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Lipid Profiling Reveals Glycerophospholipid Remodeling in Zymosan-Stimulated Macrophages[†]

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ABSTRACT: Comprehensive lipid profiling by mass spectrometry provides comparative data on the relative distribution of individual glycerophospholipids within each of the major classes. Application of this method to the analysis of glycerophospholipid remodeling in murine primary resident peritoneal macrophages (RPMs) during zymosan phagocytosis reveals significant decreases in the levels of every major arachidonic acid (20:4)-containing species of phosphatidylcholine (GPCho) and in selected 20:4-containing phosphatidylinositol (GPIIns) and phosphatidylglycerol (GPGro) species. No net changes in 20:4-containing phosphatidylethanolamine (GPEtn) species were detected. Pretreatment of RPMs with LPS resulted in subtle changes in the magnitude and kinetics of the response but had no effect on the overall pattern of zymosan-induced glycerophospholipid remodeling. Inhibition of prostaglandin (PG) synthesis with indomethacin reduced the magnitude of the changes in 20:4-containing diacyl but not alkyl acyl species. Blockade of 20:4 reacylation with thimerosal had no effect on the magnitude of the zymosan-induced changes in GPCho, GPIIns, or GPGro species but revealed decreases in the level of alkyl acyl GPEtn species. RAW264.7 cells contain much lower levels of phospholipid 20:4 than do RPMs and synthesize PGs poorly in response to zymosan. Pretreatment with granulocyte-macrophage colony stimulating factor, lipopolysaccharide, and interferon- γ substantially increased the extent of 20:4 mobilization and PG synthesis in these cells. However, under conditions of maximal zymosan-dependent PG synthesis, the only glycerophospholipid that exhibited a significant change was a 20:4-containing plasmenyl GPEtn. These results suggest that GPCho is the major ultimate source of 20:4 that is mobilized in zymosan-stimulated RPMs but that 20:4 mobilization may involve the intermediate turnover of alkyl acyl GPEtn species.

Arachidonic acid (20:4)¹ serves as the precursor for a wide variety of biologically active lipid mediators, including prostaglandins (PGs), leukotrienes, hydroxyeicosatetraenoic acids, and lipoxins. In resting cells, 20:4 is predominantly found esterified in membrane glycerophospholipids and is unavailable for lipid mediator biosynthesis. Appropriate stimuli lead to the activation of cytosolic phospholipase A₂ (cPLA₂), which is the enzyme primarily responsible for the

selective hydrolysis of the ester bond linking 20:4 to the phospholipid glycerol backbone (1–4). Free 20:4 then serves as a substrate for cyclooxygenases-1 and -2 (COX-1 and COX-2, respectively), leading to PG formation, or for multiple lipoxygenases, leading to formation of leukotrienes, hydroxyeicosatetraenoic acids, and lipoxins. The oxygenation of 20:4 results in a net irreversible removal of the fatty acid from the glycerophospholipid pool. Thus, lipid mediator biosynthesis is accompanied by a remodeling of membrane glycerophospholipids, as 20:4-containing species are hydrolyzed, and the resulting lysophospholipids are then subject to further metabolism.

Macrophages are a major source of 20:4-derived lipid mediators and have frequently served as model systems for the study of 20:4 metabolism. Early studies of eicosanoid biosynthesis in murine resident peritoneal macrophages (RPMs) indicated that these cells contain especially high levels of 20:4 in their glycerophospholipids (>20%) (5, 6). On the basis of radiolabel studies, RPMs were reported to mobilize up to 50% of their 20:4 in response to the phagocytosis of zymosan, a yeast cell wall preparation that activates multiple receptors, including dectin-1 and toll-like receptor-2 (7). More than 90% of the radioactivity that appeared in the culture medium of zymosan-stimulated cells

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¹ Abbreviations: 20:4, arachidonic acid; PG, prostaglandin; cPLA₂, cytosolic phospholipase A₂; COX, cyclooxygenase; RPM, resident peritoneal macrophage; MS, mass spectrometry; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; α -MEM, Minimum Essential Medium α ; LPS, lipopolysaccharide; IFN- γ , interferon- γ ; GM-CSF, granulocyte-macrophage colony stimulating factor; BSA, bovine serum albumin; PBS, calcium- and magnesium-free phosphate-buffered saline; GPCho, phosphatidylcholine; GPEtn, phosphatidylethanolamine; GPIIns, phosphatidylinositol; GPSe, phosphatidylserine; GPGro, phosphatidylglycerol.

had been converted to eicosanoids, with the major metabolites identified as PGE₂ and PGI₂ (identified as its stable hydrolysis product, 6-ketoPGF_{1α}), and minor quantities of hydroxyeicosatetraenoic acids. Leukotriene C₄ was identified later (5, 8–12). These studies demonstrated that eicosanoid biosynthesis is exceptionally efficient in RPMs. However, they also raised a significant question. If eicosanoid biosynthesis irreversibly removes up to 50% of 20:4, a major glycerophospholipid component, what is the effect of zymosan stimulation on the overall structure and composition of glycerophospholipids in these cells?

Prior attempts to answer this question have been hampered by the lack of a comprehensive method for evaluating all GPL species simultaneously. We have reported the use of mass spectrometry-based lipid profiling techniques for carrying out detailed studies of the glycerophospholipid compositions of RPMs and the RAW264.7 murine macrophage cell line (13, 14). Using class peak ratio analysis of the data, we have shown that this approach provides a robust means of evaluating and comparing patterns of distribution of individual glycerophospholipid species within each class. We identified major differences in glycerophospholipid profiles between RAW264.7 cells, which are deficient in 20:4-containing species as compared to RPMs, and we demonstrated the ability of the method to detect changes in lipid species distribution in RPMs as a result of exposure to bacterial lipopolysaccharide (LPS), which mobilizes 20:4 for eicosanoid biosynthesis.

Here we extend the use of global lipid profiling to study the changes in glycerophospholipid distribution that occur during zymosan phagocytosis in macrophages. We show that zymosan stimulation results in significant decreases in the relative quantities of 20:4-containing species predominantly in phosphatidylcholine (GPCCho), phosphatidylinositol (GPIIns), and phosphatidylglycerol (GPGro). We further explore the effects of LPS pretreatment, COX inhibition, and blockade of 20:4 reacylation on lipid remodeling in these cells. Finally, we demonstrate relatively few glycerophospholipid changes in zymosan-stimulated RAW264.7 cells, consistent with the much weaker PG synthetic response in these cells.

EXPERIMENTAL PROCEDURES

RPM Culture. All studies involving animals were conducted with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University. Female ICR (CD-1) mice (25–30 g) were obtained from Harlan (Indianapolis, IN). Cells were obtained by peritoneal lavage as described previously (15) and suspended at a density of $2\text{--}3 \times 10^6$ cells/mL (cells from one mouse per 2 mL) in Minimal Essential Alpha Medium supplemented with GlutaMax (Gibco), containing 10% heat-inactivated fetal calf serum (Atlas Biologicals, Norcross, GA) with 100 units/mL penicillin and 0.10 mg/mL streptomycin (Sigma, St. Louis, MO) (α -MEM/FCS). The cell suspension was plated onto 35 mm dishes at a density of 2 mL/dish (for total glycerophospholipid analysis) or onto 60 mm dishes at a density of 6 mL/dish (for glycerophospholipid fatty acid analysis) and incubated for 2 h at 37 °C in a humidified 5% CO₂ atmosphere. Nonadherent cells were removed by washing the plates four times with PBS, and the cultures were then incubated overnight in fresh α -MEM/FCS.

RAW264.7 Cell Cultures. RAW264.7 cells were obtained from American Type Culture Collection. The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with GlutaMax, high glucose, sodium pyruvate, and pyridoxine-HCl (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (DMEM/FCS). Cells were plated onto 35 mm tissue culture dishes at a density of 5×10^5 cells/dish (for assay of PG synthesis) or onto 60 mm dishes at a density of 1.5×10^6 cells/dish (for glycerophospholipid fatty acid analysis and total glycerophospholipid analysis) on the day prior to experiments. Unless otherwise stated, medium was changed to DMEM/FCS containing 20 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN) 2 h after plating, and cells were incubated overnight (22 h total).

