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Markers of Monocyte Activation Revealed by Lipidomic Profiling of Arachidonic Acid-Containing Phospholipids

David Balgoma,*† Alma M. Astudillo,*,† Gema Pérez-Chacón,*,† Olimpio Montero,*,† María A. Balboa,*† and Jesús Balsinde*†

Stimulated human monocytes undergo an intense trafficking of arachidonic acid (AA) among glycerophospholipid classes. Using HPLC coupled to electrospray ionization mass spectrometry, we have characterized changes in the levels of AA-containing phospholipid species in human monocytes. In resting cells, AA was found esterified into various molecular species of phosphatidylinositol (PI), choline glycerophospholipids (PCs), and ethanolamine glycerophospholipids (PEs). All major AA-containing PC and PI molecular species decreased in zymosan-stimulated cells; however, no PE molecular species was found to decrease. In contrast, the levels of three AA-containing species increased in zymosan-activated cells compared with resting cells: 1,2-diaraachidonyl-glycerol-3-phosphoinositol [PI(20:4/20:4)]; 1,2-diaraachidonyl-glycerol-3-phosphocholine [PC(20:4/20:4)]; and 1-palmitoleoyl-2-arachidonyl-glycero-3-phosphoethanolamine [PE(16:1/20:4)]. PI(20:4/20:4) and PC(20:4/20:4), but not PE(16:1/20:4), also significantly increased when platelet-activating factor or PMA were used instead of zymosan to stimulate the monocytes. Analysis of the pathways involved in the synthesis of these three lipids suggest that PI (20:4/20:4) and PC (20:4/20:4) were produced in a deacylation/reacylation pathway via acyl-CoA synthetase–dependent reactions, whereas PE(16:1/20:4) was generated via a CoA-independent transacylation reaction. Collectively, our results define the increases in PI(20:4/20:4) and PC(20:4/20:4) as lipid metabolic markers of human monocyte activation and establish lipidomics as a powerful tool for cell typing under various experimental conditions.


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Abbreviations used in this paper: AA, arachidonic acid; a.u., arbitrary unit; cPLA2a, group IVA cytosolic phospholipase A2; Ctrl, control; MS, mass spectrometry; m/z, mass-to-charge ratio; NL, neutral loss; PAF, platelet-activating factor; PC, choline glycerophospholipid; PC(20:4/20:4), 1,2-diaraachidonyl-glycerol-3-phosphocholine; PI, phosphatidylinositol; PC(20:4/20:4), 1-palmitoleoyl-2-arachidonyl-glycerol-3-phosphoethanolamine; PE, ethanolamine glycerophospholipid; PE(16:1/20:4), 1-palmitoleoyl-2-arachidonyl-glycerol-3-phosphoethanolamine; PI, phosphatidylinositol; PC(20:4/20:4), 1,2-diaraachidonyl-glycerol-3-phosphoinositol; PL2α, phospholipase A2.

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the unusual species 1,2-diarchidionyl-glycer-3-phosphoinositol (PI[20:4/20:4]) as a short-lived acceptor for the initial incorporation of AA into the phosphatidylinositol (PI) class (17). Using HPLC coupled to electrospray ion-trap MS, this article reports on the characterization of all molecular species of glycerophospholipids involved in AA homeostasis during the stimulation of human monocytes by zymosan. Our studies led to the identification of three AA-containing phospholipids in stimulated cells that are not present in resting cells or are found only at very low levels: PI[20:4/20:4]; 1,2-diarchidionyl-glycer-3-phosphocholine [PC(20:4/20:4)]; and 1-palmitoleoyl-2-arachidonyl-glycer-3-phosphethanolamine [PE (16:1/20:4)]. In as much, the first two species are also produced in significant amounts upon stimulation by other agonists, they can be regarded as lipid markers of human monocyte activation.

