Cytosolic phospholipase A$_2$ regulation of human monocyte NADPH oxidase activity:
cPLA$_2$ affects translocation but not phosphorylation of p67phox and p47phox*

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Abstract

The NADPH oxidase of human monocytes is activated, upon exposure to opsonized zymosan and a variety of other stimuli, to catalyze the formation of superoxide anion. Assembly of the NADPH oxidase complex is believed to be a highly regulated process and molecular mechanisms responsible for this regulation remain to be fully elucidated. We have previously reported that cytosolic phospholipase A2 (cPLA2) expression and activity are essential for superoxide anion production in activated human monocytes. In this study, we investigated the mechanisms involved in cPLA2 regulation of NADPH oxidase activation by evaluating the effects of cPLA2 on translocation and phosphorylation of p67phox and p47phox. We report that translocation and phosphorylation of p67phox, as well as p47phox, occur upon activation of human monocytes and that decreased cPLA2 protein expression, mediated by antisense oligodeoxyribonucleotides (AS-ODN) specific for cPLA2 mRNA, blocked the stimulation-induced translocation of p47phox and p67phox from the cytosol to the membrane fraction. Inhibition of translocation of both p47phox and p67phox by cPLA2 AS-ODN was above 85%. Arachidonic acid, a product of cPLA2 enzymatic activity, completely restored translocation of both of these oxidase components in the AS-ODN-treated, cPLA2-deficient human monocytes. These results represent the first report that cPLA2 activity or AA is required for p67phox and p47phox translocation in human monocytes. Although cPLA2 was required for translocation of p47phox and p67phox, it did not influence phosphorylation of these components. These results suggest that one mechanism of cPLA2 regulation of NADPH oxidase activity is to control the arachidonate-sensitive assembly of the complete oxidase complex through modulating the translocation of both p47phox and p67phox. These studies provide insight into the mechanisms by which activation signals are transduced to allow the induction of superoxide anion production in human monocytes.
Introduction

The phagocyte NADPH oxidase is a multicomponent enzyme that catalyzes the production of superoxide anion and plays a central role in the elimination of invading bacteria. Neutrophils and monocytes are among the most prominent of the phagocytic cells. Monocytes mediate several aspects of inflammation and host defense. One pathogenic process in which monocytes are believed to participate is the oxidation of low-density lipoprotein (LDL). Our laboratory was the first to report that purified human monocytes could oxidize LDL. We found that superoxide anion is a key component involved in the oxidation of LDL by activated human monocytes and that the superoxide anion, responsible for this oxidation of LDL, is produced by the monocyte NADPH oxidase. Oxidized LDL is believed to contribute to the pathogenesis of atherosclerosis due to its unregulated uptake by macrophages, resulting in the formation of lipid-laden foam cells. Thus, the regulation of the monocyte NADPH oxidase is also central to the control of this potentially pathogenic process.

The activated NADPH oxidase is a membrane-bound enzyme complex. Its composition has been defined in recent years through studies with intact or lysate fractions of neutrophils and knowledge of its function has mostly been derived from patients lacking NADPH oxidase activity; a clinical disorder called chronic granulomatous disease (CGD). In resting neutrophils, NADPH oxidase is dormant and its protein components are segregated into cytosolic components, including p47phox, p67phox, p40phox and a small GTP-binding protein, Rac, and a transmembrane-heterodimeric protein known as flavocytochrome b558 which consists of gp91phox, a glycoprotein, and p22phox. Upon stimulation, the cytosolic protein components of neutrophil NADPH oxidase translocate to the plasma membrane or phagosomal membrane, where they associate with flavocytochrome b558 to form the catalytically active oxidase. Also in response to activation, the cytosolic components of neutrophil NADPH oxidase become phosphorylated, including p67phox, p47phox, p40phox, p22phox, and both subunits of the membrane-bound flavocytochrome b558. Translocation and phosphorylation of p47phox in PMA-stimulated human monocytes has also been reported. The process of translocation is believed to be highly regulated and involve multiple binding interactions between the individual NADPH oxidase
proteins. PKC-dependent and PKC-independent pathways have been suggested to participate in the phosphorylation of p47phox and p67phox (19,20). Both translocation and phosphorylation are believed to be essential steps for activation of NADPH oxidase, however, the molecular mechanisms, particularly the up-stream signals governing the assembly and activation of the NADPH oxidase complex, remain to be clarified. The majority of what is understood about the complex formation and activity of NADPH oxidase has been derived from studies in neutrophils and much less is known about the regulation of this complex in monocytes.

In recent years, arachidonic acid (AA) has been implicated as a secondary messenger modulating many stimulation-induced cell responses such as early gene expression (21), ion-channel activity (22), apoptosis (23,24) and activation of G-protein (25). AA has also been shown to be a physiological amphilic co-factor in the in vitro activation of NADPH oxidase. For example, NADPH oxidase could only be activated in a cell-free system reconstituted with neutrophil cytochrome b558, p47phox, p67phox and the GTP-bound Rac in the presence of an anionic amphiphile such as AA or SDS (26,27). A maximal response occurred with the concentration of AA within 50 – 100 µM.

A few lines of evidence also suggest that AA regulates the production of superoxide anion in intact cells as well. For example, when the human myeloid cell line PLB-985 is rendered deficient in cPLA2 and then differentiated, exogenous AA activates the NADPH oxidase (28). AA has also been shown to increase the activation of NADPH oxidase in monocytic U937 cells by accelerating translocation of p47phox (29). These observations suggest that AA release may be a modulating event for the activation of NADPH oxidase in stimulated phagocytes. In both in vivo and in vitro activation, however, the relatively high concentration of AA (30-100 µM) has raised questions regarding its physiological relevance (30).

