Lysophosphatidylcholine generates superoxide anions through activation of phosphatidylinositol 3-kinase in human neutrophils

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Abstract Lysophosphatidylcholine (LPC) accumulates in inflammatory tissues, where neutrophils are recruited to generate superoxide anions ($O_2^{\bullet-}$). Here, we show that LPC stimulates $O_2^{\bullet-}$ generation in human neutrophils and that the activity is inhibited with phosphatidylinositol 3-kinase (PI3 kinase) inhibitors, but not with protein kinase C (PKC) inhibitors. Furthermore, we demonstrate that LPC activates PI3 kinase in neutrophils. Thus, LPC might contribute to host defense by generating $O_2^{\bullet-}$ in neutrophils through PI3 kinase activation, but not through PKC activation.

Key words: Lysophosphatidylcholine; Superoxide anion; Phosphatidylinositol 3-kinase; Protein kinase C; Neutrophil

1. Introduction

In response to appropriate stimuli, an NADPH oxidase-containing system in phagocytes is activated and generates superoxide anions ($O_2^{\bullet-}$) to kill microorganisms, known as the respiratory burst [1,2]. The importance of this system for host defense is demonstrated by the fact that recurrent severe infections occur in patients with chronic granulomatous disease caused by impairment of this system [1,2]. The oxidase activity is tightly regulated since generated $O_2^{\bullet-}$ is harmful for intact tissues. Among the upstream stimuli, at least two factors, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3 kinase), have been demonstrated to be involved in $O_2^{\bullet-}$ generation in neutrophils [3–7].

Lysophosphatidylcholine (LPC) is implicated in inflammation, since phospholipase A2 (type IIA) is secreted in inflammatory tissues [8] and catalyzes the conversion of phosphatidylcholine (PC), the most abundant phospholipid, into LPC, resulting in the local accumulation of LPC [8,9]. As for the question whether LPC could generate $O_2^{\bullet-}$ or not, Yamamoto et al. have shown that LPC generates $O_2^{\bullet-}$ in macrophages [10]. However, the signaling mechanism of LPC for $O_2^{\bullet-}$ generation remains unclear. In neutrophils, it is still controversial at the moment. One report has shown that LPC has no activity to generate $O_2^{\bullet-}$ although it enhances phorbol 12-myristate 13-acetate (PMA, a PKC stimulator)-induced $O_2^{\bullet-}$ generation [11], whereas another report has shown that LPC itself generates $O_2^{\bullet-}$ in neutrophils [12].

Here, we show that LPC generates $O_2^{\bullet-}$ in isolated human neutrophils and that the signaling pathway of LPC-mediated $O_2^{\bullet-}$ generation is through PI3 kinase activation, but not through PKC activation.

2. Materials and methods

2.1. Materials

Without specification, all the materials used here including LPC (palmitoyl, C16:0), formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), wortmannin and calphostin C were purchased from Sigma. A PKC inhibitor, GF109203X, and a PI3 kinase inhibitor, LY294002, were from Calbiochem. The anti-p85 (a subunit of PI3 kinase) polyclonal antibody was from Upstate Biotechnology, [γ-^{32}P]ATP from Amersham-Pharmacia Biotechnology, lucigenin from Molecular Probes, and diphenyliodonium chloride from Aldrich.

2.2. Isolation of neutrophils

Neutrophils were prepared by density gradient centrifugation from healthy human blood using Polymorphprep (Nycomed, Oslo, Norway) according to the manufacturer's instruction. The contaminating erythrocytes were removed by hypotonic lysis. The neutrophils were then washed and resuspended in phosphate-buffered saline (PBS) at a cell density of 5×10^6 cells/ml. The final preparations were composed of more than 95% polymorphonuclear cells by nuclear staining (data not shown) and were therefore defined as neutrophils. Typically, 1×10^7 neutrophils were obtained from 20 ml of blood. The isolated neutrophils were immediately used for experiments.