Materials and Methods

Reagents

Cell culture medium was from Invitrogen Life Technologies (Carlsbad, CA). Standard lipids were from Avanti Polar Lipids (Alabaster, AL). Triacsin C was from BIOMOL (Plymouth Meeting, PA). Chloroform, methanol, hexane, and other solvents (HPLC grade) were from Sigma-Aldrich (St. Louis, MO). Ammonium hydroxide (30%) and acetic acid were from Merck (Darmstadt, Germany). Pyrophosphate was synthesized and generously provided by Dr. Amaden Llebaria (Institute for Chemical and Environmental Research, Barcelona). All other reagents were from Sigma-Aldrich.

Cell isolation and culture

Human monocytes were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Briefly, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 g for 30 min. The mononuclear cellular layer was recovered and washed three times with PBS, resuspended in RPMI supplemented with 2 mM L-glutamine and 40 μM gentamicin, and allowed to adhere to plastic in sterile dishes for 2 h. Nonadherent cells were removed by washing extensively with PBS, and the remaining attached monocytes were used the next day. For all experiments, monocytes were cultured in a final volume of 2 ml in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine and 40 μg/ml gentamicin at 37°C. When inhibitors were used, they were added to the incubation media 30 min before stimulating the cells with zymosan.

Preparation of zymosan

Zymosan was prepared as described elsewhere (18). 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 being added to the cells. No endogenous PLA₂ activity was detected in the zymosan batches used in this study, as assessed by in vitro activity assay under a variety of conditions (see later discussion).

PLA₂ activity measurements

Ca²⁺-dependent PLA₂ activity was measured using a modification of the mammalian membrane assay described by Diez et al. (19). Briefly, monocyte homogenates were incubated for 1–2 h at 37°C in 100 mM HEPES (pH 7.5) containing 1.3 mM CaCl₂ and 100,000 dpm [3H]AA-labeled membrane, used as a substrate, in a final volume of 0.15 ml. Prior to assay, the cell membrane substrate was heated at 57°C for 5 min to inactivate CoA-independent transacylase activity (20). The assay contained 25 μM bromoenol lactone to completely inhibit endogenous Ca²⁺-independent PLA₂ activity (21). After lipid extraction, free [3H]AA was separated by thin-layer chromatography, using n-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. In some experiments, Ca²⁺-independent PLA₂ activity was also measured using a mixed-micelle substrate or the natural membrane assay. For the mixed micelle assay, Trition X-100 was added to the dried lipid substrate at a molar ratio of 4:1. Buffer was added, and the mixed micelles were made by a combination of heating >40°C, vortexing, and water bath sonication until the solution clarified. The natural membrane assay was carried out exactly as described above, except that CaCl₂ was omitted, and 5 mM EDTA was added instead. All of these assay conditions have been validated previously with regard to time, homogenate protein, and substrate concentration (16, 22–27).

HPLC-MS coupling

For HPLC separation of lipids, a Hitachi LaChrom Elite L-2130 binary pump was used, together with a Hitachi Autosampler L-2200 (Merck). The HPLC system was coupled on-line to a Bruker Esquire 6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The effluent was split, and 0.2 ml/min was introduced in the electrospray ionization interface of the mass spectrometer. The nebulizer was set to 30 psi, the dry gas to 8 l/min, and the dry temperature to 365°C.

Analysis of PI, ethanolamine glycerophospholipid, and choline glycerophospholipid species

Total lipid content, corresponding to 2·10⁶ cells, was extracted according to Bligh and Dyer (28). After evaporation of the organic solvent under vacuum, the lipids were redissolved in methanol/water (9:1) and stored under nitrogen at −80°C until analysis. 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/1% acetic acid/0.1% ammonium hydroxide, 87.5:10:5:0.5, v/v) and solvent B (methanol/1% acetic acid/1% ammonium hydroxide, 87.5:12:0.5, v/v). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A in 20 min, to 10% in 5 min, and to 0% in an additional 5 min. Flow rate was 0.5 ml/min, and 80 μl of the lipid extract was injected. PI and ethanolamine glycerophospholipid (PE) species were detected in negative ion mode with the capillary current set at +3500 V over the initial 22 min as [M−H]−. Choline glycerophospholipid (PC) species were detected over the elution interval from 22–35 min in positive ion mode, as [M+H]⁺ ion, with the capillary current set at −4000 V.

