A Phosphatidylinositol Species Acutely Generated by Activated Macrophages Regulates Innate Immune Responses

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Activation of macrophages with stimuli of the innate immune response results in the intense remodeling of arachidonate-containing phospholipids, leading to the mobilization of large quantities of this fatty acid for conversion into biologically active eicosanoids. As a consequence of this process, the arachidonate levels in membrane phospholipids markedly decrease. We have applied mass spectrometry–based lipid profiling to study the levels of arachidonate-containing phospholipids under inflammatory activation of macrophages. We identify an unusual inositol phospholipid molecule, PI(20:4/20:4), the levels of which do not decrease but actually increase by 300% after activation of the macrophages. PI(20:4/20:4) is formed and degraded rapidly, suggesting a role for this molecule in regulating cell signaling events. Using a metabolipidomic approach consisting in exposing the cells to deuterium-labeled arachidonate at the time they are exposed to stimuli, we show that PI(20:4/20:4) biosynthesis occurs via the sequential incorporation of arachidonate, first into the *sn*-2 position of a preformed phosphatidylinositol (PI) molecule, followed by the rapid introduction of a second arachidonate moiety into the *sn*-1 position. Generation requires the participation of cytosolic phospholipase $A_2\alpha$ and CoA-dependent acyltransferases. PI(20:4/20:4) formation is also detected in vivo in murine peritonitis exudates. Elevating the intracellular concentration of PI(20:4/20:4) by introducing the lipid into the cells results in enhancement of the microbicidal capacity of macrophages, as measured by reactive oxygen metabolite production and lysozyme release. These findings suggest that PI(20:4/20:4) is a novel bioactive inositol phospholipid molecule that regulates innate immune responses in macrophages. ***The Journal of Immunology*****, 2013, 190: 5169–5177.**

The mobilization of arachidonic acid (20:4) from membrane phospholipids is one of the earliest events that occur after stimulation of macrophages with innate stimuli such as the phagocytosable particle zymosan (1–3). Zymosan is a cell–wall preparation of *Saccharomyces cerevisiae* that is widely used as a model stimulus for macrophage activation (1–6). Zymosan is composed of β-glucan, mannans, mannoproteins, and chitin and is recognized by multiple macrophage receptors, including dectin-1, TLR2, complement receptor 3, mannose receptors, and scavenger receptors (7, 8).

Part of the 20:4 liberated in response to zymosan and other stimuli is oxygenated by cyclooxygenase and lipoxygenase pathways to generate a number of biologically active eicosanoids, whereas the rest is effectively incorporated back into phospholipids by the action of CoA-dependent acyltransferases (9, 10). There is now solid evidence that activation of intracellular phospholipase A_2 (PLA_2) enzymes, in particular the calcium-dependent cytosolic group IV PLA_2 ($cPLA_2\alpha$), provides the major route for effecting the 20:4 release (9–12) and that the major zymosan receptor involved in this process is dectin-1 (13–15).

$cPLA_2\alpha$ has been found to translocate to the phagosomes after stimulation of the phagocytes, and such a translocation appears to be key for eicosanoid generation and killing of the ingested microbe (16–18). The enzyme is regulated posttranslationally by increases in intracellular calcium levels and by phosphorylation (10). The intracellular calcium level regulates the ability of $cPLA_2\alpha$ to associate to cellular membranes, which is mediated by a C2 domain present at the N-terminal half of the protein. The C2 domain also contains a site for binding to ceramide-1-phosphate, which allosterically activates the enzyme and increases the residence time of the enzyme in membranes (19). The enzyme also possesses a binding site for anionic phospholipids, particularly phosphatidylinositol (PI) 4,5-bisphosphate, that may also be involved in anchoring the $cPLA_2\alpha$ to cellular membranes (20–22). Phosphorylation of $cPLA_2\alpha$ is known to be effected by members of the MAPK family of enzymes (i.e., the ERKs p42/p44, p38, and JNK), although the specific form involved appears to strikingly depend on cell type and stimulus (10). In murine macrophages, the kinases responsible for $cPLA_2\alpha$ phosphorylation/activation have been found to be p42/p44 (6, 23).

Studies on 20:4 mobilization under activation conditions traditionally have relied on the use of cells or tissues prelabeled with radioactive fatty acid and measurement of radioactivity levels in different fractions after addition of the stimulant. Although these types of studies have been very useful to delineate biochemical pathways and enzymes involved, they were not devoid of complications derived from lack of homogeneous labeling among phospholipid pools and impossibility to detect specific molecular

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Abbreviations used in this article: 20:4, arachidonic acid; $cPLA_2\alpha$, cytosolic $PLA_2\alpha$ (group IVA PLA_2); LC, liquid chromatography; MS, mass spectrometry; PAF, platelet-activating factor; PC, choline phospholipid; PE, ethanolamine phospholipid; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PS, phosphatidylserine; qPCR, quantitative PCR.

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species (9, 24, 25). The development of liquid chromatography/mass spectrometry (LC/MS)–based techniques to the study of lipids has allowed these shortcomings to be solved, because lipids in samples, even those present at relatively low concentrations, can be efficiently separated and identified with great sensitivity (9, 24–28).

Our laboratory has been applying LC/MS phospholipid profiling to the study of the metabolism of 20:4-containing phospholipids during cell activation (9, 29–34). Our goal has been to define the molecular sources for 20:4 liberation and incorporation and to relate this information with the activation of particular phospholipase forms by stimuli of the innate immune response. In this study, we have extended the use of LC/MS to characterize 20:4-containing phospholipids that increase their levels during zymosan phagocytosis in macrophages. We show the stimulated formation of an uncommon PI molecule, PI(20:4/20:4), and delineate the pathway of biosynthesis as well as its biological role in regulating innate immune responses.

Materials and Methods

Reagents

Cell culture medium was from Molecular Probes–Invitrogen (Carlsbad, CA). Chloroform and methanol (HPLC grade) were from Fisher Scientific (Hampton, NH). PI([^{2}H]20:4/20:4) and PI(20:4/[^{2}H]20:4) were purchased from Cayman Chemical (Ann Arbor, MI). Lipid standards were from Avanti Polar Lipids (Alabaster, AL) or Larodan Fine Chemicals (Malmo, Sweden). Triacsin C was from Enzo Life Sciences (Farmingdale, NY). The cPLA$_2\alpha$ inhibitor pyrrophenone was synthesized and provided by Dr. A. Llebaria (Institute for Chemical and Environmental Research, Barcelona, Spain). All other reagents were from Sigma-Aldrich.