A number of studies have suggested that AA production in stimulated neutrophils or monocytes is mediated by cytosolic phospholipase A2 (cPLA2), a member of the intracellular phospholipase A2 family. There are at least three isoforms of PLA2 in phagocytic cells, a 14-KDa secretory form (sPLA2), a 85-KDa calcium-independent PLA2 (iPLA2) and a 85-KDa isoform of cytosolic PLA2 referred to as cPLA2 which migrates as a 110-KDa protein in SDS-PAGE. cPLA2 has a high specificity for
membrane phospholipids that contain \( sn \)-2-arachidonate and is believed to be an important regulator of AA availability, thereby controlling the production of potent lipid mediators. For example, a decrease in cPLA\(_2\) in monocytes treated with AS-ODN for cPLA\(_2\) resulted in significant inhibition of \(^{3}\)H AA production induced by monocyte chemotactic protein-1 (31). In human neutrophils, experiments using PLA\(_2\) inhibitors demonstrated that generation of AA is important for activation of NADPH oxidase (32,33). An increase in phospholipase A\(_2\) activity and a release of AA have also been reported to occur in association with stimuli that activate the NADPH oxidase (34). In a permeabilized neutrophil model (35), cPLA\(_2\) was also believed to be the enzyme controlling the production of AA. Therefore, cPLA\(_2\) appears to be critical for controlling AA production in phagocytes. Our laboratory demonstrated that the expression and activity of cPLA\(_2\) are essential for superoxide anion production and related LDL oxidation in activated human monocytes. ZOP-stimulated superoxide anion production by human monocytes was inhibited by pre-treatment of the monocytes with AS-ODN specific for cPLA\(_2\) and this inhibition was negated by the addition of exogenous AA at a concentration of 3 \( \mu \)M (36). Although substantial evidence suggests that cPLA\(_2\)-derived AA plays an essential role in NADPH oxidase activation and superoxide anion production, the molecular mechanisms responsible for this activation of NADPH oxidase still need to be elucidated.

In this study, we investigated how cPLA\(_2\) and cPLA\(_2\)-derived AA regulates NADPH oxidase activity by focusing our attention on the translocation and phosphorylation of p67phox and p47phox. Our results suggest a role of cPLA\(_2\) in the translocation but not the phosphorylation of p67phox and p47phox in activated human monocytes.
Experimental procedures

Materials.

Arachidonic acid, linoleic acid, palmitic acid, phosphatase inhibitors, protease inhibitors and monoclonal antibodies specific for β-tubulin were purchased from Sigma (St. Louis, MO). Sodium okadate was obtained from Calbiochem (San Diego, CA). Zymosan, obtained from ICN Biochemicals (Cleveland, OH), was opsonized (37) and used at a concentration of 2 mg/ml to activate human monocytes. SDS-PAGE reagents and PVDF membranes were from Bio-Rad (Hercules, CA). \(^{32}\)POrthophosphate was from Perkin Elmer (Boston, MA). Rabbit polyclonal antibodies against p67phox and p47phox were described previously (38). Mouse monoclonal antibodies specific for p47phox, horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from BD Transduction Laboratories (San Diego, CA). Goat polyclonal antibodies for p67phox, p47phox, rabbit polyclonal antibodies for Tyk2 and horseradish peroxidase-conjugated pre-adsorbed anti-mouse or anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against cPLA\(_2\) were provided by Genetics Institute (Cambridge, MA).

Isolation of human monocytes

Human monocytes were isolated and purified as previously described (3). Briefly, heparinized whole blood was centrifuged over a Ficoll-Paque density solution, platelets were removed by several washes through serum and mononuclear cells were incubated in serum-coated cell culture flasks for 2 to 4 hrs. After washing away nonadherent cells, the adherent cells were released with 5 mM EDTA and plated into 6-well dishes. The isolated human monocytes rested for 2 h in Dulbecco's modified Eagle's medium with 10% bovine serum at 37°C in 10% CO\(_2\) before use in experiments.

Treatment of cells with Oligodeoxynucleotides (ODN)

The sequences of AS-ODN and S-ODN specific for cPLA\(_2\) and p47phox were identical to those described in our previous reports (3,36). Human monocytes (5 x 10\(^6\) cells/group) were cultured for 2 hrs in DMEM supplemented with 10% bovine serum. Cells were then treated with AS-ODN or S-ODN as indicated. Cells were next washed,
and the medium was changed to RPMI 1640 (Whittaker, Walkersville, MD) in the presence or absence of opsonized zymosan (2 mg/ml) for 1-h, followed by preparation of post-nuclear extracts or fractionation into cytosol and membrane fractions.

**Monocyte fractionation and preparation of post-nuclear extracts**

Fractionation of human monocytes was performed according to the method described by Clark et al (39). Briefly, cells were collected and washed twice with PBS after treatment with ODN or indicated conditions. The cell pellet was then resuspended in 0.6 ml relaxation buffer (KCl 100 mM, NaCl 3 mM, MgCl₂ 3.5 mM, EGTA 1.25 mM, 1,4-piperazine diethane sulfonic acid 10 mM, pH 7.3, added with 500 µM phenylmethylsulfonyl fluoride and 1:100 dilution of protease inhibitor mixture, Sigma). Cells were then disrupted by two 15-s cycles of sonication at 4 °C using a microprobe sonicator. Unbroken cells and nuclei were pelleted by centrifugation at 500 x g for 5 min at 4 °C. The supernatant was then centrifuged at 110,000 x g for 7 min at 4 °C. The high-speed supernatant represented the soluble, cytosolic fraction. The pellet was resuspended in 0.6 ml of relaxation buffer with vigorous mixing and again centrifuged at 110,000 x g for 7 min at 4 °C. The final pellet, representing the particulate/membrane fraction was resuspended in 0.2 ml of relaxation buffer. Hereafter it is referred to as the membrane fraction.