![FIGURE 1. Identification of AA-containing glycerophospholipids by MS³ fragmentation. A. MS³ fragmentation of PI(20:4/20:4); m/z 905, [M−H]−. B. MS³ fragmentation of PE(P-16:0/20:4); m/z 722, [M−H]−. C. MS³ fragmentation of PC(18:0/20:4); m/z 868 → 794, [M+CH₃CO₂]⁻→[M−CH₃]⁻.](image-url)
Phospholipid identification

AA-containing PI and PE species were identified by multiple reaction monitoring MS² experiments on chromatographic effluent by comparison to previously published databases (29–31). The cut-off parameter was set at m/z 150, and the fragmentation amplitude was set at 1 (arbitrary units). Because of the lability of vinyl ether linkages in acid media, plasmenyl (1-alkyl) and plasmanyl (1-alk-1'-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction (32). For the identification of acyl chains of AA-containing PC species, ionization was carried out in negative mode with postcolumn addition of acetic acid at a flow rate of 100 μl/h as [M+CH₃CO₂]⁻ adducts, and acyl chains were identified by MS³ experiments.

MS software

Mass-to-charge ratio (m/z) data were analyzed with Bruker Daltonics DataAnalysis 3.3 and MetaboliteDetect 1.1.

Statistical analysis

Assays were carried out in triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data shown are from representative experiments and are expressed as mean ± SE.

Results

Identification of AA-containing glycerophospholipid species in resting monocytes

Ion-trap MS³ experiments were performed to identify AA-containing glycerophospholipids. These lipids were unequivocally identified by looking at the production of an m/z 303 fragment in MS², which corresponds to AA (Fig. 1). When the m/z 303 fragment was detected with a high intensity, another characteristic fragment was detected at m/z 259, which corresponds to AA (Fig. 1). When the m/z 303 fragment was detected for PI and PE or MS³ experiments for PC (Fig. 1).

Using [³H]AA, we have determined that PC and PE typically constitute the major reservoirs of AA in unstimulated cells (43% and 39% of total AA in phospholipids respectively), whereas the content of AA in PI is lower (18%) (A.M. Astudillo and J. Balsinde, unpublished observations). A total of 21 AA-containing glycerophospholipid molecular species were detected in resting human monocytes by ion-trap MS (Table I). Major AA-containing species included PC(18:0/20:4), PC(18:1/20:4), PC(O-16:0/20:4), PC(O-18:1/20:4), PI(18:0/20:4), PE(P-16:0/20:4), PE(P-18:0/20:4), and PE(18:0/20:4). A few of the identified AA-containing species were not quantifiable because they were detected only in trace amounts (Table I).

A minor AA-containing species with an odd-number fatty chain in position sn-1 was detected in all monocyte samples examined (Fig. 2). This unexpected phospholipid was identified as PE(P-17:0/20:4) on the basis of the following evidence: fragmentation of this species yielded three fragments of m/z 259, 303, and 450 (Fig. 2A); fragmentation of the latter fragment yielded the minor fragment of m/z 196 that identifies this compound as a PE molecule (Fig. 2B); the species disappeared when the samples were acidified before lipid extraction, indicating that the molecule is a plasmanol; and the MS³ spectrum of a standard sample of PE(P-18:0/20:4) was almost identical to that of the unknown species, producing the m/z 196 fragment arising from the ethanolamine headgroup and two fragments arising from neutral losses 61 and 197 (Fig. 2B, 2C).

