAMINE 2000 reagent (Invitro- gen Life Technologies) was used for transient transfection of 6–7 μg of DNA into COS-phox cells grown in a 100-mm culture dish (0.5–1 × 10^6 cells per dish). Cells were analyzed 21–24 h after transfection. Transient transfection efficiency, determined by flow cytometry based on fluorescence of a cotransfected GFP, was 45–55%.

Measurement of NADPH oxidase activity

Superoxo production by COS-phox cells and neutrophils was determined by an isolinom-ELC assay (22), in 6-mm diameter wells of 96-well, flat-bottom, white tissue culture plates (E&K Scientific, Campbell, CA). COS-phox cells were harvested with enzyme-free cell-dissociation buffer (Invitrogen Life Technologies), and washed once with 0.5% BSA/BBSS. Cells were then resuspended in 0.5% BSA/RPMI 1640 buffer at the density of 3.5 × 10^6 cells/ml, and preincubated in the dark with 100 μM isoluminol and 40 U/ml HRP at room temperature for 5 min. Aliquot (200 μl) of the cells was added into the well and assayed for chemiluminescence (CL) at 37°C in a Wallace 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA). The CL counts per second (cps) was continually recorded, at 1-min intervals, for 5–15 min before and 20–30 min after stimulation with PMA or fMLP. Supernatant containing 250 U of SOD in addition to the stimulators, were run in parallel. The relative level of superoxide production was calculated based on the integrated CL during the first 20 min (COS-phox cells) or first 10 min (neutrophils) after agonist stimulation.

Analysis of protein expression

Whole-cell extracts were generated as described previously (21). In brief, the transfected COS-phox cells were lysed with 200–500 μl of PAGE buffer containing protease inhibitors (Protease Inhibitor Mixture Set I; Cal- biochem). Each sample was sonicated for 15 s on ice (60 Sonic Disembr- brator; Fisher, Hampton, NH) and heated at 95°C for 5 min. Whole-cell extracts were analyzed by 10% denaturing SDS gels, and protein profiles were then transferred to nitrocellulose membranes (Hybond ECL; Amer sham Biosciences, Piscataway, NJ) for Western blotting using ECL detec tion (Pierce, Rockford, IL).

Oxygen consumption measurements were used to determine the cell surface expression of FPR. Briefly, the transfected COS-phox cells were incubated with an anti-FPR mAb 5F1 (BD Pharmingen), washed in PBS containing 0.2% BSA, and then incubated with FITC-conjugated anti-mouse IgG (1;
The green fluorescence of each single cell was detected using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). All incubations were done on ice for 60 min.

**Rac activation assay**

Activation of Rac was determined as previously described (17), based on the affinity of Rac-GTP for the p21-binding domain (PBD) of PAK1 (23). The PBD-GST fusion protein was expressed in Escherichia coli strain HB101 and purified. Twenty-one hours posttransfection, COS-phox cells were detached with dissociation buffer, washed, and resuspended in RPMI 1640 containing 0.5% BSA to 5 × 10^6 cells/0.5 ml. The cells were then stimulated with fMLP or left untreated as indicated in the figures. Twenty micrograms of PAK1 PBD-GST recombinant protein was added, and cells were lysed by the addition of lysis/wash buffer (6 mM Na2HPO4, 4 mM NaH2PO4, 1% Nonidet P-40, 150 mM NaCl, 2.5 mM MgCl2) supplemented with 2 mM PMSF, Protease Inhibitor Cocktail III (Calbiochem), 0.1 mM Na3VO4, and 50 mM NaF. The lysate was cleared, 30 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) was added, and the binding reaction was conducted for 1 h at 4°C. Beads were pelleted and washed three times with wash buffer, and then finally resuspended in 30 μl of Laemml sample buffer. Aliquots of supernatant (Rac-GDP) and pull-down samples (Rac-GTP) were electrophoresed, and the proteins were analyzed as described above.