For preparation of post-nuclear extracts, cells were collected and washed with PBS after treatment. Cells were then resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM NaF, 50 mM Tris, pH 7.4, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 500 µM phenylmethylsulfonyl fluoride and 1:100 diluted protease inhibitor mixture). After 30 min on ice, the extract was centrifuged at 9300 x g for 15 min at 4 °C. The supernatant was used as the post-nuclear extract.

**Immunoprecipitation and Western blotting**

Samples were incubated with the relevant antibody for 2 h at 4 °C with constant rotation, then mixed with Protein G-Agarose beads (20 µl packed volume) and further rotated at 4°C overnight. The immunocomplexes were collected and washed 3 times with lysis buffer, followed by release in SDS sample buffer. For Western blotting analysis,
proteins were separated by 8% SDS-PAGE, then electrophoretically transferred to PVDF membranes. After blocking overnight with 5% milk in PBS containing 0.1% Tween-20, the membranes were probed with primary antibodies and corresponding secondary antibodies as indicated and finally developed using enhanced chemiluminescence (Pierce Biochemicals, Rockford, IL).

Treatment of cells with fatty acids

After a 24 h treatment with ODN, each indicated fatty acid was added to the cell culture and incubated for the indicated times at 37 °C with 10 % CO₂ before the stimulation of cells with ZOP. The final concentration of each fatty acid added to the cells was 3.3 µM.

Time course of ZOP induced p67phox and p47phox phosphorylation

Human monocytes (5 x 10⁶ /2ml/well) were radiolabelled with 100 µCi/ml ³²P orthophosphate for 12 h in phosphate-free DMEM. The cells were then not activated or activated with 2mg/ml ZOP for 5, 15, 30, or 60 minutes. Cells were collected, washed three times by centrifugation at 200 x g in PBS containing 100 µM sodium orthovanadate. The cells were then lysed as described above and post-nuclear extracts were incubated with goat polyclonal p67phox or p47phox antibodies. Immune complexes were collected using Sepharose G or A beads. The immune complexes were run in 8 or 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The phosphorylation of p67phox and p47phox was visualized using a PhosphorImager and quantified by ImageQuant analysis. The PVDF membranes were processed as described above using monoclonal antibodies to p67phox or p47phox.

³²P labeling of monocytes for phosphorylation study

Human monocytes were incubated with DMEM containing 10% bovine serum in the presence or absence of cPLA₂ specific AS-ODN or S-ODN (10 µM) for 24 h. Cells were washed with RPMI 1640 without sodium phosphate (Gibco BRL) and re-cultured in the same phosphate-free medium with or without cPLA₂ specific AS-ODN or S-ODN (10 µM), meanwhile cells were labeled with 100 µCi/ml [³²P]orthophosphate for 4 h.
followed by activation with ZOP for 1-h. Cells were collected and post-nuclear extracts were dissolved in the above mentioned lysis buffer containing 20 nM sodium okadate, 1 mM sodium orthovanadate and a mixture of additional phosphatase inhibitors (30 mM β-glycerophosphate, 0.5 mM phosphoserine, 0.5 mM phosphotyrosine, 1.0 mM phosphothreonine, 1.5 mM p-nitrophenylphosphate). Post-nuclear extracts were immunoprecipitated with specific antibodies for p67phox or p47phox. The final immunocomplexes were run on 8% SDS-PAGE, separated proteins were transferred to PVDF membranes, and phosphorylated p67phox or p47phox was detected using PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Membranes were further probed with specific antibodies for p67phox or p47phox and developed by methods described above.
Results

Translocation of p67phox and p47phox from cytosol to membrane upon treatment of human monocytes with ZOP

It has been reported that translocation of p67phox and p47phox of neutrophils occurs as early as one minute after PMA stimulation (17,39). However, a time course of translocation has yet to be established for these components upon activation of the NADPH oxidase in human monocytes. In this study, we first investigated the time course of p67phox and p47phox translocation in ZOP-activated human monocytes. Figure 1 shows a time-dependent manner of ZOP-induced translocation of p67phox and p47phox in human monocytes. Detectable membrane-associated p67phox and p47phox was seen as early as 5 minutes after stimulation of monocytes with ZOP. The translocation of p67phox and p47phox increased significantly at the 1-h time point (Fig. 1). This is the first report that translocation of p67phox in human monocytes occurs upon activation. We previously reported that ZOP-induced maximal production of superoxide anion in human monocytes at 1-h (2). Therefore we used the 1-h time point for further translocation studies.