2.3. Measurement of superoxide anions and cell viability

The amount of $O_2^{\bullet-}$ was measured based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* [13]. Briefly, neutrophils (3×10^5) were incubated with 1 mg/ml cytochrome *c* in the presence of an indicated stimulator with and without 20 μg/ml SOD at 37°C followed by rapid centrifugation. The optical density (OD) of the supernatant was determined at 550 nm using a spectrophotometer (Beckman DU-640). The amount of $O_2^{\bullet-}$ was expressed as the difference of the OD of those incubated with and without SOD. In the experiments using inhibitors of PI3 kinase and PKC, they were added just before the addition of stimulators without preincubation.

To measure intracellular $O_2^{\bullet-}$ and cell viability, neutrophils were incubated at 37°C with an indicated stimulator in the presence of 100 μM lucigenin, which produces chemiluminescence in response to $O_2^{\bullet-}$ [14]. Then, the cells were washed and resuspended in PBS plus 20 μg/ml propidium iodide which stains nuclei of dead cells. Intracellular $O_2^{\bullet-}$ and viability of the resuspended cells (1×10^4) were measured using a fluorescence-activated cell sorter (FACS) (Becton Dickinson FACS Vantage).

2.4. Measurement of PI3 kinase activity

The PI3 kinase activity was measured as described [15] with slight modifications. Briefly, after incubation of neutrophils (5×10^6) with LPC, PC, or PBS, they were lysed for 30 min at 4°C with a lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 30 mM Na pyrophosphate). Then, after centrifugation

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Abbreviations: LPC, lysophosphatidylcholine; $O_2^{\bullet-}$, superoxide anions; PI3 kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PC, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; fMLP, formyl-methionyl-leucyl-phenylalanine; SOD, superoxide dismutase; OD, optical density; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; PI, phosphatidylinositol; LPA, lysophosphatidic acid; PI3P, phosphatidylinositol 3-phosphate; LDL, low density lipoprotein

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of the lysate at 100 000×*g* for 30 min, the supernatant was incubated at 4°C for 60 min with protein A agarose beads (Boehringer Mannheim) coated with the anti-p85 antibody. After washing the beads twice with the lysis buffer followed by three washes with a washing buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl) to remove Triton X-100, the beads were incubated at 30°C for 10 min with 50 μM [γ-^{32}P]ATP (4000 cpm/pmol), 200 μM adenosine, and 0.2 mg/ml phosphatidylinositol (PI). The reactions were stopped by addition of 100 μl 1 M HCl and 200 μl chloroform/methanol (1:1, v/v). Then, after mixing and centrifugation, the lipid in the chloroform layer was separated on an oxalate-treated silica thin layer chromatography plate (Silica Gel 60, Merck) using a solvent system of chloroform/methanol/water/28% ammonia (70:100:25:15, v/v). The plates were then exposed to a X-ray film, and the incorporated radioactivity into the lipid was quantified by excising the corresponding portion of the plate followed by liquid scintillation counting.

3. Results

3.1. Superoxide anion generation by LPC

We first examined whether LPC could generate $O_2^{\bullet-}$ in isolated neutrophils by the cytochrome *c* reduction method. The neutrophils weakly generated $O_2^{\bullet-}$ without stimulation and LPC at 10 μM enhanced the generation by 6–8 times over control in a time-dependent manner (Fig. 1A). The production rate of $O_2^{\bullet-}$ induced by LPC was 0.5–1.5 nmol/min/10^6 neutrophils, which is comparable with that obtained with fMLP, a chemotactic peptide (Figs. 2C and 3) [5], and 15–20% of that obtained with PMA, a PKC stimulator (Figs. 2B and 3). The activity of LPC was specific among phospholipids tested here, since neither PC nor lysophosphatidic acid (LPA), which is another potent biologically-active phospholipid [16], generated $O_2^{\bullet-}$ (Fig. 1A). The activity of LPC was concentration-dependent and the maximal generation was obtained at 10–15 μM (Fig. 1B). With 20 μM LPC, however, the $O_2^{\bullet-}$ generation was 40–60% of the maximal (data not shown). This decrease might be due to the cytotoxicity of a high concentration of LPC [17]. PC or LPA did not stimulate $O_2^{\bullet-}$ generation at up to 15 μM (Fig. 1B). Both LPC- and PMA-induced $O_2^{\bullet-}$ generation was completely inhibited with 100 μM diphenyliodonium chloride, an NADPH oxidase inhibitor (data not shown), suggesting that the NADPH oxidase is responsible for the $O_2^{\bullet-}$ generation by both stimulators.