**PKC phosphorylation and translocation**

Isolated neutrophils (1–5 × 10^7 cells) were stimulated with either PMA or fMLP over a time course of 0–30 min, and stopped by addition of a 10-fold excess volume of cold HBSS. Cells were treated with 1 mM diisopropylfluorophosphate, suspended in hypotonic lysis buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgSO4, 0.5 mM EGTA, 0.1% 2-ME) supplemented with 1 mM PMSF and protease inhibitors at a concentration of 1 × 10^6 cells/ml, and sonicated (5 s, three times, at level 2) on ice. The sonicated samples were centrifuged at 800 × g for 10 min at 4°C, and the supernatants were subjected to differential centrifugation at 150,000 × g for 90 min at 4°C to yield cytosolic fractions (supernatants) and membrane/particulate fractions (pellets). Membrane/particulate fractions were subjected to Western blotting to detect PKC8 translocation by using a specific anti-PKC8 Ab (Cell Signaling Technologies). For PKC phosphorylation assay, the stimulated neutrophils were directly lysed with 200–500 μl of SDS-PAGE buffer containing protease inhibitors, sonicated for 15 s on ice, and heated at 95°C for 5 min. Whole-cell extracts were analyzed by 10% denaturing SDS gels and phosphorylated PKC8 was detected by Western blotting using a specific anti-phospho-PKC8 Ab (Cell Signaling Technologies).

**Statistics**

Paired Student’s t test was performed to determine statistical significance. A p value of <0.05 was considered to be significant.

**Results**

**Expression of FPR in COS-phox is insufficient for reconstitution of fMLP-induced O2 production**

The COS-phox cell line was generated by stable expression of gp91phox, p22phox, p47phox, and p67phox in the monkey epithelial cell line COS-7 (17). Expression of these components enables the epithelial cells to respond to arachidonic acid and PMA with potent O2 production (17) (Fig. 1). The PMA-induced O2 production was dose dependent, could be inhibited by SOD (Fig. 1A and B), and displayed kinetics similar to that of neutrophils (Fig. 1A and data not shown). To determine whether COS-phox cells respond to fMLP, we expressed the human FPR by means of liposome-mediated transfection. Twenty-four hours after transfection, 45–55% of the cells expressed FPR on cell surface as detected by an anti-FPR mAb (Fig. 1C). However, fMLP was unable to induce O2 production in the transfected cells (Fig. 1A and B), suggesting that COS-phox cells lack the necessary components for FPR-mediated signaling leading to NADPH oxidase activation.

**Multiple signaling molecules are required for optimal O2 production in fMLP-stimulated COS-phox cells**

Previous studies have shown that PLCβ2 and PI3Kγ are important for fMLP-induced neutrophil functions including O2 production (14). Because COS-phox cells do not contain these hemopoietic cell-specific enzymes, we speculated that exogenous expression of PLCβ2 and/or PI3Kγ might render the cells responsive to fMLP. To our surprise, COS-phox cells cotransfected to express PLCβ2 and FPR, PI3Kγ and FPR, or PLCβ2 and PI3Kγ with FPR still could not respond to fMLP with detectable O2 production (data not shown), suggesting that these two enzymes were insufficient for reconstitution of FPR-mediated NADPH oxidase activation.

Additional signaling molecules including the Gαi proteins, the small GTPases Rac1, and selected PKC isoforms (shown in Fig. 7...
below), were expressed in COS-phox either alone or in combinations. Although none of these molecules could rescue FPR-mediated O$_2^-$ production when expressed alone, the combined expression of FPR, Gαi2, P13Kγ, PLCβ2, Rac1, and PKCδ (FPR plus six plasmids (6PL)) resulted in the production of substantial amount of O$_2^-$ when the cells were stimulated with fMLP. After normalization with transfection efficiency (45–55%), the O$_2^-$ produced in response to 1 μM fMLP (Fig. 2B) was similar to the O$_2^-$ generated in cells stimulated with 50 ng/ml PMA (Fig. 1B). Inclusion of SOD in the assay buffer abolished fMLP-induced O$_2^-$ production as well as spontaneous production of O$_2^-$ in the 6PL-transfected COS-phox (Fig. 2A). The response to fMLP was rapid for both COS-phox cells and neutrophils, whereas the response to PMA was delayed in both cells.