Treatment of RPM or RAW264 Cultures with Various Stimuli. Cells were plated in 35 or 60 mm dishes and incubated overnight as described above. RPMs were washed twice in PBS and transferred to fresh α -MEM/FCS with or without LPS (100 ng/mL). RAW264.7 cells were transferred to fresh DMEM/FCS with or without bacterial lipopolysaccharide (LPS, 100 ng/mL, *Escherichia coli* 011:B4, Calbiochem, San Diego, CA) and/or murine recombinant interferon- γ (IFN- γ , 10 ng/mL, Sigma) as desired. GM-CSF (20 ng/mL) was also included in the medium of cells that had been preincubated overnight with that cytokine. Unless otherwise indicated, cultures were incubated for 5 h prior to stimulation.

Unopsonized zymosan A (Sigma) was prepared as described by Bonney et al. (16) and suspended at 16 mg/mL in PBS. For studies of zymosan-induced cellular responses, cultures were washed in PBS and overlaid in fresh serum-free α -MEM (for RPM) or DMEM (for RAW264.7 cells), and zymosan was added (160 μ g/35 mm dish or 480 μ g/60 mm dish). Cultures were incubated for the desired time periods. Medium was then harvested for analysis of PG formation, and cells were scraped for lipid or protein analyses as described below.

For some experiments, inhibitors were included during zymosan incubations. All inhibitors were prepared as 1000 \times stock solutions in medium or dimethyl sulfoxide and diluted into culture medium immediately prior to addition to cultures. Specific concentrations and preincubation conditions are described for individual experiments.

Assay of PGs. Following incubation with the desired stimuli, the medium was removed from cultures (35 mm dishes), placed on ice, and immediately spiked with 20 μ L of a methanol solution containing 50 pmol each of 6-ketoPGF_{1α}-d₄, PGE₂-d₄, and PGD₂-d₄ (Cayman Chemical, Ann Arbor, MI), which served as internal standards. Samples were stored at –20 °C prior to solid phase extraction and analysis by selected reaction monitoring of the ammoniated ions by liquid chromatography positive ion electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) as described previously (17). The major PGs produced by RPMs are PGE₂ and 6-ketoPGF_{1α}. In RAW264.7 cells, the major products are PGD₂ and PGE₂. Values for PG formation are the sum of the two major products in each case. Data were normalized to the protein content of the cell monolayers using a BCA protein assay kit (Pierce, Rockford, IL) and then to cell number based on a conversion factor of 1.27 mg of cell protein/10⁷ cells.

Determination of 20:4 Levels. Cell monolayers in 35 mm dishes were scraped twice into a total volume of 1 mL of ice-cold methanol, and the resulting cell lysates were combined with 1 mL of acetonitrile containing 100 ng of 20:4- d_8 (Cayman). The resulting samples were stored at -20°C prior to extraction and analysis for 20:4 by silver ion coordination LC-ESI-MS/MS as described previously (15, 18). Data were normalized to cell number as described above for PG formation.

Phospholipid Fatty Acid Analysis. Cells in 60 mm culture dishes were scraped twice into a total volume of 1.0 mL of PBS. The cells from two dishes were combined, recovered by centrifugation at 750g for 5 min, and resuspended in 0.5 mL of PBS. The resulting cell suspensions were stored at -80°C until they were analyzed by gas chromatography by the Vanderbilt University Mouse Metabolic Phenotyping Center laboratory (19).

Total Cell Glycerophospholipid Analysis. After incubation with the desired stimuli, plates (35 mm for RPMs and 60 mm for RAW264.7 cells) were placed on ice and washed with 1.5 mL of ice-cold PBS. Cell extracts were prepared via a modified Bligh/Dyer extraction procedure (20). Briefly, cells were scraped into 800 μL of a 0.1 N HCl/CH₃OH solution (1:1), and 400 μL of CHCl₃ was added to the suspension. The samples were vortexed for 1 min, and layers were separated by centrifugation at 18000g for 5 min at 4°C . The organic phase was isolated, and a solution (20 μL) of standards containing 53.4 ng of 25:0 GPCho, 53 ng of 25:0 GPEtn, 49.2 ng of GPGro, and 51.6 ng of GPIIns was added to some samples. The solvent was then evaporated in a vacuum centrifuge (Labconco Centrivap Concentrator, Kansas City, MO). Samples were redissolved in 70 μL of a CH₃OH/CHCl₃ solution (9:1), and 1 μL of concentrated aqueous (18 M) NH₄OH was added before analysis. Mass spectral analysis was performed on a Finnigan TSQ Quantum triple-quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a Harvard Apparatus syringe pump and an electrospray source. For routine analysis, samples were analyzed by direct infusion electrospray ionization mass spectrometry (ESI-MS) at an infusion rate of 10 $\mu\text{L}/\text{min}$ in both positive and negative ionization modes over the range of m/z 350–1200. Data were collected with Xcalibur (ThermoFinnigan) and analyzed with software developed in our laboratory. Identification of individual glycerophospholipids present in each peak was accomplished in separate experiments by tandem mass spectrometry (ESI-MS/MS) performed on larger samples (13, 21).

Data Analysis. Each MS sample file consisted of approximately 60 scans (instrument sweeps of one per second for 60 s) from m/z 350 to 1200 binned in 0.07 amu. The MS scans (.Raw files) were batch converted to a nonproprietary ASCII compatible form using the vendor-supplied Windows32 executable Xconvert.exe. The ASCII files were parsed, filtered for anomalous scans, and time-averaged. A peak finding algorithm was applied to the time-averaged array. The maximum ion intensity value at 1 amu intervals was used to determine the peak maximum intensity, and the associated nominal m/z and maxima were written to a file. Peaks with the same nominal m/z value were identified as equivalent across files. Files were batch-processed using a Windows executable generated in Fortran G77 (GNU) (M. O. Byrne and H. A. Brown, unpublished data). Two types

of peak normalization were applied to each MS file. For the class peak ratio analysis, the peak intensities located at the m/z values corresponding to a particular class of glycerophospholipids were normalized to the sum of the peak intensities from all the peak intensities within the class. For the internal standard ratio analysis, all of the peaks in a spectrum were normalized to the mean peak ion intensity associated with the m/z values of the internal standards added to the samples, which ionized in the relevant instrument mode. Batch normalization of peak data was performed in S-Plus version 3.3 for the Windows software suite (MathSoft, Inc., Cambridge, MA).

Statistical Analysis. All experiments were performed at least three times using triplicate samples. In each case, the triplicate values were averaged, and then the means and standard deviations of the means from individual experiments were calculated. These values were subjected to statistical analysis by Student's t tests or ANOVA as appropriate to identify significant differences. The criterion for statistical significance was a p of <0.05 .

RESULTS

Glycerophospholipid Remodeling in Zymosan-Stimulated RPMs. Following isolation and overnight incubation, RPMs were placed in serum-free medium and challenged for various periods with zymosan. Cells were harvested for direct infusion ESI-MS analysis of total glycerophospholipids, and the data were subjected to class peak ratio analysis. Specifically, for each glycerophospholipid class, the total of the mass spectral signal intensities from all species in that class was determined, and the signal intensity from each species was calculated as the percent of that total. The analysis was limited to species that have been positively identified by ESI-MS/MS fragmentation (13). It should be noted that mass spectral signal intensity decreases with increasing chain length and degree of unsaturation of the component fatty acids (22), so the relative signal intensity data are not a direct assessment of absolute quantity and tend to underestimate the absolute quantities of long chain, polyunsaturated species. However, comparisons of these relative signal patterns do allow an assessment of differences in the distribution of individual species within a class and provide a sensitive and highly reproducible method for detecting changes in the relative quantities of individual species (23).