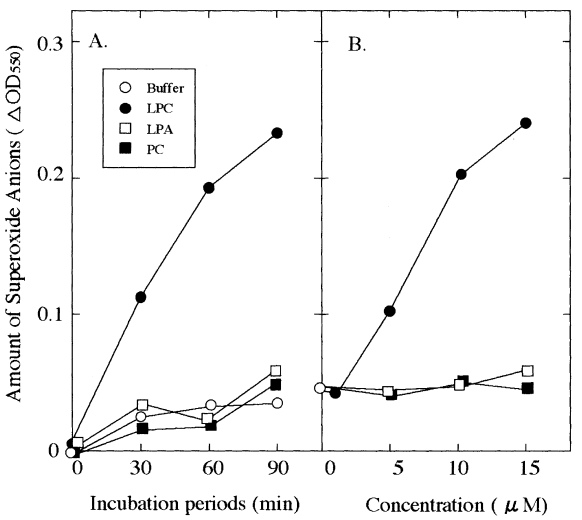

Fig. 1. Time- and concentration-dependent $O_2^{\bullet-}$ generation by LPC in human neutrophils. A: The amount of $O_2^{\bullet-}$ was measured by the cytochrome *c* reduction method as described in Section 2 after incubation of neutrophils with buffer alone (open circles), 10 μM LPC (closed circles), 10 μM LPA (open squares), or 10 μM PC (closed squares) for various periods of time at 37°C. B: Similar experiments were performed in the same way except with various concentrations of LPC, LPA, or PC for 60 min. The data shown are representative of three independent experiments with similar results.

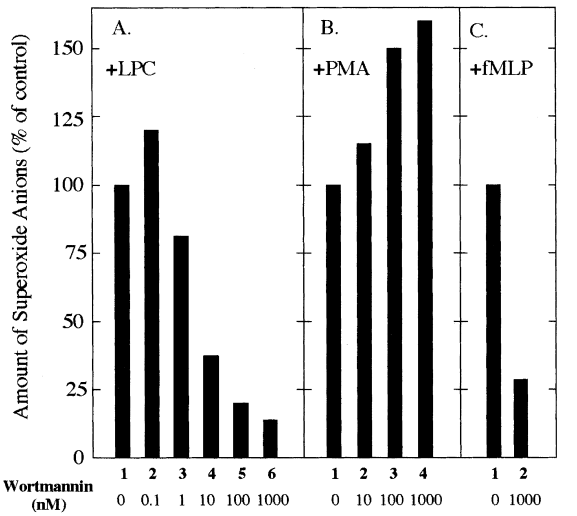

Fig. 2. A PI3 kinase inhibitor, wortmannin, inhibited LPC-induced and fMLP-induced $O_2^{\bullet-}$ generation, but not PMA-induced $O_2^{\bullet-}$ generation. $O_2^{\bullet-}$ generation was measured by the cytochrome *c* reduction method as described in Section 2 after incubation with 10 μM LPC for 30 min (A), 0.2 μM PMA for 10 min (B), or 1 μM fMLP for 10 min (C), with various concentrations of wortmannin at 37°C. The data shown are representative of three independent experiments with similar results and expressed as percentages of the amount of $O_2^{\bullet-}$ generated without wortmannin.

Next, we measured intracellular $O_2^{\bullet-}$ with lucigenin and cell viability with propidium iodide by FACS. While only 3.0% of the neutrophils were dead before incubation, 22% and 18% of them were dead after incubation for 30 min with 0.2 μM PMA and 10 μM LPC, respectively (data not shown). Thus, the effects of 0.2 μM PMA and 10 μM LPC on cell viability were comparable. We also found that only 3.5% of the neutrophils before incubation were $O_2^{\bullet-}$-positive by lucigenin fluorescence, whereas 91% and 78% of them were $O_2^{\bullet-}$-positive after incubation for 30 min with 0.2 μM PMA and 10 μM LPC, respectively (data not shown). Thus, the intracellular $O_2^{\bullet-}$ were indeed generated by LPC.