Reconstitution of fMLP-induced O$_2^-$ production in transfected COS-phox cells. COS-phox cells were transiently transfected with fMLP expression constructs coding for FPR, Gαi2, PLCβ2, P13Kγ (p110γ and p101), PKCδ, and Rac1 (FPR plus 6PL). Twenty-four hours after transfection, cells were assayed for O$_2^-$ generation. A, A representative histogram showing CL (cps) recorded after addition of 1 μM fMLP for the first 30 min. SOD (250 U) was added to the control. B, CL integrated during the first 20 min after agonist stimulation was quantified and expressed as mean ± SEM, from three independent experiments. C, Expression of the transfected components were detected by Western blotting using either specific Abs (for Gαi2 and PLCβ2) or Abs against epitope tags (HA-tagged PKCδ, myc-tagged Rac1, and myc-tagged p110γ and p101). β-Actin in the same cell lysate was used as a control for sample loading and efficiency of transfer.

Requirement of Gai coupling to FPR for the reconstitution of NADPH oxidase activity

Because FPR-mediated O$_2^-$ production requires functional coupling of the receptor to the Gαi class of G proteins in neutrophils (12, 13), we next examined a role of the Gαi proteins in the transfected COS-phox cells. Treatment with pertussis toxin that ADP-ribosylates the Gαi proteins and disrupts their interaction with FPR, abolished fMLP-induced O$_2^-$ production in the transfected COS-phox cells (Fig. 3B) as well as in human neutrophils (A). Likewise, removal of the exogenous Gαi2 expression construct from the transfection mixture reduced the fMLP-stimulated O$_2^-$ production by ~68% (Fig. 3B), confirming an important role of Gαi2 in coupling FPR to the downstream signaling pathways. We have also observed that a substitution with the exogenous Gαi3 for Gαi2 resulted in similar level of O$_2^-$ production, suggesting that FPR can couple to either protein for NADPH oxidase activation.

The relative contributions of exogenous vs endogenous PLCβ and P13K to FPR-mediated NADPH oxidase activation

We next investigated whether PLCβ2 and P13K, which by themselves reconstitute the FPR-mediated response, are necessary for the fMLP-induced O$_2^-$ production. COS-phox cells were transfected without either the PLCβ2 expression vector, or the two P13Kγ expression plasmids coding for the respective catalytic and regulatory subunits. Omission of the PLCβ2 expression vector led to a small (~23%) but statistically significant ($p < 0.05$) reduction in O$_2^-$ production. The absence of P13Kγ resulted...
in a ~75% decrease in O$_2^-$ production (Fig. 4). These findings suggest possible involvement of the two hemopoietic-specific enzymes in O$_2^-$ production by fMLP in the transfected cells. The results also indicate that the endogenous PLC$\beta$ and PI3K isoforms can partially fulfill the functions of PLC$\beta$2 and PI3K$\gamma$.

**Roles of Rac1 and Rac2 in the FPR reconstitution assay**

The small GTPase Rac is essential for NADPH oxidase activation in neutrophils and in cell-free reconstitution assays (24, 25). We investigated whether Rac also plays a critical role in fMLP-induced O$_2^-$ generation in the transfected COS-phox cells. In the experiments described below, COS-phox cells were transfected with expression constructs coding for FPR, Goi2, PI3K$\gamma$, PLC$\beta$2, and PKC$\delta$, with or without the expression vectors for human Rac1 and Rac2. Although the initial successful reconstitution included an expression construct of Rac1, substantial amount of O$_2^-$ could be produced in its absence (Fig. 5A, first group). This result suggests that FPR is able to use the endogenous Rac1 for activation of NADPH oxidase. In recent years, a critical role of Rac2 in neutrophil NADPH oxidase activation has been reported based on studies of Rac$^{2-/-}$ mice (10, 26). We investigated whether activation of Rac2 is a unique property of hemopoietic cells by exogenous expression of Rac2 in the transfected COS-phox cells. As shown in Fig. 5, Rac2 is absent from COS-phox cells and its expression resulted in a statistically significant ($p < 0.05$) enhancement of the fMLP-induced O$_2^-$ production. Activation of Rac under these conditions was determined based on the ability of the N-terminal domain of p21-activated kinase 1 to bind and pull down the activated Rac (23). Results shown in Fig. 5C demonstrate that fMLP induced rapid but transient increases in this binding as detected by Western blotting with either an anti-Rac1 Ab for the endogenous Rac1 or an anti-myc Ab for the exogenous Rac1 and Rac2 recovered from the pull-down assay. The kinetics of Rac activation remained unchanged when Rac1 is overexpressed, with peak activation detected at around 1 min after fMLP stimulation. This is consistent with the rapid generation of O$_2^-$ in fMLP-stimulated neutrophils and reconstituted COS-phox cells. Activation of Rac2 also peaked at ~1 min (Fig. 5C), and O$_2^-$ production in Rac2-transfected cells was similarly rapid as was observed in Rac1-transfected cells (data not shown).