Figures 1–5 show the time courses of the changes in lipid species distribution for GPCho, phosphatidylethanolamine (GPEtn), GPIIns, phosphatidylserine (GPSer), and GPGro, respectively, which comprise 46, 33, 5.8, 6.3, and 6.7% of RPM phospholipids, respectively (24). In each figure, panel A provides data for zymosan-treated RPM and corresponding data for control cells (incubated in the absence of zymosan) are given in panel B. In each case, the species shown were chosen because they exhibited statistically significant ($p < 0.05$) changes over at least two consecutive time points in RPM, LPS-pretreated RPM (plates C and D), or RAW264.7 cells (plates E and F), which will be discussed below. For zymosan-stimulated cells, statistical significance was tested against both the initial time point and control cells incubated for the same time period in the absence of zymosan. A change was deemed significant only if it met both criteria. For control cells, statistical significance was tested against

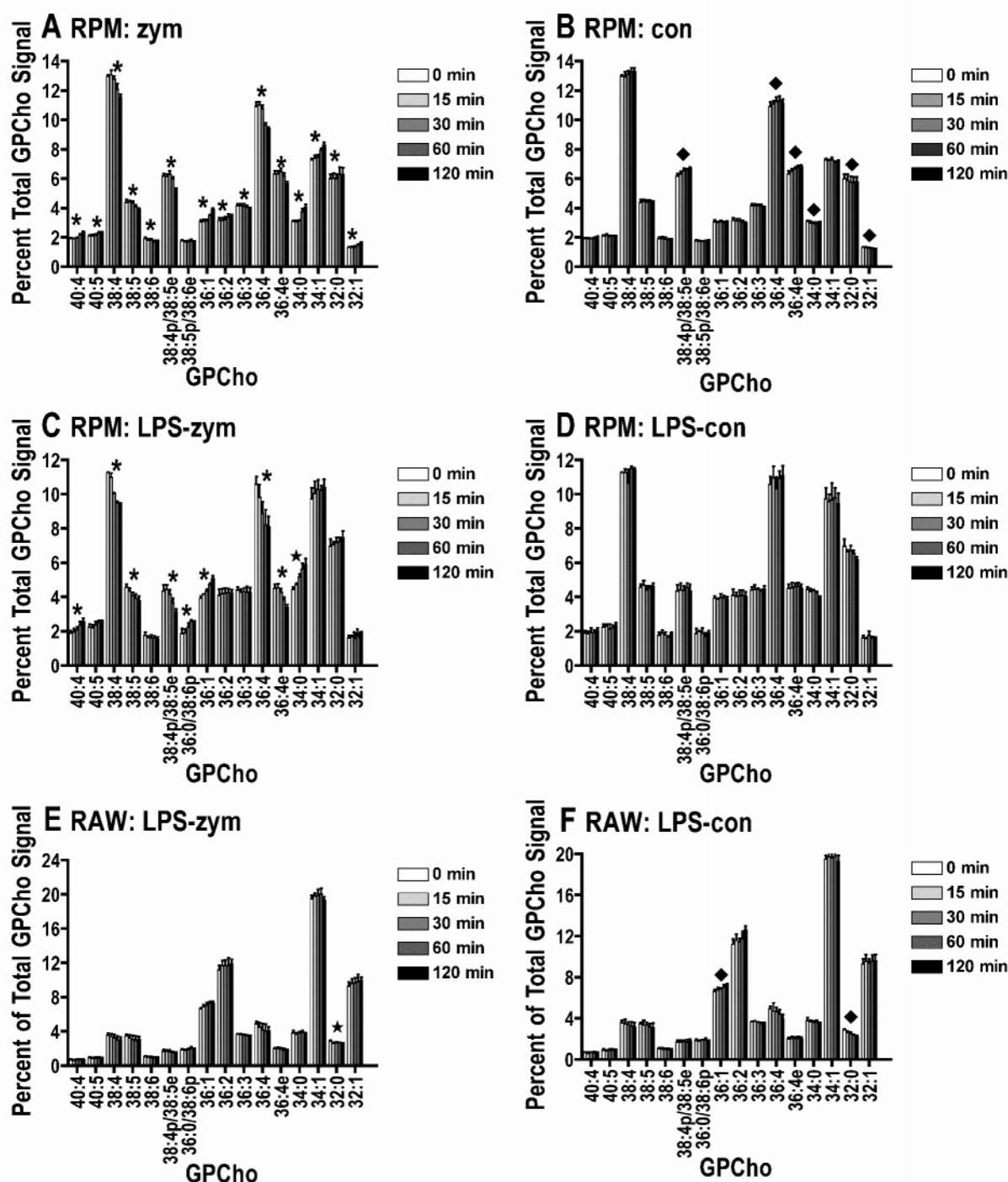


FIGURE 1: Effect of zymosan stimulation on the distribution of lipid species in GPCCho. RPMs (A–D) were isolated and incubated overnight in α -MEM/FCS (35 mm dishes). RAW264.7 cells (E and F) were plated and incubated for 22 h with 20 ng/mL GM-CSF in DMEM/FCS (60 mm dishes). Cells were washed and placed in α -MEM/FCS without (A and B) or with (C and D) 100 ng/mL LPS (RPM) or in DMEM/FCS with 100 ng/LPS, 20 ng/mL GM-CSF, and 10 ng/mL IFN- γ (RAW264.7 cells, E and F). Cells were incubated for 5 h, washed, and placed in serum-free medium with (A, C, and E) or without (B, D, and F) 160 μ g of zymosan/35 mm dish or 480 μ g of zymosan/60 mm dish. Cultures were incubated for the indicated times, and the cells were harvested for glycerophospholipid analysis by direct infusion ESI-MS. The total signal intensities of 29 distinct GPCCho species were summed, and the magnitude of the signal intensity of each species was then calculated as a percent of that total. Results are the mean \pm standard deviation of the combined results from four (A, B, E, and F) or three (C and D) separate experiments in which triplicate determinations were made. The species that are shown demonstrated statistically significant differences ($p < 0.05$) for zymosan-treated cells at two consecutive time points vs control cells (no zymosan) at the same time points and against cells at 0 min. Alternatively, species that are shown exhibited statistically significant differences ($p < 0.05$) for control cells at two consecutive time points against cells at 0 min. Data designated with a star met criteria for statistical significance in zymosan-stimulated cells at 30, 60, and 120 min. Data designated with an asterisk met criteria for statistical significance in zymosan-stimulated cells at 60 and 120 min. Data designated with a circle met criteria for statistical significance in zymosan-stimulated cells at 30 and 60 min. Data designated with a diamond met criteria for statistical significance in control cells at 60 and 120 min. Data for all 29 GPCCho species are provided in Figure S1 of the Supporting Information.