Time-dependent variation of AA-containing glycerophospholipid species upon zymosan stimulation

Stimulation of human monocytes with zymosan is known to result in the abundant release of free AA plus metabolites into the extracellular medium (33–35). Such a response is completely abrogated by the selective cPLA₂ inhibitor pyrrophenone (36) at concentrations as low as 1 μM (35), indicating that, as is true for most other systems, zymosan-stimulated AA mobilization is mediated by cPLA₂ (37). To identify specific molecular species that

Table I. AA-containing glycerophospholipids in resting monocytes

<table>
<thead>
<tr>
<th>PC Species</th>
<th>[M+H]⁺ m/z</th>
<th>Peak Area (a.u.)</th>
<th>AA in PC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-16:0/20:4</td>
<td>766</td>
<td>8.57·10⁶</td>
<td>8.4</td>
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<tr>
<td>O-16:0/20:4</td>
<td>768</td>
<td>1.74·10⁶</td>
<td>17.1</td>
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<tr>
<td>O-18:1/20:4</td>
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<td>6.7</td>
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<td>18:2/0:4</td>
<td>806</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1/20:4</td>
<td>808</td>
<td>1.99·10⁶</td>
<td>19.6</td>
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<tr>
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<td>830</td>
<td>2.74·10⁵</td>
<td>5.4</td>
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</table>

<table>
<thead>
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<th>[M—H]⁻ m/z</th>
<th>Peak Area (a.u.)</th>
<th>AA in PI (%)</th>
</tr>
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<td>857</td>
<td>4.08·10⁷</td>
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<td>18:2/0:4</td>
<td>881</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>883</td>
<td>6.15·10⁵</td>
<td>9.2</td>
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<tr>
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<td>885</td>
<td>5.59·10⁵</td>
<td>83.6</td>
</tr>
<tr>
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<td>905</td>
<td>3.59·10⁵</td>
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</table>

<table>
<thead>
<tr>
<th>PE Species</th>
<th>[M—H]⁻ m/z</th>
<th>Peak Area (a.u.)</th>
<th>AA in PE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-16:0/20:4</td>
<td>722</td>
<td>1.26·10⁶</td>
<td>33.7</td>
</tr>
<tr>
<td>P-17:0/20:4</td>
<td>736</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0/20:4</td>
<td>738</td>
<td>1.01·10⁷</td>
<td>2.7</td>
</tr>
<tr>
<td>18:2/0:4</td>
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<td>750</td>
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<td>18:1/20:4</td>
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<td>1.07·10⁷</td>
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<tr>
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<td>766</td>
<td>1.01·10⁷</td>
<td>27.1</td>
</tr>
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</table>

*Detected at levels less than the quantification limit.

a.u., arbitrary unit.

FIGURE 2. Identification of PE(P-17:0/20:4). A, MS³ spectrum of m/z 736 with retention time of 10 min. B, MS³ experiment of m/z 450 from A. C, MS³ fragmentation pattern of PE(P-18:0/20:4) m/z 750 → 464, [M—H]⁻ →[M—R²C = C = O]⁻ NL, neutral loss.
experienced net losses or gains of endogenous AA upon zymosan stimulation, we determined the time-dependent changes in the levels of all AA-containing glycerophospholipids of stimulated monocytes by HPLC-electrospray ion-trap MS.

Fig. 3 shows the effect of stimulating the cells with zymosan on the levels of AA-containing PC molecular species. All major PC species experienced dramatic decreases with time, with the notable exception of the minor species PC(20:4/20:4), whose levels increased markedly with time. Time-dependent variations in the levels of PC molecular species were also studied in cells that had been pretreated with the cPLA$_2$ inhibitor pyrrophenone (1 µM). The results indicate that pyrrophenone almost completely abrogated any change in the levels of all species detected, suggesting that cPLA$_2$ activation is responsible for the variations observed (Fig. 3). In vitro activity assays using homogenates from pyrrophenone-treated cells demonstrated that at the concentration used in this study, cellular cPLA$_2$ activity was quantitatively inhibited by pyrrophenone. In addition, no effect of pyrrophenone was detected on cellular calcium-independent PLA$_2$ activity, reflecting the specificity of the inhibitor (data not shown).