**Expression of p40$^{phox}$ enhances fMLP-induced O$_2^-$ production in reconstituted COS-phox cells**

p40$^{phox}$ is a cytosolic, SH3 domain-containing protein found in myeloid cells (27), but not in COS-7 cells (Fig. 6A). Although p40$^{phox}$ is known to form an active complex with p47$^{phox}$ and p67$^{phox}$ for their membrane translocation (27), its role in NADPH oxidase activation has not been fully established. COS-phox cells respond to PMA and arachidonic acid with potent O$_2^-$ production (17), and our initial reconstitution of fMLP-induced NADPH oxidase activity was achieved without p40$^{phox}$ (Fig. 2). These observations indicate that p40$^{phox}$ is not essential for NADPH oxidase activation in either PMA- or fMLP-stimulated COS-phox cells.

Because the current literature suggests that p40$^{phox}$ can be either stimulatory or inhibitory in NADPH oxidase activation, we examined its potential involvement in FPR-reconstituted COS-phox
PKCδ is critical to fMLP-induced O$_2^*$ production in COS-phox cells and in human neutrophils

Phosphorylation of p47$^{phox}$ by PKC is essential for translocation of the cytosolic factors and assembly of a functional NADPH oxidase complex (28). In neutrophils, the conventional PKC isoforms PKCa and PKCβII are believed to mediate PMA-induced O$_2^*$ production (29). The PKC expression profile in COS-7 cells is different from that of neutrophils. COS-7 cells contain a high level of PKCa, a moderate level of PKCζ, but only small amounts of PKCδ and PKCβI. In contrast, neutrophils express high levels of PKCβII and PKCδ, a moderate level of PKCa, and very little PKCζ (17). To determine which PKC is important for the fMLP-induced O$_2^*$ production, we expressed the four individual PKC isoforms together with other necessary components in COS-phox cells. As shown in Fig. 7, cells transfected to express FPR, Goα2, PI3Kγ, PLCβ2, and Rac1 responded to fMLP stimulation with a small increase in O$_2^*$ generation over basal level. Cotransfection with a PKCδ expression vector markedly enhanced fMLP-stimulated O$_2^*$ production (by 4-fold), whereas cotransfection with vectors encoding the other three PKC isoforms resulted in statistically insignificant ($p > 0.05$) changes in O$_2^*$ production.

Because COS-phox is a nonhemopoietic cell line and can have quite different properties than neutrophils, we next examined whether PKCδ is important for fMLP-induced NADPH oxidase activation in human neutrophils. Freshly prepared blood neutrophils were stimulated with either PMA or fMLP, and membrane translocation of PKCδ was determined at various time points after stimulation (Fig. 7C). Translocation of PKCδ was evident 10 min after PMA stimulation. In fMLP-stimulated neutrophils, PKCδ translocation appeared much earlier and was detectable after 1 min (Fig. 7C). This profile is consistent with the kinetics of fMLP-induced O$_2^*$ production.

We next determined the effects of PKC inhibitors on the fMLP-induced O$_2^*$ production. Treatment of human neutrophils with GF109203X, a broad PKC inhibitor with selectivity for PKCa and other conventional PKCs, effectively suppressed PMA-induced O$_2^*$ production at 0.1–1 μM concentrations (Fig. 8, A and B). In comparison, GF109203X was less effective on fMLP-induced O$_2^*$ production.

![FIGURE 6](image-url) Exogenous expression of p40$^{phox}$ augment fMLP-induced O$_2^*$ production. COS-phox cells were transiently transfected with expression constructs for FPR and five plasmids (minus Rac), with or without the p40$^{phox}$ expression vector. A, A representative Western blot showing expression of the transfected p40$^{phox}$ construct (p40). Vec, Vector-transfected cells. β-Actin was shown as a control for sample loading and transfer. B, The fMLP-induced change in O$_2^*$ production was shown as a function of time, in the reconstituted COS-phox cells with or without p40$^{phox}$ and stimulated with fMLP or buffer. C, Integrated CL (area under curve; shown as mean ± SEM) from experimental data shown in B and two repeating experiments.