Cell culture

Resident peritoneal macrophages from Swiss male mice (University of Valladolid Animal House, 10–12 wk old) were obtained by peritoneal lavage using 5 ml cold PBS, as described elsewhere (4, 5). The cells were plated at 2×10^6/well (6-well plates) in 2 ml RPMI 1640 medium with 10% heat-inactivated serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and allowed to adhere for 20 h in a humidified atmosphere of 5% CO_2 at 37°C. Wells were then extensively washed with PBS to remove nonadherent cells. Adherent macrophages were then used for experimentation. When inhibitors were used, they were added to the incubation media 30 min before stimulating the cells with zymosan. For inducing peritonitis, mice were injected i.p. with 1 mg zymosan in 500 μl sterile saline. Control mice received vehicle only. After the indicated times, mice were euthanized in a CO_2 chamber, and peritoneal exudates were collected by lavaging with 5 ml sterile saline. Exudate cells were obtained by centrifugation. All procedures involving animals were undertaken in accordance with the Spanish National Committee on Biosafety and Animal Care, under the guidelines established by the Spanish Ministry of Agriculture, Food, and Environment, and the European Union.

Preparation of zymosan

Zymosan was prepared as described elsewhere (35). Briefly, zymosan particles were suspended in PBS, boiled for 60 min, and washed three times. The final pellet was resuspended in PBS at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. For preparation of opsonized zymosan, zymosan particles, treated as indicated above, were incubated with fresh mouse serum (10 mg zymosan/ml serum) for 20 min at 37°C and washed three times with PBS before use. No endogenous PLA_2 activity was detected in the zymosan batches used in this study, as assessed by in vitro assay under a variety of conditions (36–39).

LC/MS analysis of 20:4-containing phospholipids

A cell extract corresponding to 10^7 cells was used for these analyses. The following internal standards were added: 600 pmol each of 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine, before lipid extraction according to the method of Bligh and Dyer (40). After evaporation of organic solvent under vacuum, the lipids were redissolved in 100 μl methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a Supelcosil LC-18 (5-μm particle size, 250 × 2.1 mm) (Sigma-Aldrich) protected with a Supelguard LC-18 (20 × 2.1 mm) guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (methanol/water/*n*-hexane/32% ammonium hydroxide, 87.5:10.5:1.5:0.5, v/v/v/v) and solvent B (methanol/*n*-hexane/32% ammonium hydroxide, 87.5:12:0.5, v/v/v). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A, 35% solvent B in 20 min, to 10% solvent A, 90% solvent B in 5 min, and to 0% solvent A, 100% solvent B in an additional 5 min. Flow rate was 0.5 ml/min, and 80 μl of the lipid extract was injected. The LC system was coupled online to a Bruker esquire6000 ion-trap MS (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas to 8 l/min, and dry temperature to 365°C. Ethanolamine phospholipid (PE), PI, and phosphatidylserine (PS) were detected in negative ion mode with the capillary current set at +3500 V over the initial 25 min as $[M-H]^-$ ions. Choline phospholipid (PC) species were detected over the elution interval from 25 to 35 min in positive ion mode, as $[M+H]^+$ ions, with the capillary current set at −3500 V.

20:4-containing PE, PI, and PS species were identified by multiple reaction monitoring MS/MS experiments on chromatographic effluent by comparison with previously published data (29–34). Cutoff parameter was set at *m/z* 150 and fragmentation amplitude at 1 arbitrary unit. Because of the lability of vinyl ether linkages in acid media, plasmanyl (1-alkyl) and plasmenyl (1-alk-1′-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction. For the identification of acyl chains of 20:4-containing PC species, ionization was carried out in negative mode with post-column addition of acetic acid at a flow rate of 100 μl/h as $[M+CH_3CO_2]^-$ adducts, and acyl chains were identified by MS^3 experiments. Stereospecific assignation of fatty acyl chains was carried out by comparing the relative intensities of the 1-lysophospholipid and 2-lysophospholipid compounds arising in the fragmentation experiments (the signal of the latter predominates over that of the former in ion-trap MS) (29–34).

Incorporation of PI(20:4/20:4) into cells

Two micrograms of phospholipid was mixed with 2 μl carrier (histone H1, 0.5 mM), resuspended in 200 μl HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$, sonicated in a water bath for 2 min, and allowed to rest at 37°C for 10 min before use. Final concentration of PI(20:4/20:4) in the solution was 10 μM.

Quantitative PCR

Quantitative PCR (qPCR) was carried out with an ABI 7500 machine (Applied Biosystems, Carlsbad, CA) using the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA) and specific primers for each gene of interest. Cycling conditions were 1 cycle at 95°C for 3 min and 40 cycles at 95°C for 12 s, 60°C for 15 s, and 72°C for 28 s. The relative mRNA abundance for a given gene was calculated using the algorithm $2^{-\Delta\Delta Ct}$, with β-actin and cyclophilin A as internal standards (41).

Superoxide anion production

The production of superoxide anion was measured by the superoxide dismutase–inhibitable reduction of ferricytochrome *c* in a discontinuous assay. A total of 2×10^6 cells were preincubated for 5 min at 37°C with cytochalasin B (5 μg/ml) in a final volume of 1 ml of a glucose-containing buffer (10 mM HEPES, 150 mM NaCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 5.5 mM glucose [pH 7.5]) containing 0.9 mg/ml cytochrome *c* in the presence or absence of 10 μg/ml superoxide dismutase. Then, the appropriate stimulus was added, and the reaction proceeded for 1 h at 37°C. When PI(20:4/20:4) was used, it was added 30 min before the addition of the stimulus. Afterward, samples were centrifuged to remove cells, and the supernatants were collected. The extent of cytochrome *c* reduction was measured at 550 nm. The difference in absorbance between superoxide dismutase-treated and -untreated samples was a measure of the amount of reduced cytochrome *c*. Controls with superoxide dismutase (10 μg/ml) were run in parallel to ensure that cytochrome *c* reduction was due only to the superoxide anion generated during cell stimulation.

Lysozyme release assay

Lysozyme content in cell supernatants was determined by measuring the rate of clearance of a suspension of *Micrococcus lysodeikticus*, as described elsewhere (42). Briefly, the cells (2×10^6 cells/ml) were placed in a glucose-containing buffer (10 mM HEPES, 150 mM NaCl, 1.2 mM $MgCl_2$,

1.3 mM $CaCl_2$, and 5.5 mM glucose [pH 7.5]). Then, the appropriate stimulus was added, and the reaction proceeded for 1 h at 37°C. When PI(20:4/20:4) was used, it was added 30 min before the addition of the stimulus. Afterward, the supernatants were collected, and 1 ml was mixed with 1 ml of a *M. lysodeikticus* suspension (0.3 mg/ml in 0.1 M sodium phosphate buffer [pH 7]). The decrease in absorbance at 450 nm was measured at room temperature. A calibration curve was constructed using chicken egg white lysozyme as a standard and run in parallel with the supernatant samples.