The effect of zymosan stimulation on the levels of AA-containing PI molecular species is shown in Fig. 4. PI(18:0/20:4), the species that accounts for ~85% of total AA in PI (Table I) experienced a strong decrease, and PI(16:0/20:4) and PI(18:1/20:4) showed little change. In contrast, the minor species PI(20:4/20:4) increased sharply, with a maximum level seen 30–60 min after stimulation. A study of the effect of pyrrophenone on these changes demonstrated that the inhibitor almost completely prevented any change in the levels of PI (16:0/20:4), PI(18:1/20:4), and PI(20:4/20:4), but an effect on the levels of PI(18:0/20:4) was only appreciated after 30 min of cell challenge.

The effect of zymosan stimulation on the levels of AA-containing PE molecular species is shown in Fig. 5. No PE species decreased significantly as a consequence of treating the cells with zymosan. Rather, the levels of these species remained fairly constant. Importantly, a species identified as PE(16:1/20:4), which was not detected in resting cells, was readily apparent in the zymosan-stimulated cells (Fig. 5). The kinetics of appearance of PE(16:1/20:4) in the stimulated cells was similar whether the cells had been pretreated or not with pyrrophenone, although the drug slightly increased the levels of this compound at all times studied (Fig. 5). These data suggest that the synthesis of PE(16:1/20:4) by the stimulated cells is essentially independent of cPLA$_2$. The levels of the other AA-containing PE species were also consistently increased in the pyrrophenone-treated cells at long incubation times (>60 min).

**FIGURE 3.** Time-dependent changes in major AA-containing PC species after zymosan stimulation. Human monocytes were preincubated for 30 min with 1 µM pyrrophenone (black circles) or carrier (open circles), and the cells were stimulated with 1 mg/ml zymosan for the time indicated.
Fig. 6 summarizes the total variations in the levels of AA-containing glycerophospholipids upon zymosan stimulation grouped by class (PC, PI, or PE) and subclass (type of linkage present at the sn-1 position of the glycerol moiety, acyl- or ether [alkyl- or alk-1’-enyl]). These data clearly indicate that PC, particularly the ether-containing subclass, is the glycerophospholipid that accounts for most of the loss of AA during stimulation of monocytes with zymosan. Losses of AA from PI were also evident, but no net losses of AA were evident from PE. In the activated cells at 60 min, the percentage of AA contained in the different classes of phospholipids was 40% for PC, 47% for PE, and 8% for PI.

Effect of triacsin C on the synthesis of PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4) by zymosan-stimulated monocytes

Experiments were conducted to characterize further the three glycerophospholipid species whose levels increased upon zymosan stimulation: PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4). To assess the involvement of CoA-dependent reactions in the synthesis of these species, we studied the effect of triacsin C, an inhibitor of AA entry into phospholipid via blockade of long-chain fatty acyl-CoA synthetases, using AA (38–41). Fig. 7 shows that incubation of the cells with triacsin C strongly blocked the appearance of PC(20:4/20:4) and PI(20:4/20:4), but it had little effect on the appearance of PE(16:1/20:4).
Effect of other stimuli on the synthesis of PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4) by monocytes

In the next series of experiments, we investigated whether PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4) also increase in response to other acute stimuli of monocyte AA release. The stimuli used were platelet-activating factor (PAF), which stimulates the cells by binding to specific surface receptors, and the pharmacological agonist PMA. Similar to zymosan, PAF and PMA have been shown to induce an immediate AA release response when applied to the cells (42–49). Fig. 8 shows that PAF and PMA induced a significant increase in the levels of PC(20:4/20:4) and PI(20:4/20:4). Interestingly, when PAF or PMA was applied to the cells at later time points (30 and 60 min) after the addition of zymosan, the level of PC(20:4/20:4) or PI(20:4/20:4) did not vary appreciably, suggesting that all of these stimuli activate the same metabolic pathway for synthesis of these phospholipids and that the pathway saturates rapidly after the addition of the first stimulus (data not shown). In contrast, neither PAF nor PMA induced an increase in the levels of PE(16:1/20:4) (Fig. 8).