![FIGURE 7](image-url) Effects of selected PKC isoforms in fMLP-induced O$_2^*$ production. COS-phox cells were transfected with FPR plus 5PL (minus PKC), and with expression construct for one of the four PKC isoforms as indicated. A, The ability of these PKC isoforms to potentiate fMLP-induced O$_2^*$ production is shown as percentage change relative to the minimal response induced by fMLP (set as 100%, with FPR plus 5PL plusPKCδ). Data were based on the integrated CL collected during the first 20 min, from three independent experiments. B, Expression of the exogenous PKC isoforms as determined by Western blotting, using an anti-HA Ab detecting the tagged PKCa, PKCδ, and PKCζ, and a specific Ab against PKCβII. Representative blots from three to four experiments are shown. C, fMLP-induced PKCδ translocation in neutrophils. Membrane translocation of PKCδ was determined in PMA (100 ng/ml)- and fMLP (100 nM)-stimulated neutrophils, as described in Materials and Methods. A representative blot, taken from three independent experiments, is shown.
used. Rottlerin inhibited fMLP-induced $O_2^*$ production by 65–100% at concentrations of 3–12 μM. Under the same experimental conditions, rottlerin had minimal effect on the PMA-induced $O_2^*$ production (Fig. 8, C and D).

Discussion

Investigation of chemoattractant-induced $O_2^*$ generation has been hampered by the lack of a whole-cell model for reconstitution of receptor-mediated signaling pathways. As a result, our current understanding of how chemoattractants activate NADPH oxidase relies heavily on information obtained with pharmacological activators such as PMA and arachidonic acids. The mechanistic information derived from these studies does not accurately reflect signaling events initiated by chemoattractant receptors. In the current study, we attempted to characterize the proximal signaling events immediately downstream of FPR activation and associate these events with kinase and GTPase activities directly responsible for NADPH oxidase activation. Our approach is based on the assumption that key signaling components for fMLP-induced $O_2^*$ production may be identified by expression of the relevant proteins in cells that lack them. The transgenic COS-phox cell line is excellent for this study, because it is not of hemopoietic origin and thus does not have the special signaling components found in neutrophils. The experimental data presented above confirmed our initial prediction and demonstrated the utility of the COS-phox cells in reconstituting NADPH oxidase activation through a cell surface receptor.

PKC-mediated phosphorylation of NADPH oxidase components is a critical step in the initiation of a series of events that led to membrane translocation of the cytosolic factors (28, 30, 31). Several PKC isoforms are found to interact with and phosphorylate p47phox in its C-terminal region containing two SH3 domains. In PMA-stimulated neutrophils, translocation of PKCα and PKCβII to the cytoskeletal fraction correlates with p47phox translocation, suggesting that these conventional PKC isoforms are involved in PMA-induced $O_2^*$ generation (29). PKC-regulated phosphorylation of p47phox is also observed in fMLP-induced NADPH oxidase activation (32–35), but the responsible PKC isoforms have not been identified. A major observation of the current study is that PKCβ plays a key role in FPR-mediated NADPH oxidase activation in both transgenic COS-phox cells and human neutrophils. In the COS-phox cells, exogenous expression of PKCβ led to a potent induction of fMLP-elicited $O_2^*$ production. In contrast, exogenous expression of PKCα, PKCβII, and PKCγ did not significantly enhance the fMLP-induced response. In human neutrophils, a role of PKCβ in fMLP-elicited $O_2^*$ generation was suggested by two findings. First, fMLP stimulates membrane translocation of this kinase with temporal correlation to the induced $O_2^*$ production. Second, the PKCβ-selective inhibitor rottlerin exerts a much stronger effect on fMLP-induced $O_2^*$ production than the PMA-stimulated response. PKCβ is a novel PKC isoform and is activated in a Ca$^{2+}$-insensitive and diacylglycerol-dependent manner. PKCβ and PKCβII are the two most abundant PKC isoforms in neutrophils, whereas COS-7 cells express only a low level of PKCβ (17). Like several other PKC isoforms, PKCβ responds to PMA stimulation with binding to p47phox (36) and translocation to the cytoskeletal fraction (29). However, the lack of temporal correlation with p47phox binding (36) and translocation (29) casts doubt on the importance of PKCβ translocation in PMA-induced neutrophil NADPH oxidase activation. More recently, Yaffe and colleagues (37) studied NADPH oxidase activation in cytosol-depleted neutrophil cores and identified PKCβ as a key component for reconstitution of PMA-stimulated NADPH oxidase activity. Their conclusion was based on the observations that rottlerin (10 μM)