Results

Profiling of 20:4-containing phospholipids in activated macrophages

The profile of 20:4-containing glycerophospholipid species of murine peritoneal macrophages was measured by LC/ion-trap MS. The lipids were unequivocally identified by determining the production of an *m/z* 303 fragment in MS^n experiments, corresponding to 20:4. Structural identification of the glycerophospholipids was achieved by looking at the fragments and/or neutral losses obtained in MS^2 experiments for PE, PI, and PS or MS^3 experiments for PC (29–34). A total of 23 different phospholipid species were detected (Fig. 1A). Fatty chains within phospholipids are designated by their number of carbons:double bonds. A designation of O- before the first fatty chain indicates that the *sn-1* position is ether-linked, whereas a P- designation indicates a plasmalogen form (*sn-1* vinyl ether linkage) (43). The major 20:4-containing species of the peritoneal macrophages were in this order: PE(P-16:0/20:4), PC(16:0/20:4), PE(18:0/20:4), PC(18:0/20:4), PE(P-18:0/20:4), PI(18:0/20:4), PS(18:0/20:4), and PE(P-18:1/20:4). Regarding classes, PE was the richest 20:4-containing class (48% of total cellular fatty acid), followed by PC (36%), PI (9%), and PS (7%) (Fig. 1B).

Stimulation of the macrophages with zymosan resulted in a significant diminution of the total cellular content of 20:4 in phospholipids. Analyses of the species that lost 20:4 after stimulation revealed that many but not all phospholipid species contributed to this release (Fig. 1C, 1D). Most PC species and the species PI(18:0/20:4) experienced similarly strong decreases, whereas PS species decreased to a much lesser extent. Conversely, no PE species showed a significant decrease (Fig. 1C, 1D). Interestingly, however, two minor 20:4-containing species not only did not decrease or remained unchanged but also actually increased after zymosan stimulation. These were two diarachidonoylated species, PC(20:4/20:4) and, most prominently, PI(20:4/20:4).

Identification of the pathway involved in PI(20:4/20:4) production

Although cellular increases in PC(20:4/20:4) were described some 25 y ago (44), the increase in PI(20:4/20:4) after cell activation is a novel and striking finding, which prompted us to characterize it in detail. The time course of production of PI(20:4/20:4) shows that PI(20:4/20:4) proceeded linearly up to 1 h after zymosan stimulation, decreasing afterward, and remaining elevated over basal even after 2 h stimulation with zymosan (Fig. 2A).

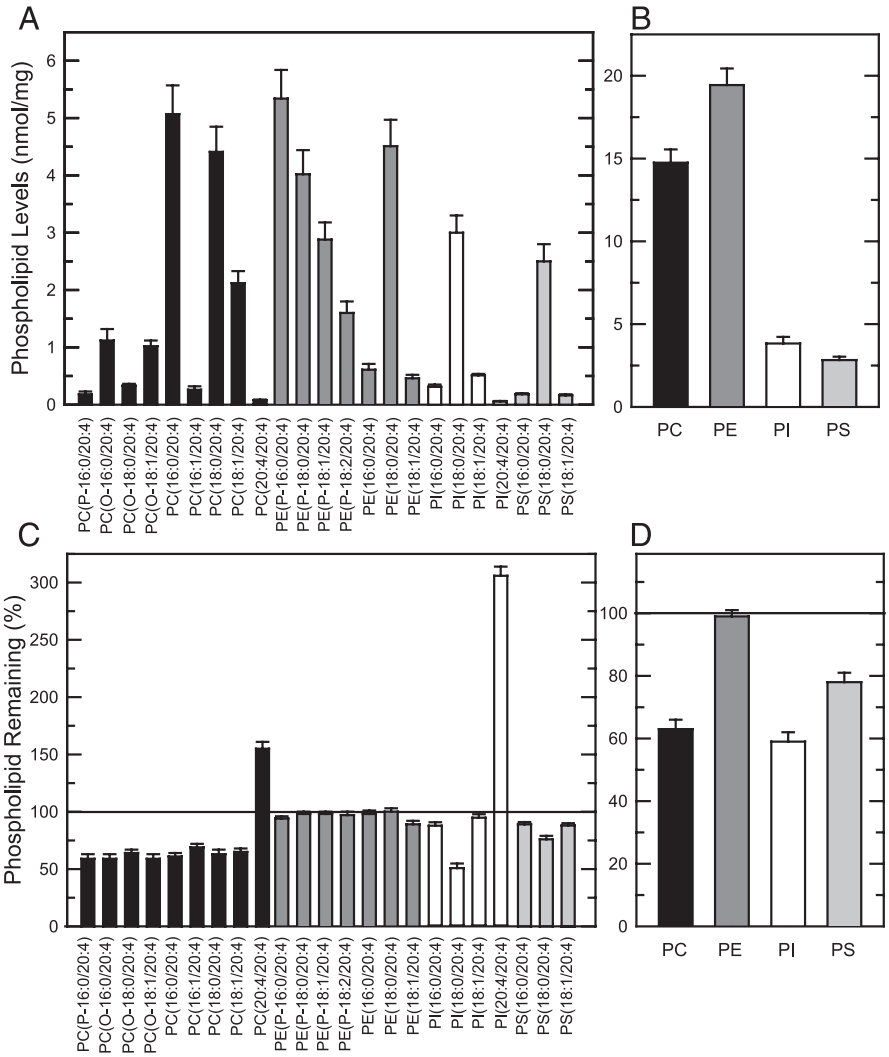

FIGURE 1. 20:4-containing phospholipid species in murine peritoneal macrophages and the effect of zymosan stimulation. (**A**) The profile of 20:4-containing PC (black), PE (dark gray), PI (white), and PS (gray) species in unstimulated cells was determined by LC/MS. (**B**) 20:4 content in phospholipids as shown by class. (**C**) 20:4 remaining in phospholipid species after a 1-h stimulation with 1 mg/ml zymosan. The 100% corresponds to the levels of each species, as shown in (**A**). (**D**) 20:4 remaining in phospholipids after a 1-h stimulation with 1 mg/ml zymosan as shown by class. Data are shown as means ± SEM of five independent determinations.