Discussion

By using HPLC coupled to ion-trap MS, we identified the glycerophospholipid species of human monocytes that contain AA, as well as the changes that occur in the levels of these species upon cell activation by the phagocytosable particle zymosan. In resting cells, eight major AA-containing species were detected: PC(16:0/20:4), PC(18:0/20:4), PC(O-18:1/20:4), PC(O-16:0/20:4), PE(18:0/20:4), PE(16:0/20:4), PE(18:0/20:4), and PI(18:0/20:4). Although the fatty acid composition of cells is strikingly dependent on the nutrients available in vivo, the major AA-containing glycerophospholipids of human monocytes identified in this study are in general agreement with studies on other primary cells, such as human neutrophils and platelets, and murine peritoneal macrophages (50–56).

As for minor glycerophospholipids, a noticeable finding was the identification of PC(20:4/20:4) and PI(20:4/20:4), which is most likely a reflection of the high AA content to which primary cells, such as monocytes, are exposed. At high free AA levels, PC(20:4/20:4) is formed by the de novo biosynthetic route, via the sequential acylation of glycerol phosphate and arachidonyl glycerol phosphate with AA (57). PI(20:4/20:4) is not produced by the de novo route, but rather via direct acylation of pre-existing lysoPI molecules when the cells are exposed to exogenous AA (17). Neither of these species is detectable in resting U937 promonocytic cells, which are known to be defective in AA (17).

Also noteworthy is the identification of a plasmalogen PE species with an odd-carbon number chain: PE(17:0/20:4). Although odd-carbon fatty acyl chains are not common in mammalian cells, they have been described previously (51, 54), and their appearance has been linked to diet (58). In particular, the appearance of heptadecanoic acid was associated with the intake of dairy products (59). Supplementation of rat chow with heptadecanoic acid produced an increased proportion of 17:0 plasmalogen...
PE in liver and kidney (60), suggesting a direct relationship between the intake of heptadecanoic acid and its incorporation into plasmalogen PE. Thus, we speculate that the presence of PE(P-17:0/20:4) in human monocytes is probably associated with the intake of dairy products by the blood donors.

Stimulation of the monocytes with yeast-derived zymosan induced significant changes in the distribution of AA among cell glycerophospholipids, both qualitative and quantitative. The stimulated cells lost 20–30% of their AA content after 60 min of stimulation, which is in accordance with our previous estimates using cells labeled with [3H]AA under equilibrium conditions (A.M. Astudillo and J. Balsinde, unpublished observations). Major losses of AA occur in PC, most prominently the ether-linked species, and to a lesser extent, PI, particularly PI(18:0/20:4). Strikingly, all of the AA losses from PC can be almost completely prevented by preincubating the cells with the cPLA2α inhibitor pyrrophenone, thus demonstrating that receptor-mediated activation of PC hydrolysis via a cPLA2α-dependent pathway is responsible for these changes. In contrast, treating the cells with pyrrophenone only partially prevented the loss of AA from PI. It seems likely that the abrupt pyrrophenone-insensitive decrease in the levels of PI(18:0/20:4) that is observed within the first 30 min of stimulation may be related to the activation of phosphoinositide kinases and the PI cycle (61, 62).

Although the majority of AA lost upon zymosan activation arises from the ether-linked PC species, a sharp decrease in the diacyl species PC(18:0/20:4) was measured as well. This finding suggests that, as long as the PC species contains AA, cPLA2α will use it efficiently as a substrate, irrespective of the type of linkage present in the sn-1 position of the glycerol backbone. This observation would be fully consistent with previous data indicating that cPLA2α does not distinguish between diacyl- and ether-linked PC subclasses (19, 63).