Treatment of monocytes with cPLA2 specific AS-ODN blocks ZOP-induced translocation of p67phox and p47phox

Translocation of p67phox and p47phox is believed to be an essential event in NADPH oxidase activation, leading to superoxide anion production. As arachidonic acid has been suggested to be involved in NADPH oxidase activation, we asked whether AA or cPLA2 played a role in the translocation of p67phox and p47phox. We treated monocytes with a cPLA2 specific AS-ODN and analyzed the membrane-associated p67phox and p47phox in cPLA2-deficient, ZOP-exposed cells. As shown in Figure 2A, the cPLA2 specific AS-ODN significantly decreased the expression of cPLA2 protein, confirming our previous data (36,40), while the S-ODN of cPLA2 had no detectable effect. Furthermore, treatment with cPLA2 AS-ODN had no effect on the expression of iPLA2 (40) or other control proteins such as Tyk2 and p47phox (Fig. 2A) or p67phox (data not shown), suggesting the specificity of cPLA2 AS-ODN. Interestingly,
monocytes treated with the cPLA₂-specific AS-ODN showed a dramatic decrease in ZOP-induced translocation of both p67phox and p47phox (Fig. 2B). The inhibition of translocation of p67phox and p47phox by cPLA₂ AS-ODN was above 85% (Fig. 2C). Meanwhile, cPLA₂ S-ODN had no inhibitory effect on translocation of p67phox and p47phox. We previously reported that treatment of human monocytes with such cPLA₂ specific AS-ODN could substantially decrease ZOP-triggered superoxide anion production (36). Taken together, these results suggest that cPLA₂ may regulate the superoxide anion production of monocytes via modulating the translocation of p67phox and p47phox from cytosol to membrane compartments, an essential step for formation of active complex of NADPH oxidase.

Arachidonic acid and Linoleic acid, but not palmitic acid, restore p67phox and p47phox translocation blocked by cPLA₂-specific AS-ODN

Our previous studies demonstrated that production of superoxide anion, in cPLA₂-deficient monocytes, could be restored by exogenous AA (36). In those studies several doses of AA were employed and 3.3 µM AA completely restored superoxide anion production in primary human monocytes in which cPLA2 expression was inhibited by treatment with antisense ODN. We therefore studied whether addition of AA, at this concentration, to cPLA₂ deficient monocytes could restore translocation of both p67phox and p47phox induced by ZOP. Results shown in Figure 3A indicate that the translocation-inhibitory effect of AS-ODN treatment could be completely negated by exogenous AA (3.3 µM). These data strongly suggest that cPLA₂ regulates translocation of p67phox and p47phox via AA. Furthermore, the data presented here suggest that cPLA₂, via the production of AA, regulates the superoxide anion production in monocytes through modulation of the assembly of active complex of NADPH oxidase.

To address the question of whether other fatty acids could also restore the translocation of p67phox and p47phox in cPLA₂-deficient monocytes, we next examined the effect of linoleic acid (LA) and palmitic acid (PA). Monocytes were treated with AS-ODN specific for cPLA₂ for 24 h, then exposed to ZOP in the presence of the same dose of LA, PA or AA (3.3 µM) in each cell culture. The membrane-associated p67phox and p47phox of each sample was examined by immunoblots. As shown in Figure 3B, LA at
3.3 µM displayed similar effects on restoration of both p67phox and p47phox translocation, as that of AA. However, PA at 3.3 µM did not restore the translocation. We observed that addition of fatty acids with ZOP caused no greater translocation than that mediated by ZOP stimulation alone. Fatty acid addition alone was less effective in promoting translocation of p47phox and p67phox than ZOP (Fig. 3C).

The association of p67phox and p47phox in human monocyte and translocation of p67phox in ZOP-induced human monocytes is affected by p47phox

During the above studies, we found that p67phox and p47phox displayed very similar patterns of translocation and inhibition of translocation by cPLA$_2$ specific AS-ODN in ZOP-stimulated human monocytes. Therefore we investigated the possible association of p67phox and p47phox in human monocytes and their association during translocation. As shown in Figure 4A, immunoprecipitation of cell lysates of untreated or ZOP-induced monocytes with p47phox specific antibody pulled down both p47phox and p67phox. Also immunoprecipitates of p67phox specific antibodies contained both p67phox and p47phox. These data suggest that at least some of p67phox and p47phox are in an associated state in human monocytes. Furthermore, ZOP treatment of human monocytes enhanced the association of the two proteins (Fig. 4A). We further found that ZOP-induced translocation of p67phox from the cytosol to the membrane could be partially blocked by pre-treating cells with a p47phox-specific AS-ODN (Fig. 4B). Treatment of cells with p47phox specific AS-ODN decreased the protein level of p47phox over 60%, without detectable effects on p67phox (Fig. 4C), and β-tubulin protein expression (Fig. 4C), in human monocytes. Meanwhile treatment of cells with p47phox specific AS-ODN blocked the translocation of p47phox itself (Fig. 4B). S-ODN specific for p47phox had no detectable effect on either translocation or protein expression as compared to control samples (Fig. 4B, 4C). These data suggest that p47phox influences the translocation of p67phox in ZOP-treated human monocytes.

Phosphorylation of p67phox and p47phox in ZOP-induced human monocytes is cPLA$_2$-independent
Phosphorylation is one of the key events involved in NADPH oxidase activation, and both p47phox and p67phox have been shown to undergo phosphorylation during the activation process in neutrophils. Recently, phosphorylation of p47phox in activated human monocytes was also reported (19). Due to our data demonstrating that cPLA$_2$ or AA regulates ZOP-induced translocation of p67phox and p47phox in human monocytes, we next examined whether cPLA$_2$ or AA also regulates the phosphorylation of p67phox and p47phox in ZOP-induced human monocytes. Figure 5A shows that the phosphorylation of p67phox and p47phox occurs as early as 5 minutes after activation with ZOP. The phosphorylation of p67phox increased significantly at the 1-h time point, whereas the phosphorylation of p47phox peaked at the 30 minute time point. We previously reported that ZOP induced superoxide anion production in human monocytes reached a maximal level at 1-h (2), therefore we used the 1-h time point for further phosphorylation studies. This is the first report to describe the phosphorylation of p67phox in human monocytes upon activation.