At 1 μM, GF109203X inhibited the PMA-induced $O_2^*$ production by ~85%, but only ~15% for the fMLP-induced $O_2^*$ production. These results contrast sharply with data derived from a parallel study in which rottlerin, a PKCβ-selective inhibitor (IC$_{50}$ = 3–6 μM), was

![FIGURE 8. Effect of GF109203X and rottlerin on $O_2^*$ production induced by fMLP and PMA. Human neutrophils were pretreated with or without the PKC inhibitors at different concentrations for 15 min, and then stimulated with fMLP (100 nM) or PMA (200 ng/ml). A and C, Histogram showing $O_2^*$ generation as a function of time, based on isoluminol-ECL, after treatment with GF109203X (GF) (A) or rottlerin (C). B and D, Integrated CL (mean ± SEM based on three independent experiments) showing different inhibitory effects on PMA vs fMLP-induced $O_2^*$ production in cells treated with GF109203X (B) or rottlerin (D).](http://www.jimmunol.org/)

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inhibits PMA-induced $O_2^\cdot$ production in neutrophil cores, and that selective depletion of PKCδ from cytosol impairs its ability to restore the PMA-stimulated response in neutrophil cores. These findings provide additional evidence for a role of PKCδ in NADPH oxidase activation. A function of PKCδ in leukotriene B4-stimulated eosinophil $O_2^\cdot$ production was also reported (38). However, eosinophils differ from neutrophils, and leukotriene B4 primes but does not directly activate neutrophil NADPH oxidase. Therefore, further investigation is necessary to determine whether PKCδ is required for neutrophil NADPH oxidase activation induced by a potent chemoattractant such as fMLP.

Although the above experimental results are important in establishing a correlation between PKCδ activation and $O_2^\cdot$ production, PKCδ may not be the only PKC isoform involved in fMLP elicited NADPH oxidase activation. Extensive cross talk exists between different kinases and may complicate the interpretation of our experimental data. The lack of highly specific inhibitors for the individual PKC isoforms also hinders studies using neutrophils that are not genetically amenable. Published reports suggest that fMLP can activate PKCβII in differentiated HL-60 cells (39) and PKCζ in human neutrophils (40). Based on these observations, it is likely that the fMLP-induced response requires more than one PKC isoform for optimal $O_2^\cdot$ generation. This possibility, as well as the relative position of PKCδ in the FPR-mediated signaling cascade, will need to be investigated in future studies.

The development of COS-phox-based reconstitution assay has enabled us to assess the roles of individual signaling molecules in NADPH oxidase activation. These signaling molecules can be divided into two groups. The first group consists of signaling molecules immediately downstream of the activated FPR. Our results demonstrated that Gq2 or Gq3 are equally capable of mediating fMLP-induced $O_2^\cdot$ generation in intact cells. Exogenous expression of Gq2 or Gq3 potentiates $O_2^\cdot$ production in response to fMLP, suggesting that the endogenous Gq proteins become a limiting factor when FPR is overexpressed. With the development of the COS-phox-based reconstitution assay, it is now possible to examine other G proteins for their potential involvement in G protein coupled receptor-mediated $O_2^\cdot$ production. PLCβ2 is a downstream effector of G proteins. Our experimental data indicate that exogenous expression of PLCβ2 is necessary for optimal reconstitution of fMLP-elicited $O_2^\cdot$ production, but significant amount of $O_2^\cdot$ can still be generated in the absence of PLCβ2 (Fig. 4). This result suggests that FPR is able to use the endogenous PLCβ3 in COS-7 cells for downstream signaling. PLCβ activation is triggered by the G protein βγ subunits that become available after the activation of Gq, which serves to link the agonist-occupied receptor with second messenger production and PKC activation. In this regard, it will be interesting to examine whether the difference in the ability to activate NADPH oxidase by FPR and chemokine receptors lies in the activation of PLCβ isoforms. Another signaling component downstream of activated G proteins is PI3K. This PI3K isoform is expressed primarily in hematopoietic cells and mediates important functions of neutrophils, macrophages, and mast cells based on loss-of-function studies involving PI3K-α−/− leukocytes (14). Our results suggest that, in the absence of PI3K, the fMLP-induced $O_2^\cdot$ production is greatly diminished. However, significant amounts of $O_2^\cdot$ were produced without the exogenous expression of PI3K-α, indicating the ability of FPR to use endogenous PI3K for signaling.

