

The Oxidation State of Phospholipids Controls the Oxidative Burst in Neutrophil Granulocytes¹

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The activation of neutrophil granulocytes has to be carefully controlled to balance desired activity against invading pathogens while avoiding overwhelming activation leading to host tissue damage. We now show that phospholipids are potential key players in this process by either enhancing or dampening the production of reactive oxygen species (ROS) during the oxidative burst. Unoxidized phospholipids induce the production of ROS, and they also work synergistically with FMLP in potentiating the oxidative burst in neutrophil granulocytes. Oxidation of these phospholipids, however, turns them into potent inhibitors of the oxidative burst. OxPLs specifically inhibit ROS production by inhibiting the assembly of the phagocyte oxidase complex but do not alter neutrophil viability, nor do they interfere with MAPK activation. Furthermore, up-regulation of the activation marker Mac-1 and phagocytosis of bacteria is not affected. Therefore, phospholipids may act as sensors of oxidative stress in tissues and either positively or negatively regulate neutrophil ROS production according to their oxidation state. *The Journal of Immunology*, 2008, 181: 4347–4353.

Neutrophil granulocytes are an integral part of the bodies innate defense mechanisms against pathogens. They represent the most abundant cell type in blood but are absent in healthy tissue. Upon infection, neutrophils are among the first cells recruited to inflamed tissue and exert indispensable microbicidal functions. Release of reactive oxygen species (ROS)³ generated by the neutrophil NADPH-oxidase complex is, along with phagocytosis and degranulation of antimicrobial proteins, the best described and most potent effector mechanism (1–4). The generation of ROS is tightly controlled. In the resting state, the multienzyme complex, also termed phagocyte oxidase (phox) complex, responsible for the generation of oxygen radicals is kept inactive by sequestering several of its components in the cytosol. Upon activation by bacterial products, the cytosolic subunits such as p47 and p67 are phosphorylated and translocated to the plasma membrane to form the active NADPH-oxidase (5–8). This leads to the production of large amounts of ROS, which contribute to killing the invading microorganism. This process, termed oxidative

burst, is a crucial element in host defense. Its importance is highlighted by the fact that loss of function mutations of subunits of the NADPH-oxidase cause the severe pathology of the chronic granulomatous disease in humans, which is characterized by overwhelming bacterial infections (9, 10). In contrast, activated neutrophils are implicated in causing severe tissue damage, e.g., in the adult respiratory distress syndrome ischemia/reperfusion injury and others (11–13). Thus, regulation of the NADPH-oxidase and ROS production is a central check-point in neutrophil homeostasis (14–16).

It has been shown that ROS produced by activated neutrophil granulocytes promotes the formation of oxidized phospholipids (OxPLs) (17, 18). OxPLs are present in inflamed tissues, oxidized low-density lipoproteins as well as apoptotic cells and were shown to exert pro- as well as anti-inflammatory effects. They are acknowledged as major culprits for chronic inflammatory processes in atherosclerosis (19) but have also been shown to antagonize acute inflammatory reactions induced by LPS as well as other proinflammatory stimuli (20–22).

In this study, we report that OxPLs, generated in inflammation by attack of ROS, are capable of inhibiting the oxidative burst in neutrophils. Inhibition is crucially dependent on oxidation. Several different OxPLs, phosphatidylcholines, phosphatidylserines, and phosphatidylglycerols, are able to block the oxidative burst. Interestingly, oxidized phosphatidylethanolamine completely lacked inhibitory activity toward the oxidative burst in neutrophils. In contrast, their nonoxidized precursors rather induced the production of ROS, and they are also capable of amplifying the response of neutrophils to FMLP. These demonstrate a regulatory role of phospholipids on neutrophil ROS production, depending on their state of oxidation.

Materials and Methods

Reagents and Abs

PMA, FMLP, LPS from *Escherichia coli* (serotype 0127-B8), peptidoglycan (PGN) from *Staphylococcus aureus*, and arachidonic acid (AA) from Sigma-Aldrich. 2'7'-dichlorodihydrofluorescein diacetate

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³ Abbreviations used in this paper: ROS, reactive oxygen species; phox, phagocyte oxidase; Ox, oxidized; OxPL, Ox phospholipid; PAPC, 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-phosphorylcholine; PLPC, 1-palmitoyl-2-linoyl-*sn*-glycero-3-phosphorylcholine; PAPS, 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-[phospho-L-serine]; PAPG, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; PAPE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; PGN, peptidoglycan; DCF-DA, 2'7'-dichlorodihydrofluorescein diacetate; AA, arachidonic acid.