To study the route of synthesis of PI(20:4/20:4) during cellular stimulation, a metabolipidomic experiment was designed where the cells were pulsed with a low [^{2}H]20:4 concentration at the time they were stimulated with zymosan. Analysis by LC/MS of deuterium-containing phospholipids formed after 30 min demonstrated the stimulated presence of PI([^{2}H]20:4/[^{2}H]20:4), along with the following species: PC(16:0/[^{2}H]20:4), PC(18:0/[^{2}H]20:4), PC(18:1/[^{2}H]20:4), PI(18:0/[^{2}H]20:4), PI(16:0/[^{2}H]20:4), and PC([^{2}H]20:4/[^{2}H]20:4). Interestingly, a PI(20:4/20:4) molecule containing deuterium in only one 20:4 chain was also found (*m/z* 913.6; Fig. 2B). This species was unequivocally identified as PI([^{2}H]20:4/20:4) in MS/MS experiments by comparison with an authentic standard. The PI species containing [^{2}H]20:4 at the *sn-2* position but not at the *sn-1* position (PI(20:4/[^{2}H]20:4)) was not detected, and there was no other phospholipid containing deuterium only at the *sn-1* position. No deuterium-containing lysophospholipids were detected either. Taken together, these findings suggest that PI(20:4/20:4) is formed via fatty acid exchange at both the *sn-2* and *sn*-1 positions through direct deacylation/reacylation reactions (i.e., the Lands cycle).

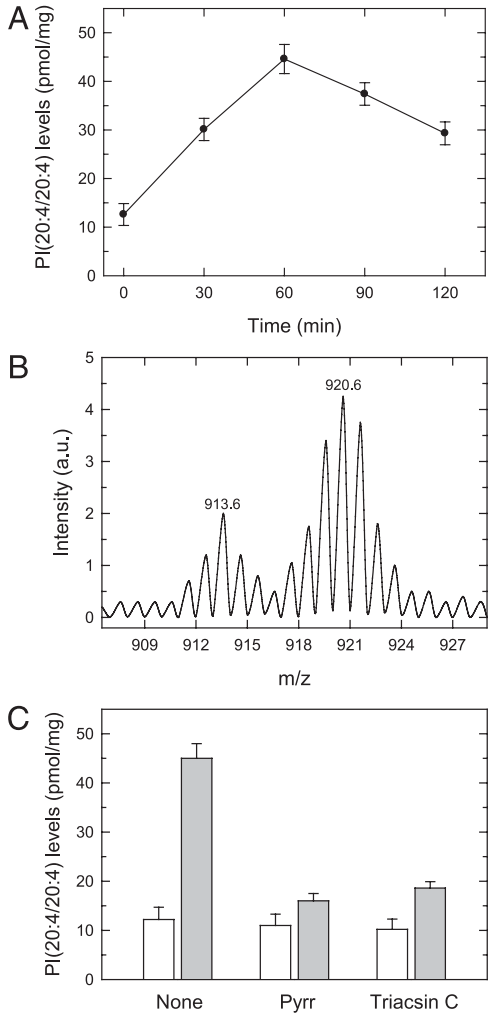

FIGURE 2. Stimulated production of PI(20:4/20:4) in murine macrophages. (**A**) Time course of PI(20:4/20:4) formation after zymosan stimulation. The cells were activated with 1 mg/ml zymosan for different times. After lipid extraction, PI(20:4/20:4) levels were determined by LC/MS. (**B**) Detection of deuterium-labeled PI(20:4/20:4) in macrophages. The cells were exposed to 1 μM [^{2}H]20:4 at the time they were stimulated with 1 mg/ml zymosan for 1 h. The figure shows the isotopic distribution of the two deuterium-labeled PI(20:4/20:4) species detected by LC/MS, namely PI([^{2}H]20:4/20:4) (set of peaks with a maximum at *m/z* 913.6) and PI([^{2}H]20:4/[^{2}H]20:4) (set of peaks with a maximum at *m/z* 920.6). (**C**) Generation of PI(20:4/20:4) after zymosan stimulation requires cPLA$_2$α- and CoA-dependent acyl transferases. The cells were treated with 1 μM pyrrophenone (Pyrr), 3 μM triacsin C, or neither (none), as indicated for 30 min. Afterward, the cells were stimulated (gray bars) or not (open bars) with 1 mg/ml zymosan for 60 min. Data are given as means ± SEM of three independent experiments.

In the Lands cycle, fatty acids at the *sn-2* position of phospholipids are cleaved by PLA$_2$ and replaced with 20:4 by the sequential action of arachidonoyl–CoA synthetase and lysophospholipid: acyl-CoA acyltransferase (10, 45). Hence, if this is the route for PI(20:4/20:4) synthesis by the zymosan-activated macrophages, production of this phospholipid should be sensitive to inhibitors of these enzymes. Fig. 2C shows that the increase in PI(20:4/20:4) levels in the activated cells was almost completely prevented when the cells were pretreated with either pyrrophenone or triacsin C, two potent and selective inhibitors of the arachidonate-releasing cPLA$_2$α and arachidonoyl–CoA synthetase, respectively. Previous data from our laboratory has demonstrated that, at the concentrations used, these inhibitors effectively block the aforementioned enzyme activities while exerting no significant effect on other PLA$_2$ or acyl–CoA synthetases (30, 34, 46, 47).

Factors regulating PI(20:4/20:4) production by macrophages

Fig. 3A shows that in addition to zymosan, other widely used macrophage stimuli also produced significant amounts of PI(20:4/20:4). Opsonized zymosan and the calcium ionophore A23187 were as potent as zymosan in inducing PI(20:4/20:4) formation. Bacterial LPS, platelet-activating factor (PAF), and PMA behaved as weak inducers of PI(20:4/20:4) formation, and ATP had no effect. Priming of the cells with LPS before stimulating the cells with the aforementioned agonists had no effect on PI(20:4/20:4) formation beyond what was already achieved with LPS alone. This is a striking finding, because stimulus-mediated cPLA$_2$α activation and subsequent 20:4 mobilization in macrophages—an event that is necessary for PI(20:4/20:4) formation, as shown in Fig. 2C—was found to be noticeably increased by LPS priming (Fig. 3B–E), in agreement with previous reports (48). Unlike free 20:4, PI(20:4/20:4) was totally retained by the cells after the zymosan stimulation (Fig. 3F).

To study whether the accumulation of PI(20:4/20:4) correlates with the intensity of 20:4 mobilization, the production of PI(20:4/20:4) was compared with that of free 20:4 in macrophages treated with different stimuli, including a combination of PMA and A23187, which is known to synergistically activate cPLA$_2$α and attendant 20:4 mobilization (4, 6, 23). The data are shown in Fig. 3G and indicate that, in general, the stimuli that mobilized more 20:4 also produced more PI(20:4/20:4). However, the combination of PMA plus A23187 induced three to four times more free 20:4 than zymosan or A23187 alone, but the amounts of PI(20:4/20:4) produced under these conditions were all comparable. PAF and PMA promoted little 20:4 release but induced significant accumulation of PI(20:4/20:4) compared with control. (Fig. 3G).