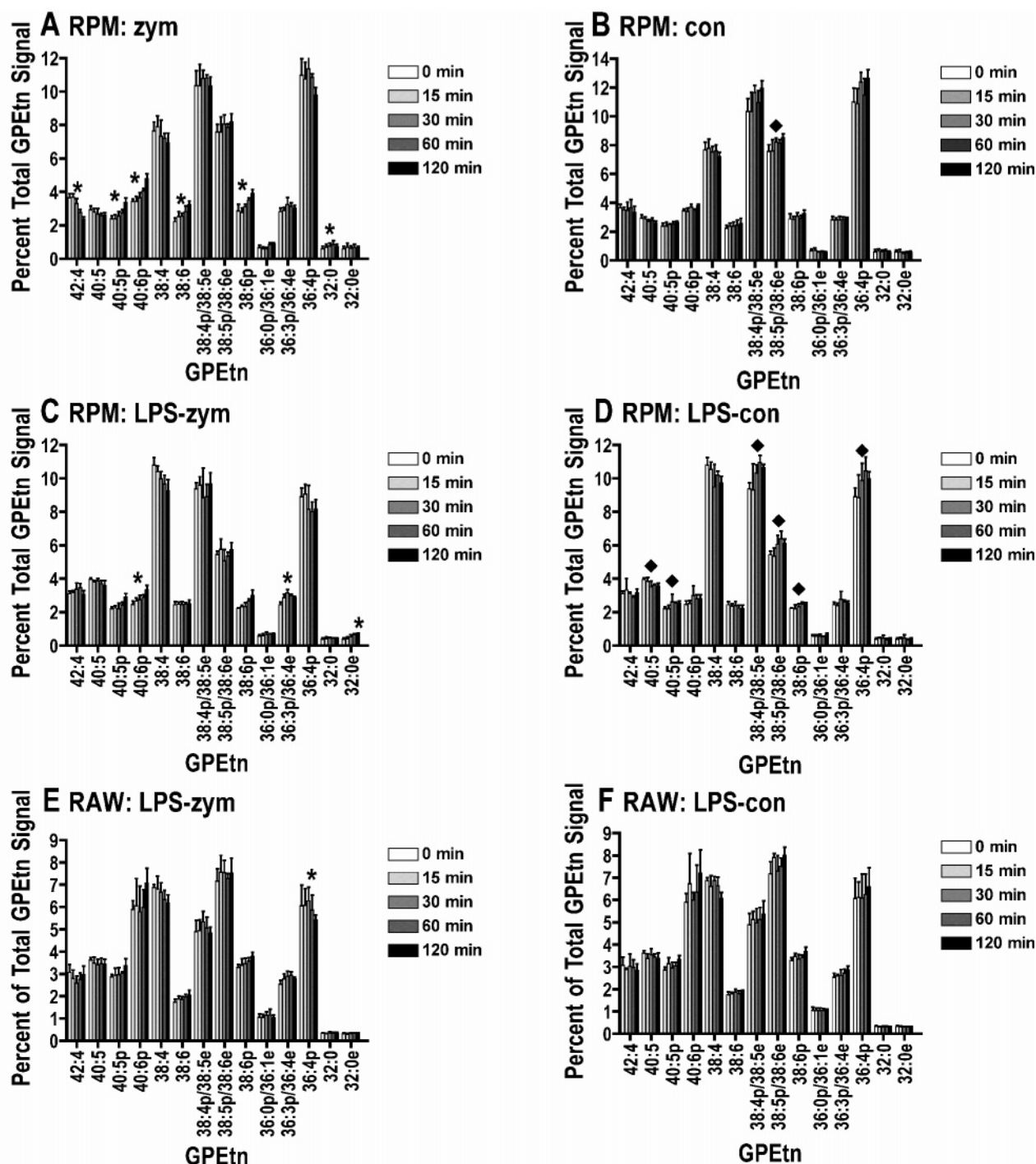


FIGURE 2: Conditions are identical to those described in the legend of Figure 1, except that data are given for GPEtn (36 distinct species analyzed). Species that are shown were chosen on the basis of the statistical significance for zymosan-stimulated or control cells and are indicated as described in the legend of Figure 1. Data for all 36 GPEtn species are provided in Figure S2 of the Supporting Information.

the initial time point only. Data for all species analyzed are provided in Figures S1–S5 of the Supporting Information. Note that in Figures 1–5, each species is designated by a number $xx:y$, where xx is the total number of carbons and y is the total number of double bonds in the two fatty acyl chains. A designation of e indicates that the fatty acid at the $sn-1$ position is ether-linked, whereas the p designation indicates a vinyl ether linkage (25). For each species, more than one combination of fatty acids may be possible. Alkyl acyl species with the composition $xx:yp$ and $xx:(y+1)e$ have the same mass and are therefore detected and listed together.

Complete tables of all species identified for each $xx:y$ designation in RPMs and RAW264.7 cells have been published previously (13).

As seen in Figure 1A, significant decreases were observed in 38:4, 38:5, 38:4p/38:5e, 36:4, and 36:4e GPCho species. All of these contain 20:4, and all remained stable or underwent a slight increase in amount in control cells during the course of the 2 h incubation (Figure 1B). In contrast, a significant decrease occurred only in the 20:4-containing 42:4 GPEtn, and no changes were observed in the major 20:4-containing 38:4, 38:4p/38:5e, 38:5p/38:6e, and 36:4p GPEtn

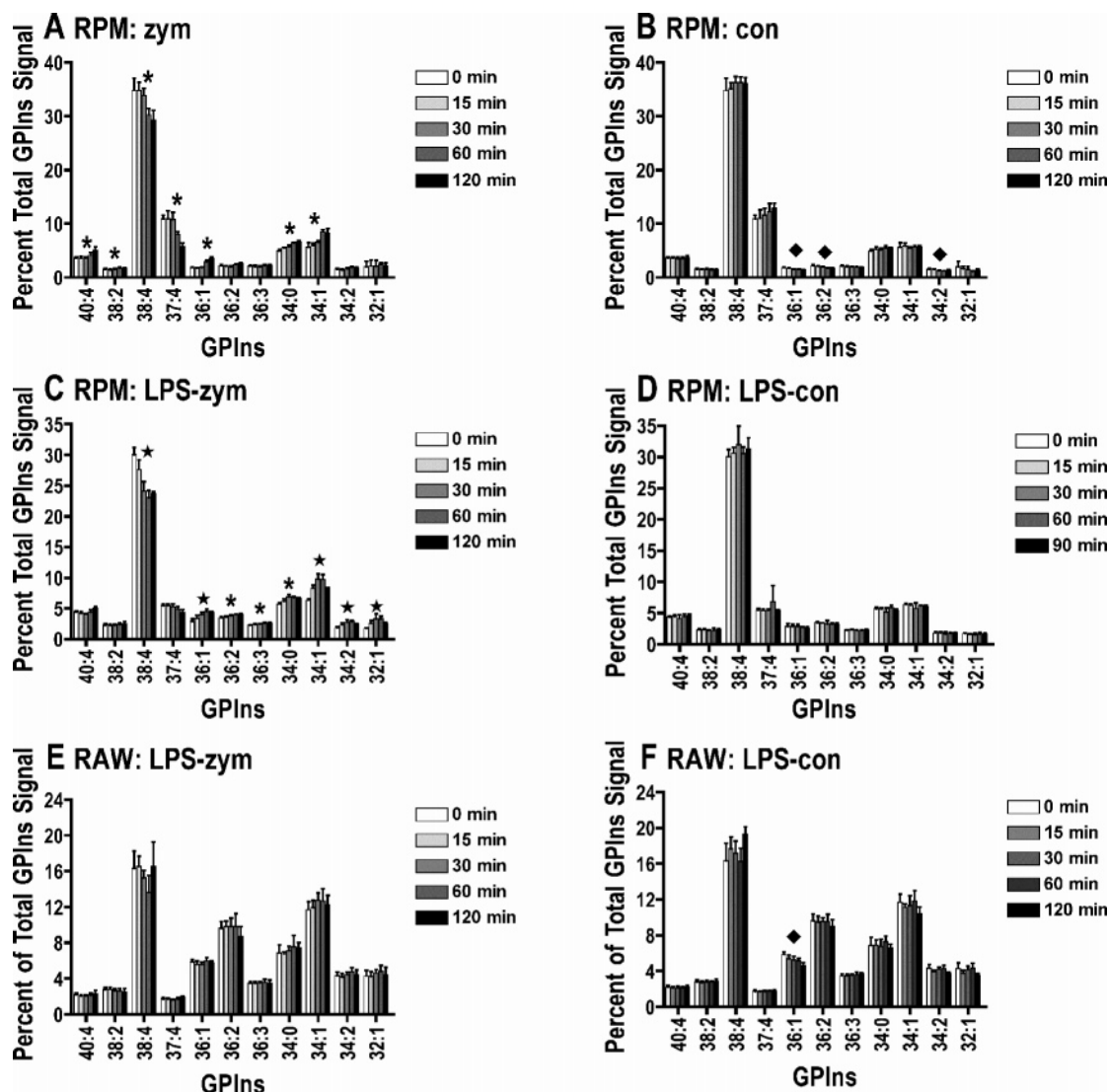


FIGURE 3: Conditions are identical to those described in the legend of Figure 1, except that data are given for GPIs (20 distinct species analyzed). Species that are shown were chosen on the basis of the statistical significance for zymosan-stimulated or control cells and are indicated as described in the legend of Figure 1. Data for all 20 GPIs species are provided in Figure S3 of the Supporting Information.

species (although 36:4p GPEtn trended down). Review of panels A and B of Figures 3–5 indicates that significant decreases occurred in 20:4-containing 38:4 and 37:4 GPIs, and 42:8, 42:9, 42:10, and 40:8 GPGro. Together, these results, combined with the high GPCho content in RPM phospholipids (24), indicate that the major pool of 20:4 that is ultimately mobilized in zymosan-stimulated RPMs is diacyl and alkyl acyl GPCho species.