Next we pre-treated monocytes with cPLA$_2$ specific AS-ODN. Cells were then metabolically radiolabelled with $[^{32}\text{P}]$orthophosphate, followed by activation with ZOP. Cell lysates were immunoprecipitated with antibodies specific for p67phox or p47phox. The immunoprecipitated proteins were separated by SDS-PAGE and then transferred onto PVDF membranes. Figure 5B shows the PhosphorImage of the membrane. The membranes used for PhosphorImager analysis were further probed with specific antibodies for p67phox or p47phox to assess the equal loading of protein (Fig. 5C upper panels). p67phox was weakly phosphorylated in resting monocytes, suggesting either a low physiological basal level of phosphorylation in resting cells or that the cells became slightly activated as a result of handing during the experiment. Repeated experiments showed a consistent increase in the incorporation of radiolabelled $^{32}\text{P}$ into both p67phox and p47phox upon monocyte activation. The level of phosphorylation increased by a factor of $3.1 \pm 0.7$ and $4.8 \pm 0.6$ for p67phox and p47phox, respectively (Fig. 5D). The difference in relative fold values for phosphorylation in Figure 5A and 5D may be due to donor variation. Treatment of monocytes with cPLA$_2$-specific AS-ODN or S-ODN did not alter the level of phosphorylation of either p67phox or p47phox as compared to that
of cells treated with ZOP only. These results suggest that phosphorylation of p67phox and p47phox in ZOP-induced human monocytes is cPLA₂-independent.

In these studies, phosphorylated p47phox was not detectable in samples immunoprecipitated by p67phox specific antibodies (Fig. 5B, left panel). Similarly, phosphorylated p67phox was undetectable in samples immunoprecipitated by p47phox specific antibodies, either (Fig. 5B, right panel). After confirming the equal loading of lysates (Fig. 5C upper panels), the membrane (left upper panel) in Figure 5C was further reprobed with specific antibodies for p47phox (left low panel) and the membrane (right upper panel) in Figure 5C was reprobed with antibodies specific for p67phox (right low panel). There was detectable association between p67phox and p47phox in all conditions. Similar to the results shown in Figure 4A, ZOP-treated monocytes showed increased association as compared to that of untreated cells, especially for samples immunoprecipitated with p47phox specific antibodies. Treatment of monocytes with cPLA₂ specific AS-ODN or S-ODN had no effect on the association of p67phox and p47phox.
Discussion

To date, most studies regarding the activation of the phagocytic NADPH oxidase have been undertaken with intact neutrophils or in vitro with isolated enzyme components and factors derived from neutrophils. Both monocytes and neutrophils play essential roles in the cellular immune response to foreign pathogens and in inflammation (41). Monocytes, unlike neutrophils, have the capacity to differentiate locally and have a long life span in tissues. Monocytes can also become activated several times. Furthermore, monocytes and not neutrophils are present and activated in atherosclerotic lesions, and one atherogenic process that monocytes are believed to contribute to atherogenesis is the oxidation of LDL by a mechanism requiring superoxide anion produced by NADPH oxidase (2,3). Oxidized LDL is believed to contribute to the pathogenesis of atherosclerosis. The monocyte activation model offers a novel opportunity to study the signal transduction and effector pathways that are linked to a pathologic function of inflammatory cells.

In the present study, we investigated the molecular mechanism linking cPLA₂ and AA with regulation of NADPH oxidase in human monocytes by focusing our attention on the translocation and phosphorylation of p67phox and p47phox, which are necessary steps for activating NADPH oxidase. First we established a time course for the events of translocation and phosphorylation of p67phox and p47phox in ZOP-induced human monocytes. Our results indicate that translocation of p67phox and p47phox in human monocytes is induced by ZOP as early as 5 minutes and that the translocation of these components continues to increase up to the 1h time point (Fig.1). These data and our previous reports, that ZOP induced superoxide anion production in human monocytes reached a maximal level at 1-h (2), indicate a correlation between the translocation of p67phox, p47phox and superoxide anion production. In Figure 5A, we also show that phosphorylation of p67phox and p47phox begins as early as five minutes. In addition, our results indicate that phosphorylation of p67phox and p47phox is induced 5 minutes after ZOP activation, whereas only a small increase of membrane associated p67phox and p47phox was observed at that time point. This may indicate that phosphorylation of these two proteins, especially p47phox, is required prior to their translocation as proposed by other studies involving neutrophils and B-lymphocytes (42,43). Also these results
suggest that ZOP-induced phosphorylation of p47phox in human monocytes likely occurs in the cytosol.

To specifically address the involvement of cPLA2 in the above processes, cPLA2-specific AS-ODN and S-ODN were used in our study. Our previous data showed that treatment of human monocytes with cPLA2-specific AS-ODN substantially inhibited cPLA2 expression and activity in human monocytes as well as superoxide anion production (36,40). Taken together with the data in Figure 2, it is reasonable to believe that blockage of translocation of p67phox and p47phox in cPLA2 AS-ODN-treated, ZOP-induced monocytes is indeed due to the inhibition of cPLA2 activity. These data suggest that cPLA2 regulates NADPH oxidase activity by controlling the assembly of the oxidase complex through modulating the translocation of both p67phox and p47phox. Corroboration for this conjecture is derived from our studies demonstrating that addition of exogenous AA in cPLA2-deficient monocytes could completely restore translocation of p67phox and p47phox (Fig. 3) and the production of superoxide anion (36). The results depicted in Figure 5B indicate that phosphorylation of p67phox and p47phox in ZOP-induced monocytes is cPLA2-independent. These data suggest a new mechanism regarding the role of cPLA2 or AA on activation of NADPH oxidase. To our knowledge, this is the first report to address the regulatory mechanism of NADPH oxidase assembly by cPLA2 or AA in intact primary monocytes.