PI(20:4/20:4) formation during murine peritonitis

To study in vivo generation of PI(20:4/20:4), a well-characterized murine model of zymosan-induced peritonitis was used (49). Zymosan was administered i.p., peritoneal cells were collected at various time points, and PI(20:4/20:4) formation was measured. PI(20:4/20:4) levels increased linearly during the first hour, decreasing rapidly afterward (Fig. 4). The time course of PI(20:4/20:4) formation is remarkably similar to that previously observed when isolated macrophages were used (Fig. 2A). Fig. 4 also shows the levels of the major 20:4-containing PI species, PI(18:0/20:4),

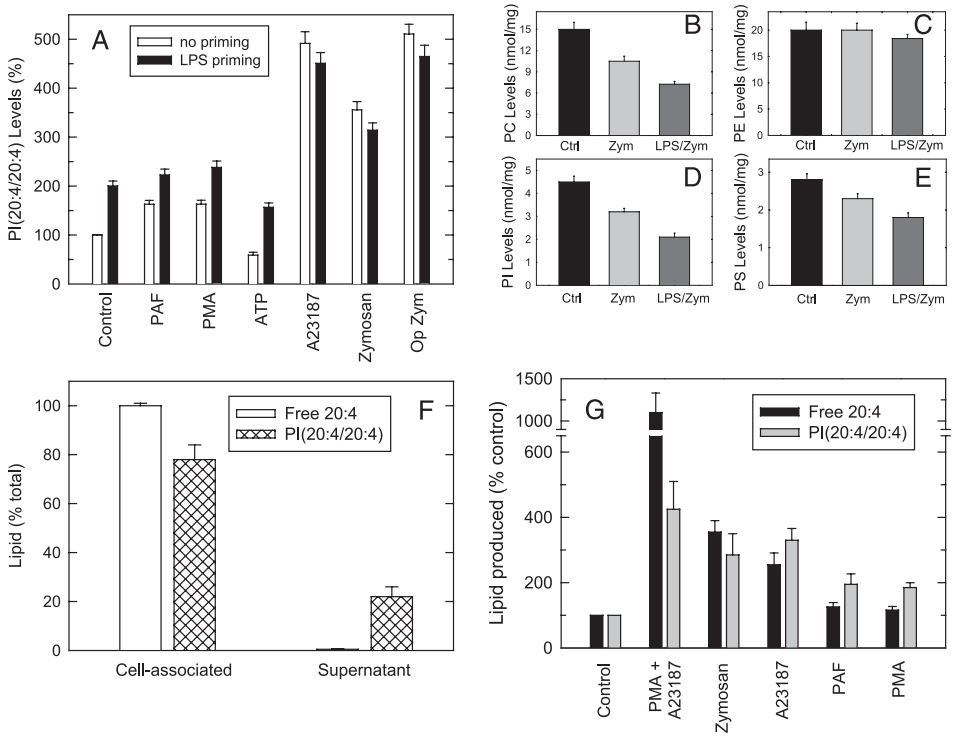

FIGURE 3. Generation of PI(20:4/20:4) in response to stimuli. (**A**) The macrophages were treated with the stimuli listed for 1 h at the following concentrations: 100 nM PAF, 100 ng/ml PMA, 100 μM ATP, 1 μM calcium ionophore A23187, 1 mg/ml zymosan, or 1 mg/ml opsonized zymosan (OpZym) (open bars). In parallel experiments, the cells were pretreated with 100 ng/ml LPS for 60 min before addition the aforementioned stimuli at the indicated concentrations (closed bars). (**B**–**E**) LPS priming enhances zymosan-stimulated 20:4 mobilization from macrophages. The cells were either untreated (Ctrl) or treated with 1 mg/ml zymosan (Zym) for 1 h, or LPS plus zymosan (1 h with 100 ng/ml LPS, followed by 1 h with 1 mg/ml zymosan). Afterward, the cellular content of 20:4-containing PC (B), 20:4-containing PE (C), 20:4-containing PI (D), or 20:4-containing PS (E) was determined by LC/MS. (**F**) PI(20:4/20:4) is associated with cells and not released after cell activation. The cells were stimulated with 1 mg/ml zymosan for 1 h. Afterward, total content of PI(20:4/20:4) (open bars) and 20:4 (stripped bars) in cells or supernatants was determined by LC/MS or gas chromatography (GC)/MS, respectively. (**G**) Comparison between the amounts of 20:4 and PI(20:4/20:4) produced by macrophages responding to various stimuli. The cells were treated with the stimuli listed at the following concentrations: 100 nM PAF, 100 ng/ml PMA, 1 μM A23187, 1 mg/ml zymosan, or 100 ng/ml PMA plus 1 μM A23187 for 60 min. Afterward, total content of 20:4 (black bars) and PI(20:4/20:4) (gray bars) was determined by GC/MS or LC/MS, respectively. Data are given as means ± SEM of three independent experiments.

which decreased with time, likely as a consequence of cPLA$_2$α activation.

Studies adding exogenous PI(20:4/20:4) to the cells

The kinetics of formation of PI(20:4/20:4), both in vitro and in vivo, showing a rapid increase followed by a decrease is consistent with this molecule acting as an intracellular signaler. To study this possibility, PI(20:4/20:4) was introduced into the cells by complexing it with cationic carriers that counteract the negative charges of the phospholipid and make it permeable to the cell membrane (22, 50). Using this procedure, we found that 35 ± 6% of the added phospholipid was incorporated into the cells during a 30-min incubation period (mean ± SEM, n = 3). Interestingly, cells metabolized the incorporated PI(20:4/20:4) rapidly, degrading ~70 ± 10% of the phospholipid within 1 h (mean ± SEM, n = 3). This is an important observation because it shows that exogenously added PI(20:4/20:4) behaves similar to that formed intracellularly (Fig. 2A), which in turn suggests that the addition of exogenous PI(20:4/20:4) appropriately mirrors a physiologically relevant condition.

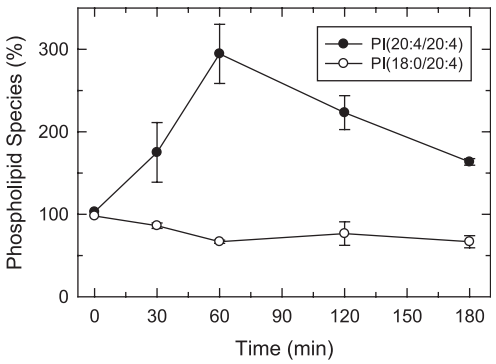

FIGURE 4. PI(20:4/20:4) is formed in vivo during murine peritonitis. Zymosan (1 mg in 500 μl sterile PBS) or control vehicle was injected i.p. to the mice. Peritoneal lavage was collected at different times, and the cells were isolated by centrifugation. After lipid extraction, PI(20:4/20:4) and PI(18:0/20:4) levels were determined by LC/MS. Data are given as means ± SE of three independent experiments, each using two different animals per condition.