3.2. Effect of PI3 kinase inhibitors on LPC-mediated $O_2^{\bullet-}$ generation

Since it has been shown that activation of PI3 kinase and PKC is involved in $O_2^{\bullet-}$ generation in neutrophils [3–7], we first examined the effects of PI3 kinase inhibitors on LPC-induced $O_2^{\bullet-}$ generation. As shown in Fig. 2A, LPC-induced $O_2^{\bullet-}$ generation was inhibited with wortmannin, a PI3 kinase

Table 1
The effect of PI3 kinase inhibitors and PKC inhibitors on superoxide anion generation induced by LPC, PMA, and fMLP

Stimulation	PI3 kinase inhibitors	PKC inhibitors
LPC	+	−
PMA	−	+
fMLP	+	+

+: inhibited; −: not inhibited.

inhibitor, in a concentration-dependent manner. The inhibition was about 50% at 10 nM of wortmannin and almost complete at 1 μM (Fig. 2A). The values are in good agreement with previous data on the inhibition of PI3 kinase with wortmannin in neutrophils [5]. On the other hand, PMA-induced $O_2^{\bullet-}$ generation was not inhibited with wortmannin, but rather enhanced (Fig. 2B). We obtained similar results with another PI3 kinase inhibitor, LY294002, at 10 μM (data not shown). These results suggest that the signaling mechanism of LPC in $O_2^{\bullet-}$ generation is through PI3 kinase activation and is different from that of PMA. As shown before [4–7], fMLP-induced $O_2^{\bullet-}$ generation was also inhibited with wortmaninn (Fig. 2C) and LY294002 (data not shown).

3.3. Effect of PKC inhibitors on LPC-mediated $O_2^{\bullet-}$ generation

Since it has been shown that LPC stimulates PKC activity in a cell free system [18], we examined a possibility that LPC might activate PKC in neutrophils, leading to $O_2^{\bullet-}$ generation. Unexpectedly, a PKC inhibitor, GF109203X at 5 μM, did not inhibit LPC-induced $O_2^{\bullet-}$ generation (Fig. 3, lanes 1 and 2) under conditions which extensively inhibited PMA- (Fig. 3, lanes 4 and 5) and fMLP-induced $O_2^{\bullet-}$ generation (Fig. 3, lanes 6 and 7). We obtained similar results with another PKC inhibitor, calphostin C, at 10 μM (data not shown). These results suggest that the signaling mechanism of LPC in $O_2^{\bullet-}$ generation is not through activation of PKC and different from that of fMLP.

Calcium ion influx induced by LPC has been suggested to cause PKC activation and $O_2^{\bullet-}$ generation in vascular smooth muscle cells [19,20]. However, exogenously added divalent cation chelator EDTA at 1 mM into the reaction mixture did not inhibit $O_2^{\bullet-}$ generation (Fig. 3, lane 3), suggesting that calcium ion influx is not involved in the LPC-mediated $O_2^{\bullet-}$ generation in neutrophils.