The studies to define the specificity for restoration of translocation of p67phox and p47phox in cPLA2-deficient monocytes by fatty acids indicate that LA, as well as AA, can also restore translocation, whereas a saturated fatty acid, palmitic acid was ineffective. These data suggest that restoration of translocation is linked with unsaturation of the fatty acid. At the doses employed, each fatty acid alone induced low levels of translocation, but no additive effect on ZOP-induced translocation of p67phox and p47phox was observed. It is not clear whether the active agent mediating restoration is AA directly or a metabolic product of the unsaturated fatty acid.

During this study, we found that p67phox and p47phox can form a complex in human monocytes (Fig. 4A). Prior studies demonstrated that in the cytosol of resting neutrophils, p47phox exists in a free form, as well as in a complex consisting of p47phox, p67phox and p40 phox (44-46). We also found that upon ZOP treatment, there was
increased association between p67phox and p47phox in human monocytes. It is not clear whether the increased association contributes to the translocation and phosphorylation, but the association between p67phox and p47phox provides an explanation for the observation that translocation of both p67phox and p47phox are regulated similarly by cPLA2 or AA. By using a specific AS-ODN to p47phox, we found that inhibition of expression of p47phox protein in human monocytes affected the translocation of p67phox (Fig. 4B), suggesting that p47phox plays a role in the translocation of p67phox in ZOP-activated human monocytes.

Previously, our laboratory reported that both Ca\(^{2+}\) influx through functional calcium channels and Ca\(^{2+}\) mobilization from intracellular stores participate in the oxidative modification of LDL by ZOP-activated human monocytes, and Ca\(^{2+}\) uptake experiments revealed profound Ca\(^{2+}\) influx during the early stages of monocyte activation by ZOP (47). We later found that PKC activity is required for LDL oxidation by ZOP-activated human monocytes (48). Our recent studies have demonstrated that a Ca\(^{2+}\)-dependent PKC isoform, PKC\(\alpha\), regulates human monocyte superoxide anion production and LDL lipid oxidation (49). We also reported that another regulator of the superoxide anion production and oxidation of LDL in human monocytes is cPLA2 (36). Furthermore, our latest data suggest that PKC\(\alpha\) regulates the phosphorylation and activation of cPLA2 (Li, Q., V. Subbulakshmi and M.K. Cathcart, submitted). Since these two proteins appear to be associated with each other and recombinant PKC\(\alpha\) can phosphorylate recombinant cPLA2, PKC\(\alpha\) is a likely candidate for mediating cPLA2 phosphorylation. Taken together with the data presented in this manuscript, we propose a simplified model illustrating the monocyte signal transduction pathways that are related to the ZOP-induced regulation of NADPH oxidase activity by cPLA2 and AA (Fig. 6). The effect of cPLA2 and AA on translocation of p67phox and p47phox but not on their phosphorylation is emphasized. Both processes are necessary for the formation of functionally activate NADPH oxidase.

Apart from the role of AA we propose here, there are also a few reports related to alternative mechanisms by which AA may contribute to the activation of NADPH oxidase. For example, the NADPH oxidase-associated H\(^+\) channel was reported to be activated by AA (50,51). Dana et al. reported that phospholipase A\(^2\) is required for...
activation of NADPH oxidase in neutrophils, but ZOP-induced translocation of p67phox and p47phox was not affected by 5-10 µM bromophenacyl bromide, which is an inhibitor of phospholipase A₂ and inhibited the release of AA by 60-75% (32). Dana et al. also found that in the human myeloid cell line PLB-985, which is rendered deficient in cPLA₂ and then differentiated by phorbol 12-myristate 13-acetate, can still be induced to translocate p67phox and p47phox (28). AA can also activate NADPH oxidase by other means. In a cell-free system AA (1-5 µM) synergized with the phosphorylation of p47phox to facilitate the interaction with p22 phox and induce activation of the oxidase (52). This was hypothesized to perhaps be due to observed conformational changes in p47phox upon exposure to AA (52-54). Kadri-Hassani et al. (55) reported that 13-methyltetradecanoic acid and AA have bimodal effects on superoxide anion production by human adherent monocytes activated by phorbol 12-myristate 13-acetate or diacylglycerol through the modulation of protein kinase C as well as p47phox translocation. AA (30–50 µM) also increased NADPH oxidase activity in monocytic U937 cells by accelerating translocation of p47phox and co-stimulation of protein kinase C (29). Therefore cPLA₂ or AA may have multiple effects on regulation of NADPH oxidase. Different observed roles for arachidonic acid in regulating NADPH oxidase activity are likely due to the differences of the cell types. Our data strongly suggest that cPLA₂-dependent translocation of p67phox and p47phox, in ZOP-induced primary human monocytes, is an essential step for activation of NADPH oxidase.