To investigate the metabolism of PI(20:4/20:4) by the macrophages, we used phospholipids labeled with deuterium in the 20:4 moiety at either the *sn-1* or *sn-2* positions, namely PI([^{2}H]20:4/20:4) and PI(20:4/[^{2}H]20:4). LC/MS analyses of deuterium-containing lipids at different times after exposure of the cells to these deuterium-labeled PI(20:4/20:4) molecules revealed no significant incorporation of label into any other cellular phospholipid species, thus suggesting that PI(20:4/20:4) is not an intermediate in the formation and/or replenishment of membrane phospholipid pools. Of note, no [^{2}H]20:4-labeled lysoPI was detected under any condition tested, indicating that the loss of the two 20:4 moieties from PI(20:4/20:4) occurs either simultaneously or sequentially in such a rapid manner that a 20:4-containing lysoPI intermediate

does not accumulate. It is worth noting in this regard that the two major intracellular PLA_2 present in cells (i.e., $cPLA_2\alpha$ and group VIA PLA_2) possess significant lysophospholipase activity (51), and thus, both enzymes could degrade PI(20:4/20:4) without generating a lyso intermediate.

In contrast to the lack of deuterium label in phospholipid species, abundant label was detected in neutral lipids (triacylglycerol) when the cells were incubated with deuterium-labeled PI(20:4/20:4) at either the *sn-1* or *sn-2* positions. This is consistent with the notion that macrophages, as primary cells, have their 20:4 phospholipid pools replete, and thus, any excess of this fatty acid is stored in neutral lipids (10, 45).

Cellular role of PI(20:4/20:4)

Previous results have suggested that the PC analog of PI(20:4/20:4) (i.e., PC(20:4/20:4)) may serve a metabolic intermediary role by constituting a rapid reacylation pool for free 20:4 once the level of this fatty acid raises within the cell (44, 45). Formation of PC(20:4/20:4) under these conditions occurs primarily via the de novo pathway of lipid synthesis (44, 45). To investigate whether this can also be a metabolic fate for PI(20:4/20:4), experiments were carried out in which macrophages were incubated with a high free 20:4 concentration (20 μM, 10 min). The data indicated that PI(20:4/20:4) was indeed formed under these conditions as was, expectedly, PC(20:4/20:4). Confirming that production of these diarachidonoylated phospholipids most likely arises from the de novo pathway, the intermediates diarachidonoyl phosphatidic acid and diarachidonoyl glycerol were also readily detected under these conditions (data not shown).

Previous results indicated that PC(20:4/20:4) may provide the 20:4 moiety used for anandamide synthesis in rat brain (52). To investigate whether PI(20:4/20:4) may serve a similar role, we used phospholipids labeled with deuterium in the 20:4 moiety at either the *sn-1* or *sn-2* positions. After the phospholipid was introduced into the cells, they were stimulated with zymosan for various periods of time, and anandamide synthesis was determined by LC/MS under the conditions described by Dennis and coworkers (53). We failed to detect deuterium-labeled anandamide at any condition tested up to 2-h incubation of the cells. Likewise, no water-soluble deuterium-containing eicosanoid-like material could be identified by LC/MS. Collectively, these results indicate that, unlike PC(20:4/20:4), PI(20:4/20:4) does not provide 20:4 moieties for the synthesis of anandamide or other bioactive lipid compounds in murine macrophages.

Recent results have shown that certain phospholipid species such as PC(16:0/18:1) or PC(12:0/12:0) have the capacity to regulate gene induction by modulating the activity of transcription factors (54, 55). To evaluate whether PI(20:4/20:4) serves a similar biological function in macrophages, we studied by qPCR the possible effects of this phospholipid on the induction of a number of genes that are known to be involved in inflammation. The genes tested were *Arg1*, *Ccl2*, *Cox2*, *Fizz1*, *Il1b*, *Il6*, *Il10*, *Il12a*, *Il12b*, *Il23*, *Mrc1*, *Mrc2*, *Nos2*, *Tgfb*, *Tnfa*, and *Ym1*. Of these, a strong upregulation of *Il10*, *Cox2*, *Il23*, and *Tnfa* was clearly detected in cells stimulated with zymosan, but addition of PI(20:4/20:4) to the cells failed to exert any effect, whether stimulatory or inhibitory, on the induction of all genes tested. Combinations of the phospholipid with zymosan did not produce any effect beyond what was already observed with zymosan alone (Fig. 5).

The inability of PI(20:4/20:4) to regulate long-term responses such as gene transcription in macrophages prompted us to investigate possible effects of the phospholipid on immediate responses. Among those, generation of reactive oxygen species such as superoxide anion and hydrogen peroxide is a critical response of macrophages for pathogen killing and maintaining efficient host defense (56). The effect of PI(20:4/20:4) on superoxide anion production by the macrophages is shown in Fig. 6A. There was a tendency for PI(20:4/20:4) to induce superoxide anion when added alone to the cells, but it did not reach statistical significance. However, PI(20:4/20:4) did significantly enhance the responses to both PMA and zymosan. In these experiments, PI(20:4/20:4) was added 30 min before either PMA or zymosan. As a control of these experiments, we used a PI extract from bovine liver (Sigma-Aldrich; consisting primarily of species PI(18:0/20:4), PI(18:0/20:3), PI(18:1/18:1), and PI(18:0/18:1)) as assessed by LC/MS. 20:4 comprised 20–25% of total fatty acids in this liver PI extract, and no PI(20:4/20:4) was found in it. Under the same conditions as those used for PI(20:4/20:4), liver PI had no effect on superoxide production under any condition tested, highlighting the specificity of action of PI(20:4/20:4) (Fig. 6A). When zymosan and PMA were added to the cells 2 h after addition of PI(20:4/20:4)—a time long enough to allow the cells to completely metabolize the phospholipid (vide supra)—the enhancing effect of PI(20:4/20:4) was no longer observed (data not shown).