Effect of LPS Pretreatment on Glycerophospholipid Remodeling. Exposure of RPMs to LPS leads to an increased level of expression of COX-2 and cPLA₂, both of which promote PG biosynthesis (26). However, LPS is itself a stimulus of PG biosynthesis, and exposure results in a 9% decrease in the level of glycerophospholipid 20:4 (13, 26). As previously reported, class peak ratio analysis indicates significant decreases in the relative proportions of 20:4-containing 38:4, 38:4p/38:5e, 36:4, and 36:4e GPCho, 36:4p GPEtn, and 38:4 GPIs as a result of LPS treatment (13). Together, these changes might be expected to affect zymosan-dependent glycerophospholipid remodeling in RPMs pretreated with LPS. We tested this hypothesis by incubating RPMs for 5 h in the presence of LPS, followed by zymosan

exposure. The LPS-pretreated cells demonstrated a statistically significant ($p < 0.001$) 21% increase in the level of zymosan-dependent PG biosynthesis from 6.5 ± 0.2 nmol/ 10^7 cells (in cells not pretreated with LPS) to 7.9 ± 0.2 nmol/ 10^7 cells (in LPS-pretreated cells). The effects of zymosan on the distribution of glycerophospholipid species in LPS-pretreated cells are shown in Figures 1C–5C. For comparison, data are presented for LPS-pretreated control cells (no zymosan) in Figures 1D–5D. In general, the overall pattern of changes is strikingly similar between LPS-pretreated RPMs and those that had not been pretreated. However, some subtle differences are notable. Because of the changes resulting from the LPS pretreatment, levels of some 20:4-containing species were lower at the beginning of the time course in LPS-pretreated cells as compared to those in cells that had not been pretreated. Decreases in the levels of 42:8, 42:9, 42:10, and 40:8 GPGro and 37:4 GPIs that were observed in the absence of LPS pretreatment were blunted in the pretreated cells. Finally, visible trends occurred at early time points in LPS-pretreated cells, consistent with the fact that PG biosynthesis also occurs with a more rapid time course as a result of LPS pretreatment (15).

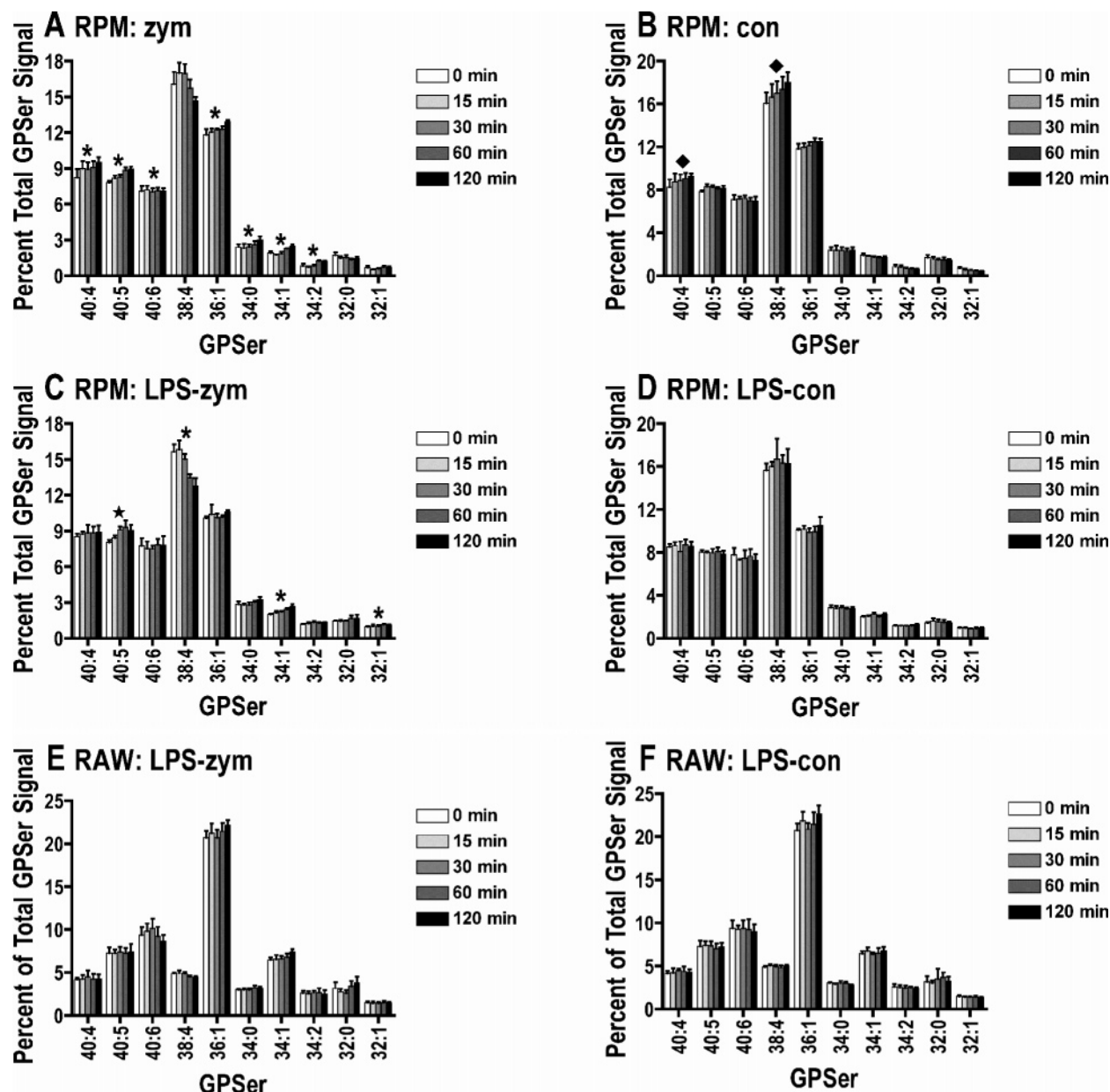


FIGURE 4: Conditions are identical to those described in the legend of Figure 1, except that data are given for GPSeR (21 distinct species analyzed). Species that are shown were chosen on the basis of the statistical significance for zymosan-stimulated or control cells and are indicated as described in the legend of Figure 1. Data for all 21 GPSeR species are provided in Figure S4 of the Supporting Information.