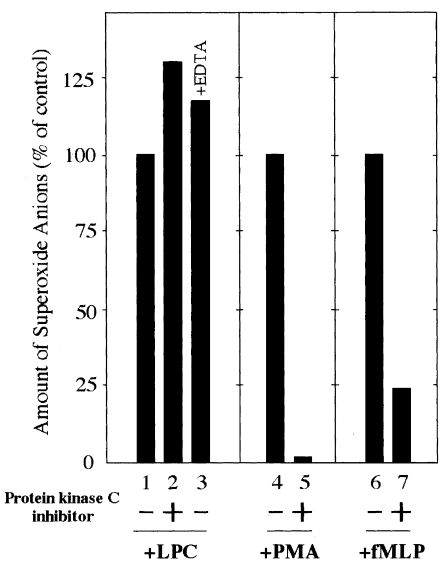

Fig. 3. A PKC inhibitor, GF109203X, inhibited PMA-induced and fMLP-induced $O_2^{\bullet-}$ generation, but not LPC-induced $O_2^{\bullet-}$ generation. $O_2^{\bullet-}$ generation was measured by the cytochrome *c* reduction method as described in Section 2 after incubation with 10 μM LPC for 30 min (lanes 1 and 2), 0.1 μM PMA for 10 min (lanes 4 and 5), or 1 μM fMLP for 10 min (lanes 6 and 7), in the absence (lanes 1, 4, and 6) or in the presence (lanes 2, 5 and 7) of 5 μM GF109203X at 37°C. In lane 3, LPC-induced $O_2^{\bullet-}$ generation was measured in the same way in the absence of GF109203X and in the presence of 1 mM EDTA. The data shown are representative of three independent experiments with similar results and expressed as percentages of the amount of $O_2^{\bullet-}$ generated without GF109203X.

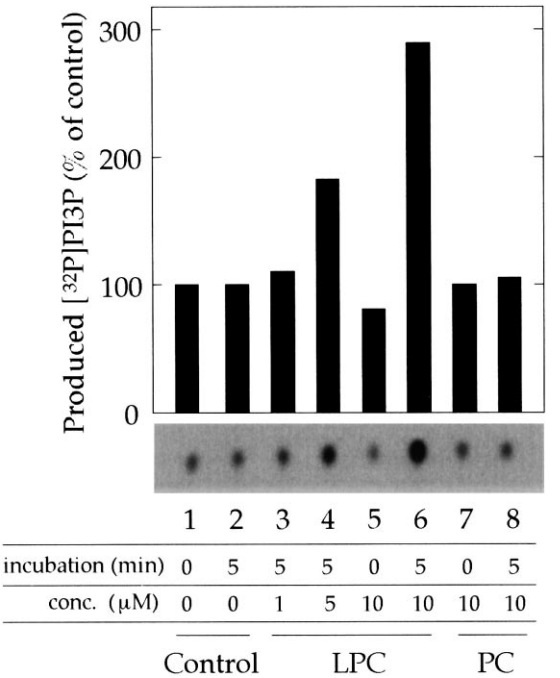

Fig. 4. LPC activates PI3 kinase in neutrophils. PI3 kinase was immunoprecipitated with the anti-p85 antibody-coated beads from the lysates of neutrophils incubated at 37°C for 0 min (lanes 1, 5 and 7) or 5 min (lanes 2–4, 6 and 8), without stimulation (lanes 1 and 2), or with 1 (lane 3), 5 (lane 4) or 10 μM LPC (lanes 5 and 6) or with 10 μM PC (lanes 7 and 8), and the beads were incubated with PI and [γ-^{32}P]ATP at 30°C for 10 min as described in Section 2. Then, the ^{32}P-incorporated lipid ([^{32}P]PI3P) was separated by thin layer chromatography. The radioactive spots of the autoradiography were shown (lower panel) and the radioactivity in the spots was counted and expressed as % of control (lane 1) (upper graph). The data shown are representative of three independent experiments with similar results.

3.4. LPC activates PI3 kinase in neutrophils

Since wortmannin inhibited LPC-mediated $O_2^{\bullet-}$ generation, we examined whether LPC activates PI3 kinase in isolated neutrophils. We quantified [^{32}P]phosphate-incorporated PI catalyzed by the PI3 kinases immnoprecipitated with the anti-p85 antibody in the neutrophil lysates, since it is believed that the production of [^{32}P]PI3 phosphate (PI3P) reflects the PI3 kinase activity [15]. As shown in Fig. 4, at 5 min of incubation at 37°C LPC increased [^{32}P]PI3P production in a concentration dependent manner, by 1.8 times at 5 μM (lane 4) and by 2.9 times at 10 μM (lane 6) compared with the basal (lane 1). However, LPC at 1 μM (lane 3) or PC at 10 μM (lane 8) did not increase [^{32}P]PI3P production. The level of PI3 kinase activity obtained with 10 μM LPC at 10 min of incubation was similar to that at 5 min of incubation, however, it returned to the basal level at 15 min of incubation (data not shown).