To further delineate the effect of PI(20:4/20:4) on innate immune responses, we next measured lysozyme secretion by macrophages treated or not with the phospholipid. Lysozyme hydrolyzes a specific glycoside bond in the peptidoglycan that forms bacterial cell walls, thereby constituting a key component of innate immune defense against bacterial infection (57). Although PI(20:4/20:4) did not show a measurable effect on its own on lysozyme secretion, it did significantly enhance lysozyme secretion by activated

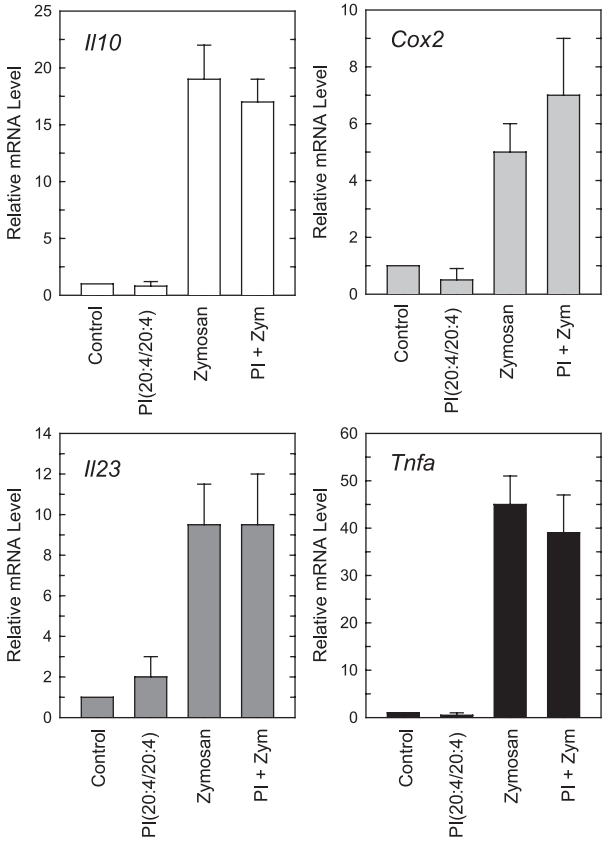

FIGURE 5. PI(20:4/20:4) does not regulate the expression of inflammatory genes in macrophages. The cells were treated with 10 μM PI(20:4/20:4), 1 mg/ml zymosan, both or neither (control) as indicated for 6 h. Afterward, the expression of *Il10*, *Cox2*, *Il23*, and *Tnfa* genes was studied by qPCR. Data are from an experiment with triplicate determinations (means ± SEM) that is representative of three independent experiments.

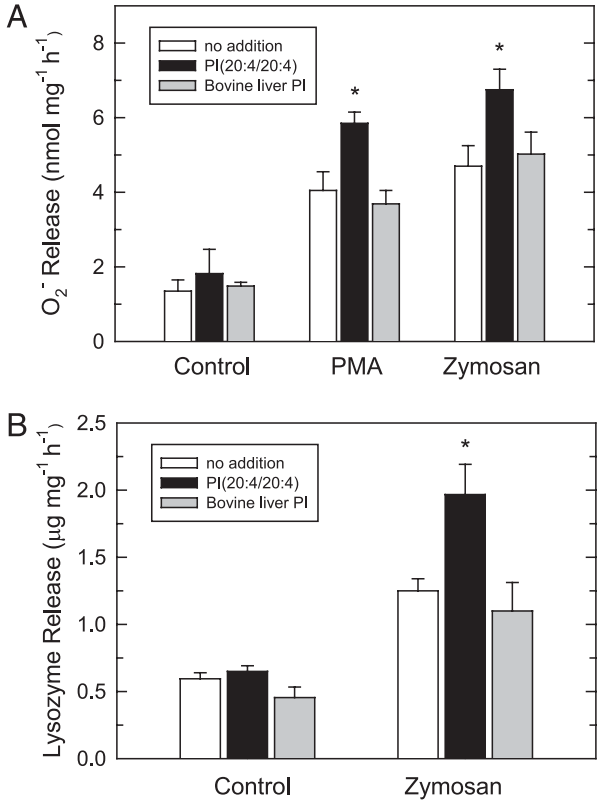

FIGURE 6. PI(20:4/20:4) regulates superoxide production (**A**) and lysozyme secretion (**B**) in macrophages. Superoxide anion and lysozyme were measured in control cells or cells treated with the stimuli listed (100 ng/ml PMA and 1 mg/ml zymosan) that had been incubated previously with PI(20:4/20:4) (closed bars), bovine liver PI extract (gray bars), or neither (open bars) for 30 min. Data are given as means ± SEM of three independent experiments. *$p < 0.05$, significantly different from their respective controls.

macrophages, and this effect was not mimicked by the liver PI extract used as a control (Fig. 6B).

Discussion

LC/MS provides a comprehensive frame for the analysis of changes in the composition of unique glycerophospholipid species present in cells (9, 25). In this study, we have applied this technology to study changes in the levels of all major 20:4-containing glycerophospholipids of murine macrophages responding to zymosan. Our results show that the major phospholipid species for 20:4 mobilization in zymosan-stimulated macrophages are PC (all major species) and PI (largely the species PI(18:0/20:4)), with little contribution from PS species and virtually no contribution from any PE species. It is interesting to note that, among the major contributors to 20:4 release, the percent hydrolysis was very similar; irrespective of their relative mass levels, all these species are consistently hydrolyzed by 50–55%. These findings are fully consistent with the notion that it is the presence of 20:4 at the *sn*-2 position of phospholipids and not the substituent and type of linkage (acyl-, alkyl- or alkenyl-) at the *sn*-1 position that determines their hydrolysis by cPLA$_2\alpha$ (45, 51).

The lack of contribution of any PE species, which collectively constitute the major reservoir of intracellular 20:4 in these cells, is striking. In this regard, it should be noted that in our experiments, the levels of 20:4-containing phospholipids are measured after zymosan challenge, which represents a balance between what is released by phospholipases minus what is reincorporated back into phospholipids by the highly active CoA-dependent acyltransferases present in these cells (10, 45). Thus, the lack of net release of 20:4 from PE species might not necessarily reflect that these phospholipids are not substrates for the release but rather that, once attacked by cPLA$_2\alpha$, they are rapidly reformed/replenished via the concerted action of CoA-dependent acyltransferases and CoA-independent transacylases.

Of all the 20:4-containing species measured, only two of them, namely PC(20:4/20:4) and PI(20:4/20:4), increased their levels after zymosan stimulation. The stimulated production of the two species was almost completely prevented by treating the cells with the cPLA$_2\alpha$ and arachidonoyl–CoA synthetase inhibitors pyrrophenone and triacsin C, suggesting that both compounds are formed via CoA-dependent reacylation reactions, in which the 20:4 acting as a donor arises from the cPLA$_2\alpha$-mediated hydrolysis of other 20:4-containing glycerophospholipids.