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(DCF-DA) was from Invitrogen. 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-phosphorylcholine (PAPC), 1-palmitoyl-2-linoyl-*sn*-glycero-3-phosphorylcholine (PLPC), 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-[phospho-L-serine] (PAPS), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PAPG), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PAPE), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were from Avanti Polar Lipids. Immobilon-P transfer membranes were from Millipore. Polyclonal Abs against phosphorylated and unphosphorylated ERK 1/2 and p38 were from Cell Signaling Technology. Abs against NADPH-subunits (gp91: clone 53; p47: 1/p47Phox; p67: clone 29) were from BD Biosciences. Peroxidase-conjugated secondary Abs were from Amersham Biosciences.

Cells

Mononuclear cells and polymorphonuclear granulocytes isolated from healthy donors were separated using discontinuous Ficoll Hypaque (Pharmacia) density gradient centrifugation. Mononuclear cells were recovered from the interface, and granulocytes in the pellet were depleted of contaminating erythrocytes by NH_4Cl lysis. Cell preparations were washed in PBS and then resuspended at 1×10^6 cells/ml in PBS.

Lipid oxidation

Lipids were oxidized by exposure of dry lipid to air for 72 h. The extent of oxidation was monitored by positive ion electrospray mass spectrometry as described previously (20). OxPLs used for experiments contained less than 50 $\mu\text{g/ml}$ endotoxin as determined by the *Limulus* amoebocyte assay (BioWhittaker).

Oxidative burst

Human neutrophil granulocytes ($1 \times 10^6/\text{ml}$) were incubated for 10 min at 37°C with lipids at concentrations indicated in the figure legends. They were loaded with DCF-DA (1 mg/ml) at 37°C for 5 min. After addition of either PMA (10^{-7} M) or FMLP (10^{-6} M), they were placed in a pre-warmed water bath (37°C) for 10 min. Stimulation was stopped by addition of ice cold 0.5% BSA in PBS and, after centrifugation, green fluorescence was immediately measured by flow cytometry. Where indicated, AA was added at a concentration of 30 $\mu\text{g/ml}$.

Immunofluorescence analysis

For CD11b (LM-2; ATCC) membrane staining, cells (5×10^5) were incubated for 30 min at 4°C with unlabeled mAbs at a concentration of 20 $\mu\text{g/ml}$ in the presence of human IgG Abs (20 mg/ml; Beriglobin; Aventis Behring). The primary mAb was visualized using Oregon Green-conjugated goat anti-mouse Ab from Molecular Probes and analyzed on a FACSCalibur flow cytometer (BD Biosciences) supported by CellQuest software (BD Biosciences). The exclusion of dead cells was performed by the addition of propidium iodide.

Subcellular fractionation

A total of 3×10^7 neutrophils were stimulated as indicated in the figure legend. After stimulation, cells were washed once in ice-cold PBS and cytoplasmic and membrane fractions prepared. In brief, the cells were resuspended in 500 μl of homogenization buffer A (20 mM Tris-HCl 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, and 1 mM PMSF). The cell suspension was sonicated twice on ice for 10 s at output 4 with a Bandelin Sonoplus 70 Sonicator. The cell lysate was centrifuged for 3 min at $3000 \times g$. Postnuclear supernatants were centrifuged at $100,000 \times g$ for 1 h in a TLA 100.4 rotor of a Beckman ultracentrifuge (Beckman Coulter). The supernatant was collected as the cytoplasmic fraction. The remaining pellet was collected as the membrane fraction. A total of 100 μl of Laemmli buffer was added to each sample, boiled at 95°C for 5 min, and 10 μl were loaded on SDS-PAGE.

Western blotting

After stimulation, polymorphonuclear granulocytes were lysed in Laemmli buffer (Bio-Rad) and proteins separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride membrane and, after blocking with 5% dry milk/0.1% Tween 20, incubated with primary Abs in the same solution. Bound Abs were detected by anti-IgG conjugated with peroxidase (Amersham Biosciences) and subsequent chemiluminescent detection. Western blots were quantified using the ImageJ 1.32 software (National Institutes of Health).