The link between protein phosphorylation and NADPH oxidase activation in neutrophils was first documented with p47phox (7). Phosphorylation of p67phox during neutrophil activation was reported later (17). In the present study, our results clearly show that ZOP induces the phosphorylation of both p67phox and p47phox in human monocytes. We demonstrate that cPLA₂ has no detectable effect on the phosphorylation of either of these oxidase components in human monocytes (Fig. 5B) even though cPLA₂ regulated their translocation. Phosphorylation of p47phox appears to precede translocation. All of these data suggest that translocation and phosphorylation are two separate events in human monocytes and that the role of cPLA₂ or AA is downstream of the phosphorylation of p67phox and p47phox. In neutrophils, recent studies suggested that phosphorylation of p67phox occurs in cytosol and is independent of p47phox (38).
Further these investigators detected no association between phosphorylated p67phox and phosphorylated p47phox in the cytosol fraction (38). In contrast, Benna et al. reported that phosphorylated p67 and phosphorylated p47phox form a complex in neutrophils (20). There was no clear association between phosphorylated p67phox and phosphorylated p47phox in our experimental system (Fig. 5B), but the association between p67phox and p47phox is clear (Fig. 4A and 5C). We can not exclude the possibility that association between phosphorylated p67phox and phosphorylated p47phox may occur in human monocytes although this association was undetectable in our studies. One explanation may be that the phosphorylated p67phox and phosphorylated p47phox are likely a small fraction of the total p67phox or p47phox protein, and phosphorylated p67phox may form a complex with either phosphorylated or non-phosphorylated p47phox (and vice versa). Thus competitive binding may limit the interaction of the two phosphorylated species.

In summary, our data indicate that cPLA$_2$ and AA regulate NADPH oxidase activity in primary human monocytes via modulating the assembly of the active enzyme complex through controlling the translocation but not phosphorylation of p67phox and p47phox. These studies should provide insight into the mechanisms by which activation signals are transduced to allow the induction of superoxide anion and acquisition of the capacity to oxidize LDL lipids. The detailed mechanisms for how cPLA$_2$ and AA regulates the translocation of p67phox and p47phox are the focus of our further investigations.
Acknowledgement

We thank Drs. H. Malech and T. Leto for kindly providing the goat polyclonal antibodies specific for p47phox. We also thank B. Xu and C. Horton for critical reading of the manuscript.
References


Footnotes

* Supported by funding to MKC from NIH-HL61972.

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Key Words: Human monocytes, cPLA\textsubscript{2}, arachidonic acid, p67phox/p47phox, translocation, phosphorylation.

1 Abbreviations used in this paper: cPLA\textsubscript{2}, cytosolic phospholipase A\textsubscript{2}; AA, arachidonic acid; ODN, oligodeoxyribonucleotide; AS-ODN, anti-sense ODN; S-ODN, sense ODN; ZOP, opsonized zymosan; LA, linoleic acid; PA, palmitic acid; LDL, low density lipoprotein; PKC, protein kinase C.
Figure legends

Figure 1. Monocyte activation induces the translocation of p47phox and p67phox.
Human monocytes were plated in 6 well plates at a concentration of 5 x 10^6 /2ml/well. The monocytes were not activated or activated for 5, 15, 30 and 60 minutes with 2mg/ml ZOP. Cells were then collected and fractionated as described in “Materials & Methods”. The membrane fractions were separated by 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane. Human p47phox was detected using a polyclonal goat anti-human p47phox antibody followed by incubation with horseradish peroxidase conjugated rabbit-anti-goat IgG. The immunoblot was then developed by ECL and exposed to film. The same membrane was reprobed with an affinity purified polyclonal rabbit anti-human p67phox antibody.

Figure 2. Inhibition of cPLA₂ expression blocks ZOP-induced translocation of p67phox and p47phox in human monocytes. A. Human monocytes (5 x 10^6) were incubated in the absence or presence of cPLA₂-specific AS-ODN or S-ODN (10 µM) for 24 h, post-nuclear extracts were prepared and 100 µg protein of each lysate was used to determine the expression of cPLA₂ protein by Western blotting according to protocols described in “Materials & Methods.” This same membrane was stripped and reprobed with polyclonal antibodies specific for Tyk2 and p47phox to assess the equal loading of protein. B. A representative result shows the membrane-associated p67phox and p47phox in each indicated condition. Human monocytes (5 x 10^6) were incubated in the absence or presence of cPLA₂ specific AS-ODN or S-ODN (10 µM) for 24 h, then exposed to ZOP (2 mg/ml) for 1-h. Cells were fractionated and the membrane fractions were separated on an 8% SDS-PAGE. The proteins were transferred to a PVDF membrane and detected with rabbit polyclonal antibodies against p67phox or p47phox. C. The integrated densities of p67phox and p47phox bands from the light-exposed films shown in Fig. 2B were determined using the software program NIH image. Data are averages of three similar experiments and error bars indicate S.D.
Figure 3. **Arachidonic acid and linoleic acid, but not palmitic acid, restored translocation of p67phox and p47phox in monocytes depleted of cPLA2.** A. Human monocytes (5 x 10^6) were incubated in the absence or presence of cPLA2 specific AS-ODN or S-ODN (10 µM) for 24 h. Cells were then activated with ZOP (2 mg/ml), in the presence or absence of 3.3 µM arachidonic acid. After ZOP treatment, membrane fractions were isolated and the membrane-associated p67phox and p47phox were blotted with each specific rabbit polyclonal antibody. B. Human monocytes were pretreated with cPLA2 specific AS-ODN or S-ODN as described in Fig. 2B. Cells were then exposed to ZOP in the presence or absence of each indicated fatty acid (3.3 µM). The membrane fraction of each sample was analyzed for their p67phox and p47phox as described in Fig. 2B. C. Cells were treated with each indicated fatty acid (3.3 µM) alone or in the presence of ZOP. The membrane-associated p67phox and p47phox of each sample were then examined by Western blotting using each specific rabbit polyclonal antibody.

