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

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

David Balgoma,† alm 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 to various molecular species of phosphatidylcholine (PC), 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-diacylgllycerol-3-phosphoinositol [PI(20:4/20:4)]; 1,2-diacylgllycerol-3-phosphocholine [PC(20:4/20:4)]; and 1-palmitoyl-2-arachidonyl-glycerol-3-phosphoethanolamine [PE(16:1/20:4)]. PI(20:4/20:4) and PE(16:1/20:4), but not PC(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 suggests 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. The Journal of Immunology, 2010, 184: 3857–3865.

The key role that monocytes play in inflammation and related diseases is well established (1, 2), and these cells can also constitute a target for therapeutic strategies in cancer (3). The role of monocytes as immunomodulatory cells has been related, in part, to their capacity to synthesize and release large amounts of arachidonic acid (AA)-derived eicosanoid mediators (4).

AA is an intermediate of a deacylation/transacylation pathway of membrane glycerophospholipids, the so-called “Lands cycle” (5–7), in which the fatty acid is cleaved from the sn-2 position of glycerophospholipids by the action of phospholipase A2 (PLA2) and is reincorporated by CoA-dependent acyltransferases. Under resting conditions, reacylation dominates over deacylation; thus, free AA is kept at very low levels. Stimulation of the cells by receptor agonists results in the activation of the group IVA cytosolic PLA2α (cPLA2α) (8–12). Under these conditions, the rate of AA release clearly exceeds that of reincorporation into phospholipids; hence, net accumulation of AA occurs that is followed by its conversion into different oxygenated compounds, collectively called the eicosanoids. Nevertheless, increased AA reacylation during cellular activation is still very significant, as manifested by the fact that only a minor portion of the free AA released by cPLA2α is converted into eicosanoids; the remainder is effectively reincorporated back into phospholipids.

Two model pathways have been described for the incorporation of AA into glycerophospholipids (6, 7). The first one is a high-capacity, low-affinity pathway that operates when the concentration of available free AA is low. In this pathway, AA is linked to CoA by long-chain fatty acyl CoA synthetases, is incorporated mainly into choline glycerophospholipids by a CoA-dependent acyltransferase, and is then transferred to ethanolamine lyso-phospholipids by a CoA-independent transacylase (6, 7). This route is active in resting and activated cells (13–15), and the availability of PLζ-derived lysophospholipid acceptors seems to be a key regulatory point (16). The second pathway for AA incorporation into phospholipids is a low-affinity, high-capacity one that seems to operate when the high-affinity/low-capacity pathway is saturated by the high concentrations of available free AA (6, 7). In this second pathway, the fatty acid is incorporated via the de novo route of glycerophospholipid synthesis (6, 7).

Study of AA trafficking by combining conventional radiolabeling approaches with thin-layer chromatography does not allow the identification of specific phospholipid molecular species involved in the process. Within the framework of the emerging field of lipidomics, and by applying HPLC/mass spectrometry (MS) to the study of lipids, it is now possible to distinguish among various molecular species within each glycerophospholipid class, and tricky derivation is avoided. Recently, we applied this technique to the study of the incorporation of deuterium-labeled AA in inositol glycerophospholipids in resting human monocytes; we identified...
ethyl ether/acetic acid (70:30:1) as a mobile phase. In some experiments, palmitic acid was separated by thin-layer chromatography, using volumes of chloroform/methanol (1:2). After lipid extraction, free [3H] and the remaining attached monocytes were used the next day. For all for 2 h. Nonadherent cells were removed by washing extensively with PBS, and 40

incubation media 30 min before stimulating the cells with zymosan.

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

Ca\textsuperscript{2+}-independent PLA2 activity was also measured using a mixed-micelle substrate or the natural membrane assay. For the mixed micelle assay, Triton 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 CaCl\textsubscript{2} 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 glycerolphospholipid, and choline glycerolphospholipid species

Total lipid content, corresponding to 2·10\textsuperscript{6} 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/triethylamine/30% ammonium hydroxide, 87.5:10:5.1:5.0:5.5, v/v) and solvent B (methanol/triethylamine/30% 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 glycerolphospholipid (PE) species were detected in negative ion mode with the capillary current set at +5500 V over the initial 22 min as [M—H]−. Choline glycerolphospholipid (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 glycerolphospholipids by MS\textsuperscript{n} fragmentation. A. MS\textsuperscript{2} fragmentation of PI(20:4/20:4), m/z 905. [M—H]− → B, MS\textsuperscript{2} fragmentation of PE(P-16:0/20:4), m/z 722, [M—H]−. C. MS\textsuperscript{3} fragmentation of PC(18:0/20:4) m/z 868 →794, [M+CH\textsubscript{3}CO\textsubscript{2}]− → [M—CH\textsubscript{3}]−.
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 liability of vinyl ether linkages in acid media, plasmenyl (1-alkyl and plasmalogen (1-alk-1'-ene)) glycerophospholipids were distinguishe

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 MS² 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).

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 cPLA2α (37). To identify specific molecular species that 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).

Identification of PE(P-17:0/20:4) 2. 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–R² C=C=O⁻]. NL, neutral loss.

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>
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<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>1.74·10⁹</td>
<td>17.1</td>
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<tr>
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<td>794</td>
<td>1.47·10⁹</td>
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<td>796</td>
<td>6.79·10⁹</td>
<td>6.7</td>
</tr>
<tr>
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<td>806</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>18:1/20:4</td>
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<td>19.6</td>
</tr>
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</thead>
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<td>—</td>
</tr>
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<td>883</td>
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<table>
<thead>
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<th>[M–H]⁻ m/z</th>
<th>Peak Area (a.u.)</th>
<th>AA in PE (%)</th>
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<tbody>
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<td>1.26·10⁹</td>
<td>33.7</td>
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<td>—</td>
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<tr>
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<td>—</td>
</tr>
<tr>
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<td>748</td>
<td>—</td>
<td>—</td>
</tr>
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<td>766</td>
<td>1.01·10⁹</td>
<td>27.1</td>
</tr>
</tbody>
</table>

*Detected at levels less than the quantification limit. a.u., arbitrary unit.
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 cPLA2 inhibitor pyrrophenone (1 µM). The results indicate that pyrrophenone almost completely abrogated any change in the levels of all species detected, suggesting that cPLA2 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 cPLA2 activity was quantitatively inhibited by pyrrophenone. In addition, no effect of pyrrophenone was detected on cellular calcium-independent PLA2 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 cPLA2. 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(P-18:0/20:4), PE(P-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(P-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 pre-incubating 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).

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.
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 pyrophenone or triacsin C. 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 bio-synthetic 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 pyrophenone 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 bio-synthetic pathways suggests that they may be regarded as lipid markers of the activation state of human monocytes. In turn, the data validate lipidomics as a useful approach to identify specific molecular markers in resting versus activated cells that may complement other biochemical and/or immunological approaches.

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