Comparison of Class Peak Ratio Analysis and Internal Standard Ratio Analysis. The class peak ratio method of data analysis provides data of high quality with low variance. One reason for this is that mass spectral peak intensities within a sample are compared to one another so that variability due to differences in cell number or early stages of cell harvesting between samples does not contribute to the variance. However, the use of ratio analysis raises a concern. Although it is clear that this method reliably reflects changes in the relative distribution of species within a lipid class, it is not clear whether these relative changes reflect absolute changes in the quantities of individual species. Furthermore, inspection of Figure 1A, 3A, and 5A reveals that the decreases in the levels of 20:4-containing GPCho, GPIIn, and GPGro are accompanied by relative increases in the levels of species comprised of 16- and 18-carbon fatty acids. It is of interest to know whether these changes reflect absolute increases in

these species or simply result from the fact that in the relative calculation, if some species decrease others must increase (so that the sum of all is 100%). To address this question, we conducted an experiment in which we incubated RPMs for 5 h in the presence or absence of LPS and then for 2 h in the presence or absence of zymosan. Cells were harvested for global lipid profiling, and an internal standard cocktail containing 25:0 GPCho, GPEtn, GPGro, and GPIIn was added during the sample workup. Mass spectral data were analyzed by class peak ratio analysis and by calculating the ratio of the absolute peak intensity for each species, relative to that of the internal standards as described in Experimental Procedures. The latter internal standard ratio method allows a comparison of the absolute quantity of a given species between samples. The data, shown in Figure 6 for class peak ratio analysis and in Figure 7 for the internal standard ratio method, clearly indicate that the majority of zymosan-

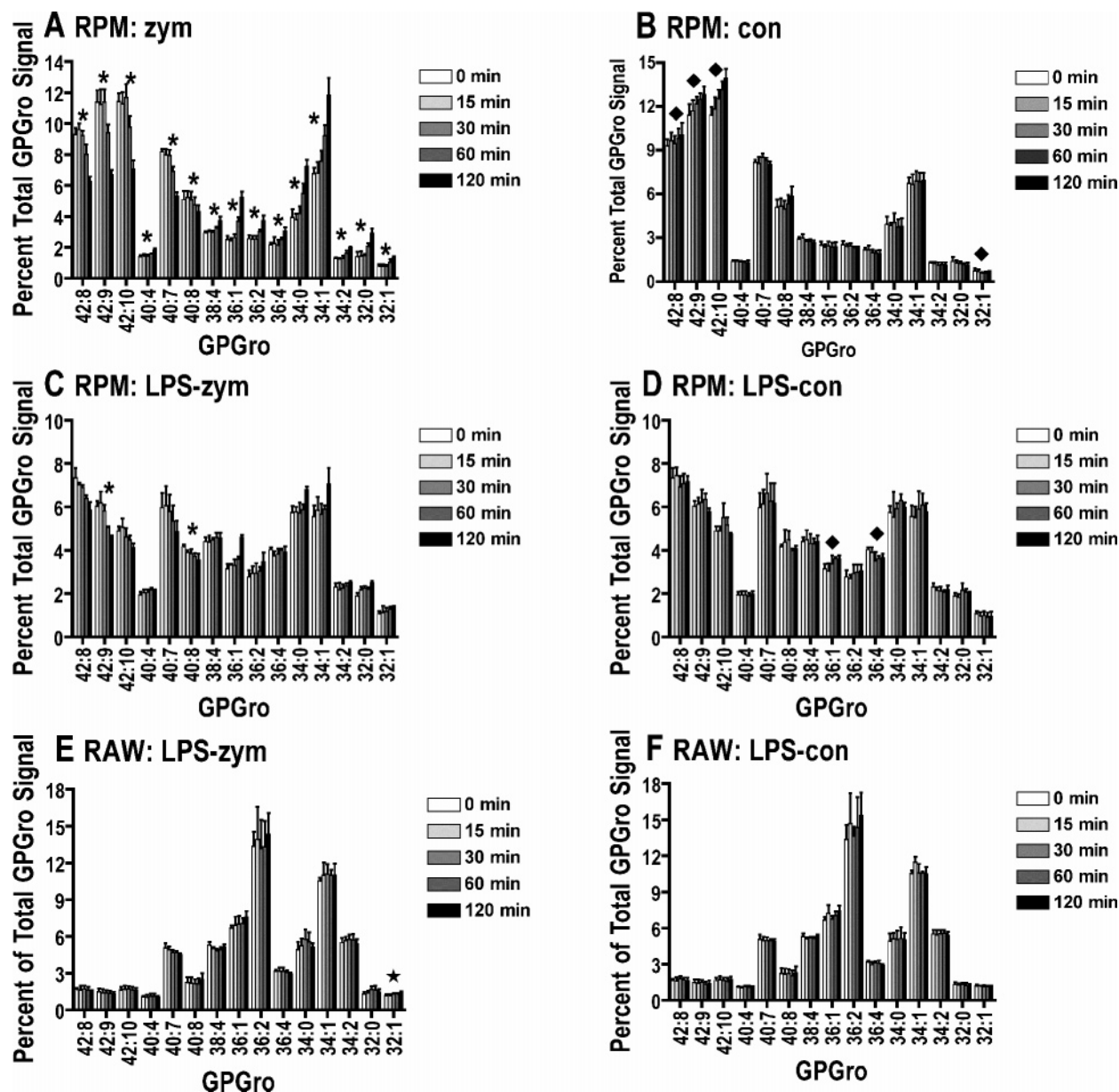


FIGURE 5: Conditions are identical to those described in the legend of Figure 1, except that data are given for GPGro (25 distinct species analyzed). Species that are shown were chosen on the basis of the statistical significance for zymosan-stimulated or control cells and are indicated as described in the legend of Figure 1. Data for all 25 GPGro species are provided in Figure S5 of the Supporting Information.

dependent decreases in the levels of 20:4-containing species that are detected by peak ratio analysis reflect changes in absolute quantities of lipids. This is not uniformly true for differences that result from LPS treatment. For example, peak ratio analysis detects statistically significant differences in the levels of 36:4 GPCho and 38:4 GPIs between untreated cells and cells treated with LPS alone (★) and between zymosan-treated cells in the presence versus absence of LPS pretreatment (●). These differences are not detected by the internal standard ratio analysis. It is also evident that many of the increases in lipid species detected by ratio analysis are either less pronounced or not significant for the internal standard ratio method. Examples are 36:2, 34:1, and 32:0 GPCho, the levels of all of which appear to increase significantly with zymosan treatment by class peak ratio analysis, but not by the internal standard ratio method. As expected, the variances are larger using the internal standard ratio method as opposed to class peak ratio analysis, and

this certainly contributes to the smaller number of changes detected as significant. However, it is also likely that the mathematical treatment used in peak ratio analysis overestimates the magnitude of the changes for the reasons mentioned above.

The data in Figures 6 and 7 provide a direct comparison of the changes resulting from LPS pretreatment and zymosan stimulation at the final time points. Note that some changes are designated as statistically significant on these figures that are not shown to be significant in Figures 1–5 because in Figures 6 and 7, a single time point was compared whereas significance criteria for Figures 1–5 were more stringent. In general, there is excellent agreement between the class peak ratio method and the internal standard ratio method of data analysis with regard to identification of 20:4-containing lipid species that undergo a decrease in amount in response to zymosan. Both methods suggest increases in the levels of 16- and 18-carbon fatty acid-containing lipids; however, since