Figure 4. **p67phox and p47phox can form a complex in human monocytes and ZOP-induced translocation of p67phox is affected by p47phox.** A. Association of p67phox and p47phox in human monocytes. Cells (5 x 10^6) were cultured in the absence or presence of ZOP (2 mg/ml) for 1h, post-nuclear extracts were prepared as described in “Materials & Methods”, followed by immunoprecipitation with goat polyclonal antibodies against p67phox or goat polyclonal antibodies against p47phox as described in “Materials & Methods”. Samples were run on 8% SDS-PAGE and transferred to PVDF membrane. Samples, immunoprecipitated with anti-p67phox antibodies, were blotted with rabbit polyclonal antibodies specific for p67phox, the membrane was then re-probed with antibodies specific for p47phox. Samples immunoprecipitated with anti-p47phox antibodies were blotted with mouse monoclonal antibodies specific for p47phox at first, followed by re-probing it with antibodies specific for p67phox. Pre-adsorbed anti-mouse IgG HRP or anti-rabbit IgG HRP was used for these experiments. B. Human monocytes (5 x 10^6) were incubated in the absence or presence of human p47phox specific AS-ODN or S-ODN (5 µM) for 72 h with a refeeding at 48 h, then exposed to ZOP (2 mg/ml) for 1-h. Cells were fractionated and membrane-associated p67phox and p47phox were determined as described in Fig. 2B. C. Human monocytes were treated with p47phox
specific AS-ODN or S-ODN as described in Fig. 4B. 50 µg of each post-nuclear extract was analyzed for p47phox expression with monoclonal antibodies against p47phox. The membrane was then stripped and reprobed first with monoclonal antibodies specific to β-tubulin to confirm equal loading. Blot was then reprobed with polyclonal antibodies for p67phox.

Figure 5. **ZOP-induced phosphorylation of p67phox and p47phox in human monocytes is cPLA2-independent.** A. Time-dependent phosphorylation of p67phox and p47phox in ZOP-activated human monocytes was investigated. Phosphorylated p67phox and p47phox protein bands were visualized using a PhosphorImager. The phosphorylation in these bands was quantified using the program ImageQuant. The ImageQuant values were normalized to the integrated density values of each the protein bands obtained by subjecting the membrane to Western blotting. The normalized p67phox and p47phox phosphorylation values are representative results of two or more similar experiments. B. Human monocytes (5 x 10^6) were cultured in the absence or presence of cPLA2 specific AS-ODN or S-ODN (10 µM) for 20 h, then changed to phosphate-free medium with the same dose of cPLA2 specific AS-ODN or S-ODN. Cells were further cultured and labeled with [32P]orthophosphate (200 µCi) for 4 h followed by activation with ZOP (2 mg/ml) for 1-h. Post-nuclear extracts were prepared and immunoprecipitated with goat polyclonal antibodies specific for p67phox or rabbit polyclonal antibodies specific for p47phox. Immunocomplexes were subjected to 8% SDS-PAGE, then transferred to PVDF membrane. Phosphorylated p67phox and p47phox were detected using PhosphorImager analysis. Unfilled arrowheads indicate the corresponding position of p47phox and p67phox, respectively. C. The membranes that were analyzed on the PhosphorImager were subjected to Western blotting with rabbit polyclonal antibodies against p67phox and mouse monoclonal antibodies against p47phox, respectively, to confirm the equal loading of protein. Membrane was further reprobed with antibodies specific for p47phox or p67phox, respectively, as indicated (low panels). Pre-adsorbed anti-mouse IgG HRP or anti-rabbit IgG HRP was used in these studies. D. Quantitative results of phosphorylation of p67phox and p47phox upon activation are compared to unactivated cells. Data are averages of three similar
experiments and error bars indicate S.D. Values were derived from band densitometry of the phosphorylated protein signal (Fig. 5B) and were corrected for the amount of protein detected by Western blotting (Fig. 5C upper panels).

Figure 6. Proposed model of the human monocytes signal transduction pathways upon ZOP-induced activation of NADPH oxidase.
<table>
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<tr>
<td>15</td>
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<td>30</td>
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<td>60</td>
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**Western Blot Analysis**

- **p67phox**
- **p47phox**
A

<table>
<thead>
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<th>S-ODNs</th>
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<tbody>
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<td>Tyk2</td>
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B

Membrane fraction

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ZOP cPLA₂ AS-ODN cPLA₂ S-ODN

- - + + -
- - + - +

C

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<td>ZOP S-ODN</td>
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Integrated Density
A

Normalized relative fold increase in phosphorylation

Activation time (min)

5 15 30 60

p67phox

p47phox

B

Phosphorylation

IP: p67phox

IP: p47phox

p67phox

p47phox

ZOP

− + + +

cPLA2 AS-ODN

− − + −

cPLA2 S-ODN

− − + +

C

Western Blot

p67phox

rep probe p47phox

p47phox

rep probe p67phox

D

Normalized relative fold increase in phosphorylation

p67phox

p47phox

ZOP

− + + +

cPLA2 AS-ODN

− − + −

cPLA2 S-ODN

− − + +
Cytosolic phospholipase A2 regulation of human monocyte NADPH oxidase activity: cPLA2 affects translocation but not phosphorylation of p67phox and p47phox
Xiaoxian Zhao, Erik A. Bey, Frans B. Wientjes and Martha K. Cathcart

J. Biol. Chem. published online May 6, 2002

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