Cell-free activation assay

The rate of superoxide generation was determined as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* measured at 550 nm in

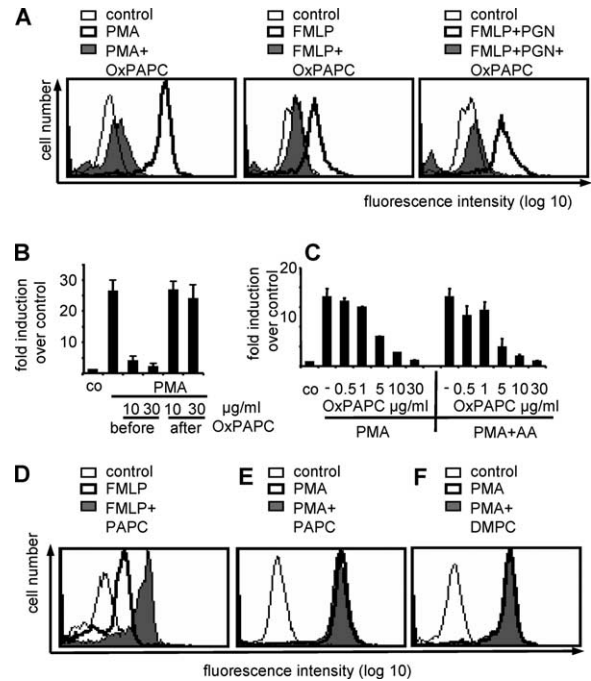


FIGURE 1. OxPAPC inhibits the oxidative burst in neutrophils induced by various stimuli. *A*, Neutrophils were stimulated with PMA (*left panel*), FMLP (*middle panel*), and FMLP and PGN (*right panel*) with (gray filled histograms) or without (open histograms, thick line) preincubation with OxPAPC. Unstimulated controls are represented by open histograms, thin line. Representative histograms of five independent experiments are shown. *B*, neutrophil granulocytes were either preincubated for 10 min with the indicated amounts of OxPAPC or with PBS and stimulated with PMA (before) or OxPAPC was added 10 min after stimulation with PMA (after) and analyzed by flow cytometry. Data are presented as mean values \pm SD of two independent experiments. *C*, Neutrophils were incubated with the indicated amounts of OxPAPC and stimulated with PMA with or without addition of AA and analyzed by flow cytometry. Data are presented as mean values \pm SD of two independent experiments. *D*, Neutrophils were stimulated with FMLP with (gray filled histogram) or without (open histogram, thick line) preincubation with PAPC and analyzed by flow cytometry. Unstimulated controls are represented by open histograms, thin line. *E*, Neutrophils were stimulated with PMA with (gray filled histogram) or without (open histogram, thick line) preincubation with PAPC and analyzed by flow cytometry. Unstimulated controls are represented by open histograms, thin line. *F*, Neutrophils were preincubated with DMPC (gray filled histograms) or PBS (open histograms, thick line) with subsequent addition of PMA and analyzed by flow cytometry. Unstimulated controls are represented by open histograms, thin line.

a Labsystems iEMS Microplate Reader as described in Ref. 23. In brief, isolated membrane (5 μg) and cytosol (50 μg) fractions were incubated in the presence of 165 μM cytochrome *c* in a final volume of 100 μl PBS containing 1 mM MgCl_2 and 20 μM FAD. AA was applied as amphiphile and GTP γ S was added in 3- μM concentration. Incubation was conducted at room temperature for 5 min. Thereafter, O_2^- production was initiated by the addition of 200 μM NADPH and followed for 20 min. The initial linear portion of the absorption curves (lasting for 10–15 min) was used for calculation of the rate of O_2^- production. Parallel samples were run in the presence of 100 μg superoxide dismutase. The absorption coefficient of ferricytochrome *c* of $21,000 \text{ M}^{-1} \times \text{cm}^{-1}$ was used for calculation of O_2^- production. Phospholipids were added in the indicated concentration either before or at the end of the incubation period.

Statistical analysis

Statistical analysis was performed on GraphPadPrism4 software with the unpaired Student's *t* test. All *p* values ≤ 0.05 were considered significant. *, *p* ≤ 0.05 . Error bars denote the SD.