Although previous studies have focused on the formation and possible biological roles of PC(20:4/20:4) in cells (44, 52), with the exception of our own recent work identifying PI(20:4/20:4) as a minor 20:4-containing phospholipid of human monocytes (29, 30), to the best of our knowledge, we are not aware of previous studies dealing with this relatively uncommon species. By using a metabolipidomic approach consisting of stimulating the cells with zymosan in the presence of a low dose of [^{2}H]20:4 to "trap" possible intermediates, we studied in detail the route of biosynthesis of PI(20:4/20:4). Two main pathways exist for the incorporation of 20:4 in phospholipids. These are the de novo pathway for phospholipid biosynthesis and the Lands cycle of phospholipid deacylation/reacylation (10, 44). In the Lands cycle, a preformed phospholipid is acted upon by intracellular phospholipases of the A-type to generate a one- or two-lysophospholipid that can be reacylated with 20:4 by the action of CoA-dependent acyl transferases. A major difference between the de novo route and the Lands cycle is that the latter, but not the former, is sensitive to PLA$_2$ inhibitors. Thus, the results showing that PI(20:4/20:4) is strongly sensitive to inhibition of the signal-activated cPLA$_2\alpha$ by pyrrophenone are fully consistent with the Lands pathway being the major route of biosynthesis for this compound.

From our metabolipidomic experiments, further mechanistical considerations can be made, as follows: 1) the finding that not one but two species of deuterium-labeled PI(20:4/20:4), namely PI([^{2}H]20:4/[^{2}H]20:4) and PI([^{2}H]20:4/20:4), are detected indicates that, if the PI molecule already contains 20:4 in the *sn*-2 position, recycling only takes place at the *sn*-1 position; 2) the finding that if a PI molecule contains only one 20:4 moiety it is invariably found at the sn-2 position suggests that recycling at the *sn*-1 position with 20:4 only takes place if the *sn*-2 position is already occupied by 20:4; and 3) the conspicuous absence of deuterium-containing lysoPI suggests that incorporation of a second 20:4 molecule proceeds at a very high rate, thus lyso intermediates do not accumulate. Collectively, these considerations prompt us to propose a sequential mechanism for PI(20:4/20:4) synthesis in activated cells, whereby a preformed PI molecule is first acted upon by cPLA$_2\alpha$—and perhaps also by other intracellular PLA$_2$ (58–60)—to generate a 2-lysoPI. This 2-lysoPI will be rapidly reacylated with 20:4 before a phospholipase A$_1$ attacks the newly formed 20:4-containing PI to produce 1-lysoPI that will be reacylated again with 20:4 to form PI(20:4/20:4). From this model, it can be predicted that the phospholipase A$_1$ activity participating in this sequence of events shows specificity for PI molecules containing 20:4 at the *sn*-2 position. An enzyme with such an exquisite selectivity could constitute an excellent target for pharmacologic control of PI(20:4/20:4) formation, and we have initiated studies aimed at characterizing it in macrophages.

The rapid rate of formation of PI(20:4/20:4) in zymosan-stimulated cells and its cellular retention suggest that this molecule may play a role in regulating cell signaling events. Moreover, increased PI(20:4/20:4) formation is shown to be of pathophysiological significance because it is also detected in vivo during murine peritonitis. To define the biological roles of PI(20:4/20:4), we devised strategies for introducing this phospholipid in cells. We achieved success in this regard by complexing the phospholipid with the cationic carrier protein histone H1, adapting protocols originally described by Prestwich and coworkers (50) for the introduction of polyphosphoinositides in cells.

Considerable evidence has accumulated over the years to indicate that cPLA$_2\alpha$ and attendant 20:4 mobilization are involved in the activation of the NADPH oxidase complex of phagocytes (61). The NADPH oxidase is a multicomponent electron transport chain that transfers electrons from NADPH to molecular oxygen to generate superoxide anion, a critical step for pathogen killing and the maintenance of efficient host defense (56). Activation of the oxidase involves assembly of the membrane component flavocytochrome b_{558} (consisting of gp91phox and p22 phox), with the cytosolic components p67phox, p47phox, p40phox, and the small GTP-binding protein Rac-1 (56). Although the exact target sites for 20:4 on the assembled NADPH are not known, there is evidence that the fatty acid serves a role in activating the oxidase after the assembly of the oxidase complex has taken place (61). Much of the evidence implicating 20:4 in these events has been obtained from studies adding the fatty acid exogenously to the cells and/or by inhibiting cPLA$_2\alpha$ activity by different means. However, conclusions drawn from this type of studies have sometimes yielded conflicting results and are complicated by the lack of knowledge of the molecular mechanism through which 20:4 mediates these activating functions.

Given that 20:4 incorporates very rapidly into various phospholipids in activated cells (9, 10, 46), the possibility exists that at least part of the effects on innate immune responses observed when 20:4 is added to the cells may be mediated by its incorporation into discrete phospholipid species such as PI(20:4/20:4), which would be the actual molecular entity that carries biological activity. In analogy with this idea, it has recently been reported that some of the stimulatory effects of exogenous palmitoleic acid on murine fibroblast-like cell lines might be due not to the fatty acid itself but to the fatty acid accumulating into phosphatidylinositol species (62). Moreover, pharmacological manipulations that lead to inhibition of PI(20:4/20:4) production such as use of pyrrophenone or triacsin C are long known to strongly blunt NADPH oxidase activation and superoxide anion generation in receptor-activated cells (61, 63). Because triacsin C acts to prevent incorporation of 20:4 into phospholipids, this inhibitor would not be expected to blunt NADPH oxidase activation if the activating stimulus was primarily the exogenous free 20:4 (63).

The activating effect of PI(20:4/20:4) on superoxide anion production and lysozyme secretion appears to be specific for this particular phospholipid, because addition of a mixture of PI species from bovine liver—in which 20:4 makes up for 20–25% of total fatty acid, and no PI(20:4/20:4) is present—does not reproduce the stimulatory effects of PI(20:4/20:4). These findings raise the intriguing question of how much of the biological activity of PI(20:4/20:4) is related to the presence of two arachidonyl residues. This is an interesting concept, because the structurally related and much more studied analog PC(20:4/20:4) has been found to serve primarily a metabolic role, both as a "defensive" means for the cells to protect themselves from the excessive availability of exogenous free 20:4 (44, 45) and as a 20:4 donor for anandamide synthesis under certain conditions (52). According to the data reported in this paper, the former role but not the latter may also be served by PI(20:4/20:4). It is also noteworthy that, despite PE being the major 20:4-containing class in macrophages and in phagocytic cells in general, these cells do not appear to synthesize PE(20:4/20:4). We have consistently failed to detect such a species under a variety of conditions and using different types of phagocytic cells (30–34), and to the best of our knowledge, we are not aware of studies by other groups documenting the formation of PE(20:4/20:4). Taken together, we speculate that not only the presence of the two 20:4 residues but likely also the polar headgroup may be a key determinant of the biological activity associated to PI(20:4/20:4).

In summary, we conclude that comprehensive profiling of 20:4-containing phospholipids by LC/MS during zymosan phagocytosis has uncovered an unexpected role for a discrete phospholipid species, PI(20:4/20:4), in regulating innate immune responses. Our studies suggest that this phospholipid adds to the growing family of lipids with active roles in cell signaling.

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Disclosures

The authors have no financial conflicts of interest.

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