4. Discussion

We have shown here that LPC generates $O_2^{\bullet-}$ in isolated human neutrophils through PI3 kinase activation, but not

through PKC activation. It is well known that phospholipase A2 is secreted in inflammatory tissues, and catalyzes PC into LPC, resulting in the local accumulation of LPC [8,9]. Therefore, LPC could be utilized by neutrophils recruited into the inflammatory tissues for generating $O_2^{\bullet-}$ to kill living microorganisms. Although LPC is not the only factor which generates $O_2^{\bullet-}$ in neutrophils, it is conceivable that LPC contributes to host defense, at least in part, with this activity.

We demonstrate that LPC activates PI3 kinase in neutrophils by showing that PI3 kinase in neutrophils incubated with LPC catalyzed more PI3P from PI than the control (Fig. 4). In neutrophils, the fMLP-induced PI3 kinase activation occurs and reaches the maximal level within 30 s after stimulation and then returns to the almost basal level at 4 min of incubation [5]. However, fMLP-induced $O_2^{\bullet-}$ generation is seen at 15 min [5]. Similarly, we showed here that LPC activated PI3 kinase for 5–10 min (data not shown) and generated $O_2^{\bullet-}$ for 30–90 min (Fig. 1A). An atherogenic lipoprotein, oxidized low density lipoprotein (LDL), has also been shown to stimulate PI3 kinase in macrophages for 5–15 min [21] and generate $O_2^{\bullet-}$ for 10–40 min [22]. Thus, transient PI3 kinase activation is followd by $O_2^{\bullet-}$ generation. Since it has been shown that $O_2^{\bullet-}$ generation is efficiently reconstituted in a cell free system which is not affected by a PI3 kinase inhibitor [7,23]; PI3 kinase seems to be an upstream regulator of the $O_2^{\bullet-}$-generating machinery in the cells. Furthermore, since LPC is a markedly increased component in oxidized LDL compared with that in LDL, the effect of oxidized LDL on the stimulation of PI3 kinase and the generation of $O_2^{\bullet-}$ might be due to LPC.

As for the signaling pathway of $O_2^{\bullet-}$ generation in neutrophils, at least two factors, namely PI3 kinase and PKC, have been demonstrated to be involved so far [3–7]. $O_2^{\bullet-}$ generation induced by direct stimulation of PKC by PMA was not inhibited with PI3 kinase inhibitors (Fig. 2B) [7], while $O_2^{\bullet-}$ generation induced by fMLP was inhibited with PI3 kinase inhibitors as well as PKC inhibitors (Fig. 2C, Fig. 3) [4–7]. Therefore, PI3 kinase has been suggested to be upstream of PKC in the signaling pathway [7]. However, our present results, summarized in Table 1, show that the LPC's activity is mediated through PI3 kinase activation, but not through PKC activation. This demonstrates that PI3 kinase is not upstream of PKC in the LPC-mediated $O_2^{\bullet-}$ generation in neutrophils, which is different from $O_2^{\bullet-}$ generation mediated by fMLP, the best characterized generator of $O_2^{\bullet-}$.

In the case of vascular smooth muscle cells, LPC has been shown to generate $O_2^{\bullet-}$ through PKC activation [19] and the activity might be mediated by calcium ion influx caused by formation of small holes in the plasma membrane [20]. However, it is unlikely in this case since no calcium ions were added throughout the experimental procedure for $O_2^{\bullet-}$ generation. Moreover, exogenously added divalent cation chelator EDTA into the reaction mixture did not inhibit the generation (Fig. 3, lane 3), suggesting that calcium ion influx is not involved in the LPC-induced $O_2^{\bullet-}$ generation in neutrophils.

In summary, we have shown here that LPC activates PI3 kinase in human neutrophils, resulting in $O_2^{\bullet-}$ generation. The signaling mechanism of LPC seems unique since LPC-mediated $O_2^{\bullet-}$ generation is through PI3 kinase activation, but not through PKC activation, whereas those of well characterized $O_2^{\bullet-}$ generators, PMA and fMLP, are through PKC activation.

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