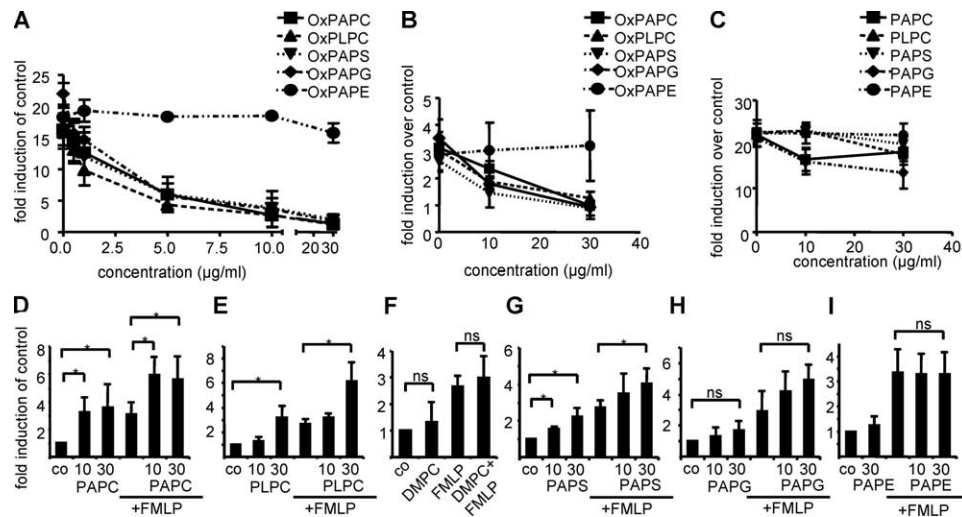


FIGURE 2. Different classes of phospholipids have a dual role in influencing the oxidative burst. *A* and *B*, Freshly isolated neutrophil granulocytes were preincubated for 10 min with the indicated amounts of the different OxPIs (OxPAPC; OxPLPC; OxPAPS; OxPAPG; OxPAPE), stimulated with PMA (10^{-7} M) (*A*) or FMLP (*B*), and subsequently analyzed by flow cytometry. Plots are mean values \pm SD of geometrical means calculated as fold induction above control level from at least three independent experiments. *C*, Neutrophils were preincubated with 10 or 30 μ g/ml of the indicated unoxidized phospholipid with or without subsequent addition of PMA and analyzed by flow cytometry. Plots are mean values \pm SD of geometrical means calculated as fold induction above control level from at least three independent experiments. *D–I*, Neutrophils were preincubated with 10 or 30 μ g/ml of the indicated unoxidized phospholipid with or without subsequent addition of FMLP (10^{-6} M) and the analyzed by flow cytometry. Plots are mean values \pm SD of geometrical means calculated as fold induction above control level from at least three independent experiments. (*, $p \leq 0.05$).

Results

OxPAPC inhibits whereas unoxidized PAPC induce the oxidative burst in neutrophil granulocytes

To test the effects of phospholipids on ROS generation, neutrophil granulocytes were incubated with OxPAPC, a well described mixture of OxPIs, and loaded with the redox-sensitive dye DCF-DA (24). Treatment of neutrophils with OxPAPC inhibited the production of ROS induced by PMA and FMLP (Fig. 1*A*). Ligation of TLRs on neutrophils is known to amplify the reaction of neutrophils in response to FMLP (25, 26). OxPAPC also inhibited the PGN- or LPS-primed oxidative burst induced by FMLP (Fig. 1*A* and data not shown). OxPIs did not quench the fluorescence signal of the dye because their addition just before measuring the cells in the flow cytometer did not diminish the signal (Fig. 1*B*). Furthermore, OxPAPC-stimulated neutrophils were able to take up Calcein-AM, a nonredox-sensitive dye, whose intracellular metabolism is similar to DCF-DA, as well as control cells (not shown). Taken together, OxPAPC inhibited the formation of the oxidative burst in neutrophils induced via different stimuli.

Generation of AA from phospholipid-precursors is an important step in the oxidative burst formation of neutrophils. To test whether OxPAPC interfered with AA generation leading to insufficient generation of free AA, we exogenously added it together with the OxPIs to the assay. However, we were not able to restore ROS production in neutrophils (Fig. 1*C*).

We next analyzed the effect of the unoxidized precursor molecule PAPC on neutrophils ROS production. We found that PAPC induced an oxidative burst in neutrophil granulocytes and that it also acted synergistically with FMLP in that respect (Fig. 1*D*). It did not, however, substantially alter PMA-induced oxidative burst formation (Fig. 1*E*). DMPC, a phospholipid bearing a phosphatidylcholine head group but has two saturated fatty acid residues at the sn-1 and the sn-2 position and, therefore, cannot be oxidized, did not influence PMA-induced oxidative burst activity (Fig. 1*F*).

Dual role of various classes of phospholipids on oxidative burst formation

We next tested whether structurally different phospholipids would also influence oxidative burst formation in neutrophils. As shown in Fig. 2*A*, OxPLPC, which has at the sn-2 position a linoleic acid instead of AA, potentially inhibited PMA-induced ROS production, comparable to OxPAPC. We also tested OxPIs bearing other head groups than PC and found that OxPAPS as well as OxPAPG showed similar dose-dependent inhibitory effects on ROS production in PMA-stimulated neutrophils as OxPAPC. Neither of these lipids showed significant effects on unstimulated ROS production (not shown). Also, these lipid species inhibited FMLP-induced production of ROS to a similar extent as OxPAPC (Fig. 2*B*). Notably, oxidized PAPE was not capable of inhibiting PMA- or FMLP-induced oxidative burst formation in neutrophils (Fig. 2, *A* and *B*). This was surprising, since the lipid composition at the glycerol backbone of PAPE is the same as that of PAPC, PAPS, and PAPG. Therefore, the head group of phospholipids as well as oxidation is an important factor determining the ability to inhibit the oxidative burst.