An unexpected finding from this work is that no net changes in AA-containing PE were detected. Previous studies using [3H]AA-labeled cells under equilibrium-labeling conditions indicated that PE is a source of free AA during cell activation (64–70). Thus, the simplest explanation for these findings is that the levels of AA-containing PE in the activated cells are maintained by a tight, opposing balance between hydrolysis (cPLA2α-mediated) and transacylation (CoA independent transacylase mediated). Thus, hydrolysis of an AA-containing PE species would be followed by its rapid reacylation using AA, likely from PC. The stimulated entry of AA into PE via CoA-independent transacylase reactions using AA-containing PC as an AA donor has been demonstrated to occur in human neutrophils and murine mast cells (13, 65). Consistent with this model, there seems to be a net gain of AA in PE at later times in the pyrrophenone-treated cells, which could be explained by the continuous incorporation of AA via CoA-independent transacylase at the expense of AA-containing PC.

**FIGURE 7.** Influence of triacsin C on the levels of PC(20:4/20:4); PI (20:4/20:4), and PE(16:1/20:4) after zymosan stimulation. Human monocytes were untreated (open bars) or treated (hatched bars) with 3 μM triacsin C for 30 min. Afterward, the cells were stimulated or not (control) with 1 mg/ml zymosan for 60 min.

**FIGURE 8.** Effect of various stimuli on the production of PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4) by monocytes. The cells were untreated (Ctrl) or treated with 1 mg/ml zymosan, 100 nM PAF, or 100 ng/ml PMA for 60 min. Afterward, the cellular content of PC(20:4/20:4), PI(20:4/20:4), and PE(16:1/20:4) was determined by ion-trap MS. Ctrl, control.
Two particular AA-containing glycerophospholipid species, PC (20:4/20:4) and PI (20:4/20:4), showed remarkable behavior during stimulation of the monocytes by various agonists. The appearance of these two species was abrogated by preincubating the cells with pyrophosphonates. This pharmacological profile suggests that PC (20:4/20:4) and PI (20:4/20:4) are formed via CoA-dependent reacylation reactions, in which the AA acting as a donor arises from the cPLA₂-mediated hydrolysis of AA-containing glycerophospholipids. Analogous with the routes of biosynthesis for these compounds that have been drawn from studies in resting cells exposed to exogenous AA (7, 17), it is likely that the acceptors for PC (20:4/20:4) synthesis arise from the de novo biosynthetic route, whereas PI (20:4/20:4) is produced at the expense of pre-existing lysoPI acceptors.

A third distinctive species of activated cells, PE(16:1/20:4), is not detectable in resting cells, and its kinetics of appearance is quite different from those of PC (20:4/20:4) and PI (20:4/20:4). Importantly, this phospholipid is produced when the cells are stimulated by zymosan but not by PAF or PMA. Thus, the appearance of PE (16:1/20:4) could constitute a specific marker of zymosan-stimulated monocytes, but not a general activation marker, as would be the case for the increase in PC (20:4/20:4) and PI (20:4/20:4). The stimulated appearance of PE (16:1/20:4) is not inhibited by pyrophorone or triacsin C, suggesting that this species is produced from pre-existing lysoPE (16:1) via a CoA-independent transacylation reaction using an AA-containing phospholipid, not free AA, as an acyl donor. Collectively, our finding that two particular AA-containing species that are detected at low levels, PC (20:4/20:4) and PI (20:4/20:4), significantly increase in activated cells via selected biosynthetic pathways suggests that they may be regarded as lipid markers of the activation state of human monocytes. In turn, the data validate lipids as a useful approach to identify specific markers of the activation state of human monocytes. In turn, the synthetic pathways suggest that they may be regarded as lipid markers of the activation state of human monocytes.

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Disclosures
The authors have no financial conflicts of interest.

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