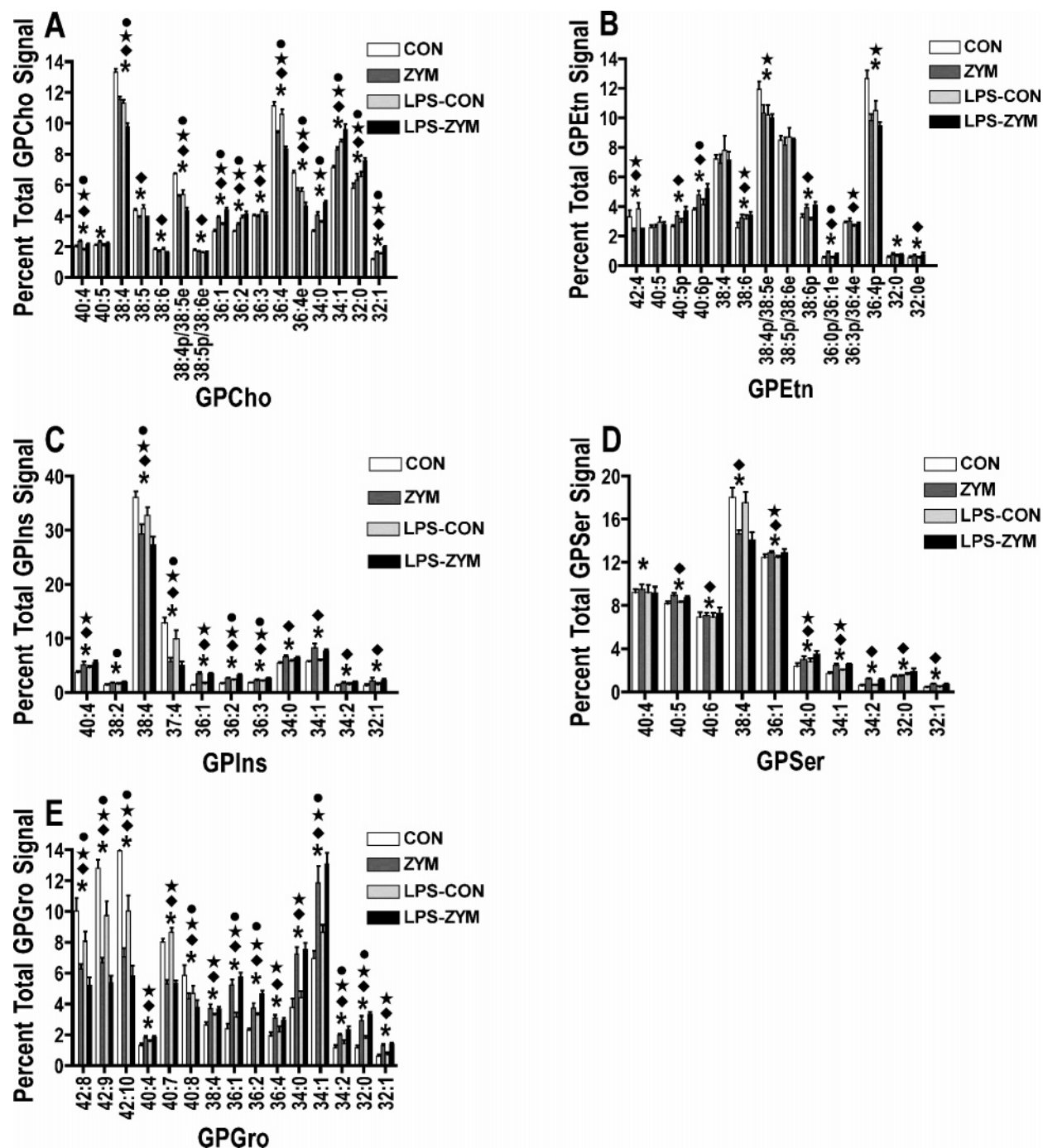


FIGURE 6: Effect of LPS and zymosan on the distribution of species within major glycerophospholipid classes in RPMs as detected by class peak ratio analysis. RPMs that had been isolated and incubated overnight (35 mm dishes) were transferred to fresh α -MEM/FCS with or without 100 ng/mL LPS. Cells were incubated for 5 h, washed, and overlaid with serum-free medium with or without 160 μ g of zymosan. Cells were incubated for 2 h and then harvested for global lipid analysis by direct infusion ESI-MS. Data were analyzed by the class peak ratio analysis method. Results are the mean \pm standard deviation from four separate experiments in which triplicate determinations were made. Species labeled with an asterisk showed significant differences between cells not pretreated with LPS or zymosan (CON) and cells not pretreated with LPS but exposed to zymosan (ZYM). Species labeled with a diamond showed significant differences between cells pretreated with LPS but not zymosan (LPS-CON) and cells pretreated with LPS and exposed to zymosan (LPS-ZYM). Species labeled with a star showed significant differences between CON and CON-LPS cells. Species labeled with a circle showed significant differences between ZYM and LPS-ZYM cells.

many of these changes are small and/or not statistically significant by the internal standard ratio method, it is not clear that they represent actual increases in absolute quantities of lipid species. Clearly, however, the class peak ratio analysis detects changes in relative lipid composition, which may be as important as absolute changes in determining the functional status of the macrophage membrane.

Effect of COX Inhibition on Zymosan-Dependent Glycerophospholipid Remodeling. Glycerophospholipid composition is the result of a dynamic balance between synthesis and degradation of individual species. During zymosan phagocytosis, the decrease in the level of 20:4-containing glycerophospholipid species indicates that the degradation rate has increased and/or the synthesis rate has decreased,

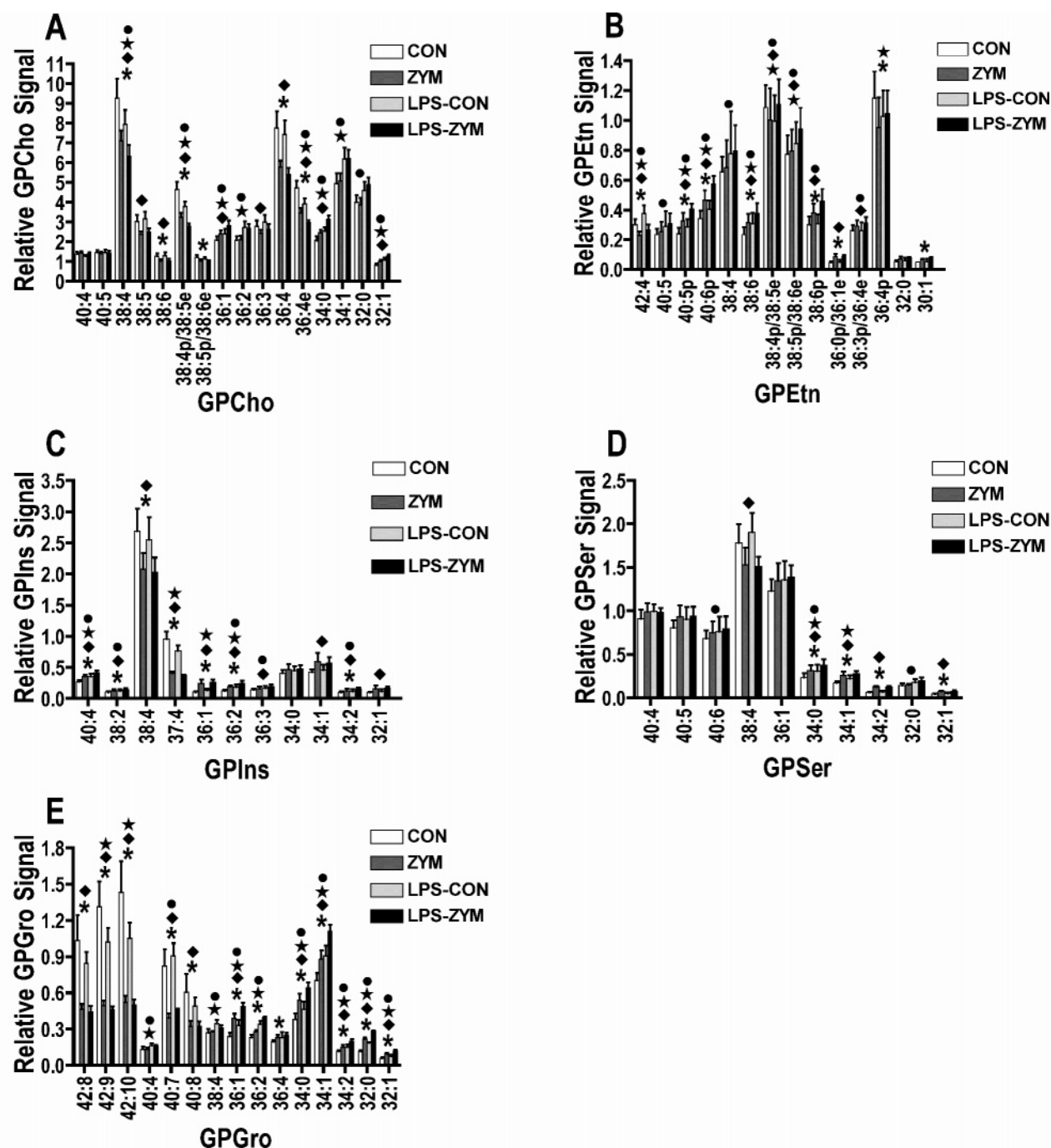


FIGURE 7: Effect of LPS and zymosan on the distribution of species within major glycerophospholipid classes in RPMs as detected by internal standard ratio analysis. Experimental conditions are exactly as described in the legend of Figure 6, except that MS data were analyzed by the internal standard ratio method.