Next, we tested the effect of unoxidized phospholipids also on neutrophil oxidative burst formation. We could not detect any significant differences in PMA-stimulated neutrophils pretreated with unoxidized PAPC, PLPC, PAPS, PAPG, and PAPE compared with PMA stimulation alone (Fig. 2*C*). We found, however, that unoxidized phospholipids were able to dose dependently increase the production of ROS (Fig. 2, *D–H*). Again, PAPE was the exception since it did not alter ROS production in neutrophils (Fig. 2*I*). Furthermore, unoxidized phospholipids primed neutrophils for an enhanced response to FMLP, comparable to the effect of the well described priming agent LPS.

Also, in this regard, DMPC, which has saturated fatty acids on the sn-1 as well as on the sn-2 position, had no detectable influence on either basal or stimulated neutrophil ROS production (Fig. 2*F*).

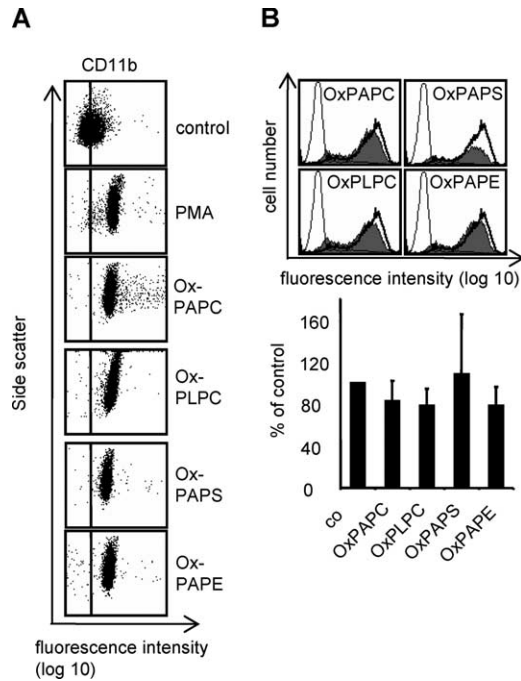


FIGURE 3. OxPLs do not inhibit up-regulation of CD11b or phagocytosis of bacteria. *A*, Neutrophil granulocytes were preincubated for 10 min with 10 $\mu\text{g/ml}$ of the indicated OxPI or with PBS and stimulated with PMA (10^{-7} M). Cell surface expression of CD11b was measured by flow cytometry. Plots are representative of at least three independent experiments. *B*, Neutrophil granulocytes were incubated with fluorescently labeled *E. coli* at 0°C (open histograms, thin line) or at 37°C with (gray filled histograms) or without (open histograms, thick line) the addition of the indicated OxPLs (30 $\mu\text{g/ml}$). Representative overlays are shown. Statistical analysis of mean values \pm SD are shown.

Taken together, we find that there is an oxidation state-dependent regulatory role for OxPLs on neutrophil oxidative burst formation.