Another group of proteins studied in the COS-phox cells includes PKC, Rac, and p40phox, factors that directly participate in the modification and assembly of the phox proteins. In addition to demonstrating a role of PKCδ in the fMLP-elicited response, we have shown that the FPR-mediated signaling pathway is able to trigger activation of endogenous Rac1. Exogenous expression of Rac1 further increased the level of Rac activation as well as $O_2^\cdot$ production, suggesting that Rac1 is one of the limiting factors in this pathway. This finding does not conflict with previous observations that Rac2 plays an important role in NADPH oxidase activation in human neutrophils, but suggest that FPR-mediated signaling pathway can lead to Rac1 activation in an epithelial cell line. fMLP may stimulate Rac1 dissociation from Rho GDP-dissociation inhibitor, as seen in primary human monocytes (41), thereby promoting NADPH oxidase activation. We have further demonstrated the ability of fMLP to induce Rac2 activation and to potentiate $O_2^\cdot$ generation in the reconstituted COS-phox cells expressing human Rac2. This finding suggests that Rac2 activation is not an exclusive property of hematopoietic cells where it is naturally expressed; instead, it can be achieved by the guanine nucleotide factor(s) present in epithelial cells such as COS-7. Further studies will be necessary to compare the different Rac guanine nucleotide exchange factors for their ability to mediate fMLP-induced $O_2^\cdot$ production.

In addition to the four essential phox proteins and Rac, recent studies suggest that p40phox may be involved in regulating NADPH oxidase activation. Published results indicate that p40phox can enhance $O_2^\cdot$ generation in cell-free assays (42–44) and in K562 cells (45). The PX domain of p40phox is considered important in the interaction with the lipid products of PI3Ks (44, 46, 47), which may be one of the mechanisms for the potentiation of $O_2^\cdot$ production. However, there are also published reports indicating that p40phox is an inhibitory molecule for NADPH oxidase activation, in both K562 cells (48) and cell-free assays (49, 50). In light of the controversy surrounding the exact role of p40phox in NADPH oxidase activation, we sought to examine this cytosolic protein, which is absent from the epithelial COS-phox cells. By exogenous expression, we observed that p40phox enhances the level of fMLP-induced $O_2^\cdot$ production in the reconstituted COS-phox cell, suggesting that p40phox can be stimulatory for chemoattractant-induced NADPH oxidase activation in intact cells. Expression of p40phox does not alter the kinetics of fMLP-induced $O_2^\cdot$ production, because the initial rise to peak and subsequent fall of $O_2^\cdot$ levels follow a similar pattern in the presence or absence of p40phox (Fig. 6B). Overexpression of p40phox affects the basal level of NADPH oxidase activity as well. Because COS-phox cells can be readily transfected with DNA expression constructs, the reconstitution system is suitable for further delineation of the mechanism of p40phox action through analysis of mutants and chimeric proteins.

In summary, the FPR-reconstituted COS-phox cells share many functional features with neutrophils and provide a genetically amenable system for characterization of the individual signaling components and phox proteins. Using the COS-phox reconstitution system, we have identified PKCδ as an important kinase for fMLP-induced $O_2^\cdot$ production, demonstrated the ability of FPR to activate both Rac1 and Rac2 in a nonhemopoietic cell line, and confirmed the requirement of PLCβ2 and PI3K for optimal NADPH oxidase activity. We further demonstrated that p40phox is a positive regulator of fMLP-induced $O_2^\cdot$ production. This whole-cell-based reconstitution system is expected to complement the existing cell-free system for more detailed analysis of receptor-mediated signaling pathways leading to $O_2^\cdot$ generation.

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