OxPLs do not inhibit up-regulation of CR3 or phagocytosis

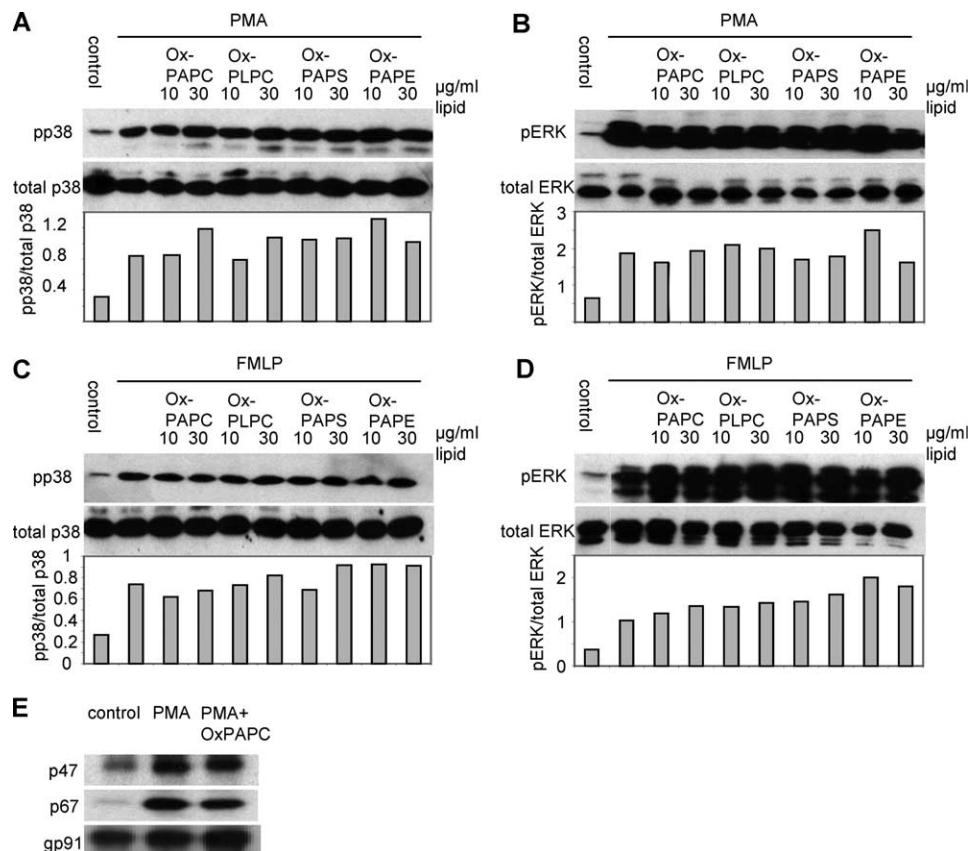
In addition to production of ROS, stimulation of neutrophils leads to the up-regulation of Mac-1 (CR3, CD11b/CD18), which is involved in adhesion of activated neutrophils to the endothelium (11, 27). As shown in Fig. 3*A*, OxPLs did not interfere with PMA-induced up-regulation of CD11b. Also, FMLP-induced up-regulation of CD11b was not inhibited (not shown).

Phagocytosis of bacteria is a very important function of neutrophil granulocytes in host defense. Analyzing the effects of OxPLs on ingestion of fluorescently labeled *E. coli*, we found, that all OxPLs except for OxPAPS slightly inhibited the uptake of bacteria, which was not statistically significant (Fig. 3*B*). These data show that OxPLs rather specifically interfere with oxidative burst formation but are not toxic to neutrophils nor do they generally block their activation.

OxPLs do not block activation of intracellular signaling events or translocation of phox components to the plasma membrane

Several steps in the signal-transduction pathway leading to phosphorylation and subsequent assembly of the neutrophil phox complex have been identified. Although the roles of the MAPK ERK1/2 and p38 in the generation of the oxidative burst of neutrophils are not fully understood, there is evidence that these kinases are activated during the oxidative burst and that there is involvement of those kinases in the production of ROS (28–32). To test whether OxPLs interfered with activation of these signal transduction pathways, we stimulated neutrophils with either PMA or FMLP and determined the phosphorylation state of p38 and ERK1/2 with phospho-specific Abs in Western blots. Addition of OxPLs alone did not lead to a detectable phosphorylation of ERK

FIGURE 4. OxPLs do not inhibit MAPK activation or assembly of the phox complex on the cell membrane. *A* and *B*, Neutrophils were preincubated with 10 or 30 $\mu\text{g/ml}$ of the indicated OxPI and stimulated for 3 min with PMA (10^{-7} M), harvested, and analyzed by Western blot for phosphorylated p38 MAPK (*A*) and phosphorylated ERK1/2 (*B*). *C* and *D*, Neutrophils were preincubated with 10 or 30 $\mu\text{g/ml}$ of the indicated OxPI and stimulated for 3 min with FMLP (10^{-6} M), harvested, and analyzed by Western blot for phosphorylated p38 MAPK (*C*) and phosphorylated ERK1/2 (*D*). All blots were further analyzed densitometrically using Image J software. Data are presented as ratio of phosphorylated to total protein. *E*, Neutrophils were preincubated with 10 $\mu\text{g/ml}$ OxPAPC and stimulated for 5 min with PMA (10^{-7} M). Cells were harvested, and the membrane fraction was isolated and analyzed for the presence of the p47- and p67-phox component. Blots are representative of three independent experiments.



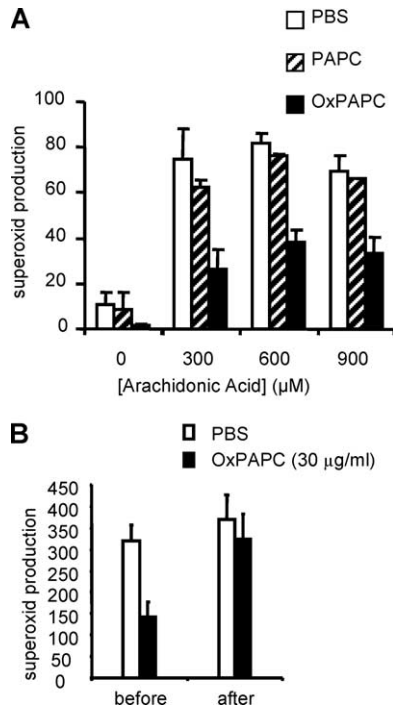


FIGURE 5. OxPLs inhibit ROS production in a cell-free assay system. *A*, PAPC or OxPAPC was added at a concentration of 200 $\mu\text{g/ml}$ before the incubation period. Mean \pm SEM of three independent experiments conducted on different membrane preparations are shown. *B*, OxPAPC (30 $\mu\text{g/ml}$) was added either before or at the end of the incubation period. Mean \pm SEM of three experiment conducted on the same membrane preparation is shown.

(not shown), and, when added together with PMA or FMLP, densitometrical analysis revealed that neither lipid inhibited the activation of ERK1/2 and p38 in neutrophils stimulated with either PMA or FMLP (Fig. 4, *A–D*).

Since translocation of cytosolic subunits to the membrane is a prerequisite for ROS production, we analyzed whether OxPLs inhibited PMA-induced membrane translocation of the p47 and p67 subunit of the phox complex. To this end, we made subcellular fractions of neutrophils stimulated with PMA with or without addition of OxPAPC. As shown in Fig. 4*E*, OxPAPC did not inhibit translocation of the cytosolic components of the p47 as well as of the p67 subunit of the phox complex to the membrane. In parallel measurements of neutrophil oxidative burst, OxPAPC showed potent inhibitory effects.

OxPLs inhibit ROS-generation in a cell-free system

To test, whether the inhibition of ROS-production by OxPLs was dependent on living cells or functioning signaling, we used a cell-free system of ROS generation (23). In these experiments, a pronounced inhibition of ROS production was detected when adding OxPAPC, whereas PAPC hardly showed an effect. In this assay, the rate of superoxide production is strongly dependent on the applied concentration of AA. Several factors were shown to shift the optimal AA concentration and mimic thereby inhibition of superoxide production (33, 34). To exclude this possibility, we tested the effect of PAPC and OxPAPC in a broad range of AA. As shown in Fig. 5*A*, both lipids induced a detectable shift of the optimal AA concentration (from 300 to 600 μM), but, at each point, there was a clear inhibition of superoxide production in the presence of OxPAPC. The nonoxidized lipid had only minimal effect. The inhibitory action of OxPAPC was not influenced by

preincubation of either the membrane or the cytosolic fraction with the modified lipid (data not shown). Similar to the results obtained on intact cells, OxPAPE had negligible effect (data not shown). The inhibitory effect of OxPAPC was only visible when it was added before the assembly of the phox complex. No effect was seen when OxPAPC was added after activation of the ROS-producing complex (Fig. 5*B*). These observations suggest that OxPLs inhibit the proper assembly of the complex but do not interfere with the enzymatic activity.

Discussion

In this paper, we describe a novel role for phospholipids oxidation products in the regulation of the oxidative burst in neutrophil granulocytes. Recently, we could demonstrate that OxPLs are potent modulators of innate immune activation and also inhibit the initiation of adaptive immune responses (20, 21, 35). We show here that OxPLs potently inhibit the generation of ROS induced by several stimuli, but they do not generally interfere with activation of neutrophils as evidenced by a normal up-regulation of the activation marker CD11b and they also do not interfere with phagocytosis of bacteria. Furthermore, OxPLs do not inhibit proximal signaling events, since activation of ERK and p38 MAPK induced by PMA or FMLP is not affected by OxPLs. In addition, OxPLs do not interfere with the initial steps of assembly of the ROS-producing phox complex, since translocation of the pivotal subunits p47 and p67 is not inhibited by OxPLs. Therefore, OxPLs seem to selectively target the generation of ROS, which are on their part believed to be responsible for the generation of OxPLs in an inflammatory setting (36, 37).

We find that oxidation is a prerequisite for phospholipids to become inhibitors of the neutrophil oxidative burst. Several oxidized lipids, phosphatidylcholines, phosphatidylserines, and phosphatidylglycerols, which have a (poly-) unsaturated fatty acid at their sn-2 position, are capable of inhibiting the neutrophil oxidative burst. Interestingly, their unoxidized precursor molecules had the opposite effect. Unoxidized PAPC as well as PLPC and PAPS are inducing ROS production in neutrophils. They are also capable of enhancing the response of neutrophils to FMLP, in a similar magnitude as ligands of TLRs. We could not, however, detect any statistically significant effects of unoxidized phospholipids on PMA-induced ROS. A similar phenomenon has also been reported for another priming agent, leukotriene B₄ (38). Of note, phosphatidylethanolamine did not show these effects. Neither was OxPAPE able to inhibit the oxidative burst in isolated neutrophils or in cell-free experiments nor was the unoxidized form able to induce ROS-production. When we tested the influence of a phospholipid with a phosphatidylcholine head group, but with saturated fatty acids on the sn-1 as well as the sn-2 position, DMPC, we did not detect any significant influence on the oxidative burst formation, be it positive or negative regulation. These experiments showed that both oxidation as well as the head group of the phospholipids was important determining the effect of the lipids.

It has long been known that unsaturated fatty acids are able to increase the oxidative burst activity, most well known for this activity is AA, but also other fatty acids (39–41). Our results now suggest that unsaturated fatty acids integrated in phospholipids are also capable of activating the oxidative burst. Structural integrity of the double bonds is a prerequisite to do so, since oxidation of these phospholipids not only abrogates their stimulatory properties but also turns them into potent inhibitors of oxidant production.

In addition to fatty acids, other lipid mediators such as platelet-activating factor (PAF) and leukotrienes have been shown to enhance neutrophils ROS production also. PGs, in contrast, were

demonstrated to inhibit neutrophil functions including the oxidative burst (38, 42, 43). All of those lipid mediators have known receptors. There are no reports available today describing a cellular receptor for unoxidized phospholipids. Yet some of the effects of PAPC, in particular the coinduction of oxidative burst formation with FMLP, are reminiscent for other lipid compounds such as PAF, which bind to defined cellular receptor (PAF-R). Thus, it is intriguing that the activating properties of PAPC are mediated, at least in part, via a so far undefined cell surface receptor with similar functional features like PAF-R.

In contrast to unoxidized phospholipids, a number of potential receptors have been reported for OxPLs. Li et al. (44) demonstrated that PGE₂ receptor subtype 2 (EP2) is a receptor for OxPAPC on endothelial cells. Also on endothelial cells, 20 different binding proteins for OxPAPC were identified (45). Pegorier et al. (46) demonstrated that PAF-R is a cellular receptor for POVPC, a major compound of OxPAPC, on human macrophages. We have recently demonstrated that OxPAPC interacts with the TLR-4 receptor complex and blocks LPS recognition (21). However, a cellular receptor for OxPLs on neutrophils has not been reported so far. Since most of the so far identified receptors for OxPLs are expressed on human neutrophils, it is intriguing that OxPAPC might bind to these receptors. However, our results provide evidence that the OxPLs inhibit oxidative burst formation may not critically involve signaling via cell surface receptors, since we observed that OxPAPC inhibited Phox activity also in a cell-free assay system.

Furthermore, our observations suggest that these inhibitory effects of OxPLs are quite specific for the generation of ROS. Up-regulation of the activation marker CD11b, known to originate from intracellular pools and which has been shown to correlate with the oxidative burst response in neutrophils (47), is not affected by OxPLs. Phagocytosis of bacteria is also not affected, indicating that essential functions of neutrophils are still operable in the presence of OxPLs.

Moreover, well described signaling events leading to ROS production, like phosphorylation of ERK and p38 MAPK, remain intact. Even translocation of cytosolic components of the phox complex to the plasma membrane is still functioning in the presence of OxPLs.

Mechanistically, we found that OxPLs inhibit ROS production in a cell-free assay when the lipids are added before the correct assembly of the phox complex, but they do not inhibit the already functioning complex. The same results were noted using live cells. This indicates that the enzymatic activity of the phox complex is not inhibited, but suggests that OxPLs interfere with the correct assembly of the various components. We did note, however, that the effect of OxPLs was more pronounced in the assays where live cells were used. The fact that OxPLs inhibited ROS production also in the cell-free assay argues against a major involvement of cell surface receptor-mediated effects.

Lipids have long been recognized as important modulators of neutrophil functions (48–50), but the effects of phospholipids described here are unique. Our results suggest that phospholipids could act as biosensors of oxidative stress in tissues. Unsaturated fatty acid moieties in phospholipids, which are prone to oxidation, might constitute sensitive indicators of the oxidative damage regulating the activation of neutrophil granulocytes at the site of inflammation. Unoxidized phospholipids would issue a positive signal for activation of the microbicidal ROS-producing machinery, whereas accumulation of their oxidized forms is delivering a strong inhibiting signal. Thus, intact phospholipids at the site of inflammation would enhance neutrophil oxidative burst and allow a full-blown activation of the microbicidal machinery. At later time points, when the oxidative stress increases, oxidatively mod-

ified lipids would lead to an attenuation of the neutrophil oxidative burst, limiting collateral damage to host tissues induced by inflammatory processes.

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Disclosures

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