leading to an imbalance and a net loss. Under our experimental conditions, there is no source of exogenous 20:4 during the zymosan exposure period. Consequently, the use of 20:4 for eicosanoid biosynthesis must contribute to the net loss of 20:4-containing glycerophospholipid species, as this component is removed from the pool of 20:4 available for glycerophospholipid synthesis. To determine the degree to which PG biosynthesis contributes to the alterations in the distribution of glycerophospholipid species observed in zymosan-stimulated RPMs, we carried out an experiment in which cells were incubated with zymosan in the presence and absence of 5 μ M indomethacin, which effected a 97% inhibition of PG biosynthesis (data not shown). For these

experiments, LPS-pretreated cells were used to maximize PG biosynthesis and its contribution to glycerophospholipid remodeling. The results (Figure 8) demonstrate that indomethacin significantly reduced the decreases in the relative amounts of 38:4, 38:5, and 36:4 GPCho (by 37, 48, and 45%, respectively), 38:4 GPIs (by 35%), and 42:9, 42:10, and 40:8 GPGro (by 13.5, 25, and >100%, respectively). Notably, indomethacin had no effect on decreases in the levels of 38:4p/38:5e and 36:4e GPCho.

Effect of Thimerosal on Zymosan-Dependent Glycerophospholipid Remodeling. 20:4 is incorporated into glycerophospholipids by lysophosphatidylcholine acyltransferase (CoA-dependent) (EC 2.3.1.23) which transfers 20:4 from

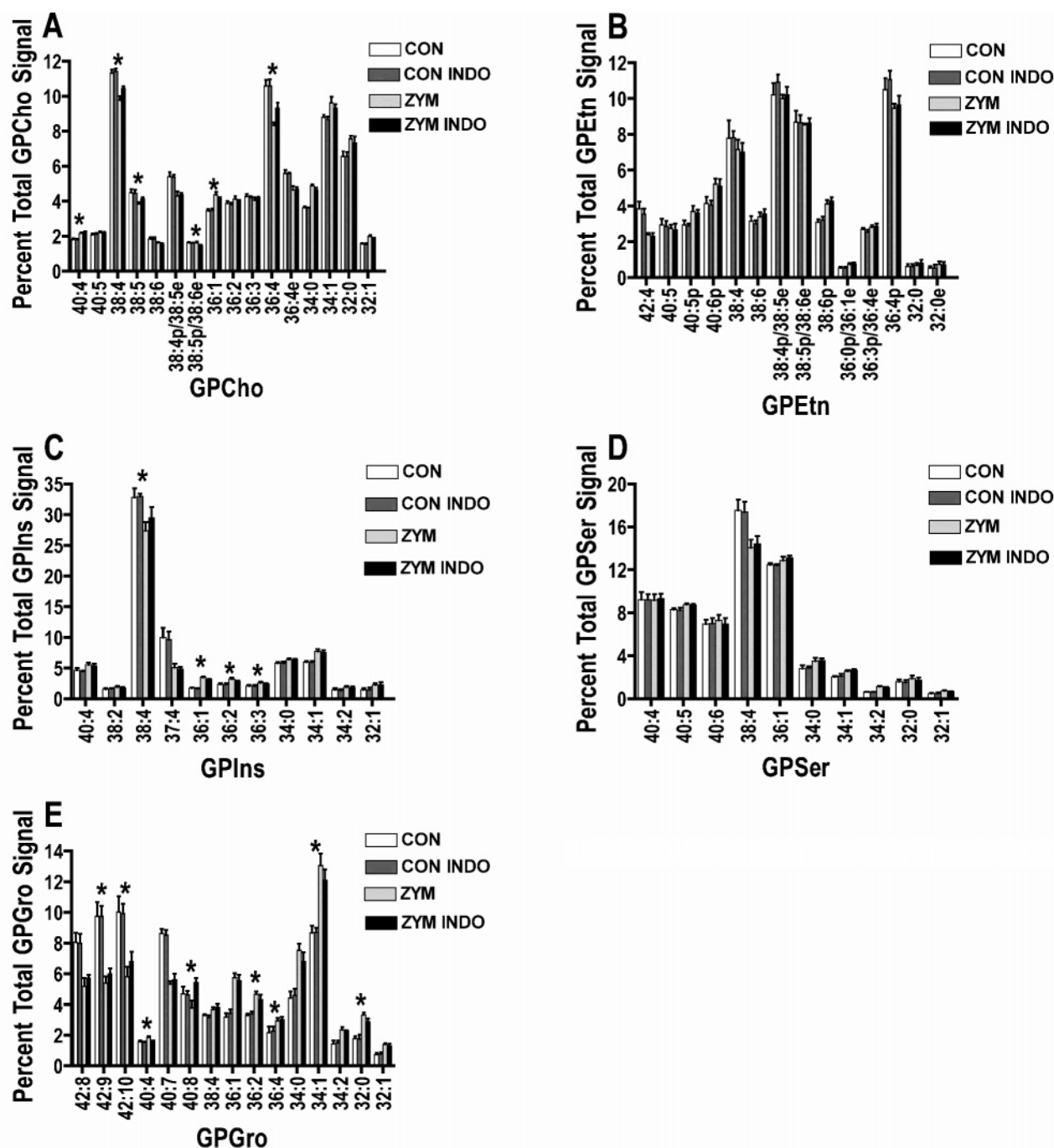


FIGURE 8: Effect of indomethacin on glycerophospholipid remodeling in response to zymosan. RPMs that had been isolated and incubated overnight (35 mm dishes) were transferred to fresh α -MEM/FCS containing 100 ng/mL LPS. Cells were incubated for 4.5 h, and then indomethacin was added as a stock solution in DMSO to yield a final concentration of 5 μ M. Control cells received DMSO at the same final concentration (0.1%). Cells were incubated for an additional 30 min and were then washed and overlaid with serum-free medium with or without indomethacin and 160 μ g of zymosan. Cells were incubated for 2 h and then harvested for global lipid analysis by direct infusion ESI-MS. Data were analyzed by the class peak ratio analysis method. Results are the mean \pm standard deviation from four separate experiments in which triplicate determinations were made for cells incubated in the absence of indomethacin or zymosan (CON), cells incubated with indomethacin but not zymosan (CON INDO), cells incubated with zymosan in the absence of indomethacin (ZYM), and cells incubated with zymosan in the presence of indomethacin (ZYM INDO). Species labeled with an asterisk showed significant differences between ZYM and ZYM INDO cells.

CoA to a lyso-GPCho or lyso-GPIIns. 20:4 is transferred between phospholipids primarily by glycerophospholipid arachidonoyltransferase (CoA-independent) (EC 2.3.1.147) (27, 28). One or more of these pathways may function in zymosan-stimulated cells to reincorporate free 20:4 that is not used for eicosanoid synthesis into glycerophospholipids

and replenish specific 20:4-containing pools. Thimerosal is a mercury-containing salicylic acid derivative that has been shown to covalently modify protein sulfhydryl groups. It is a well-established inhibitor of the CoA-dependent acyltransferase and has been reported to inhibit CoA-independent acyltransferase in some tissues (29, 30). It also induces

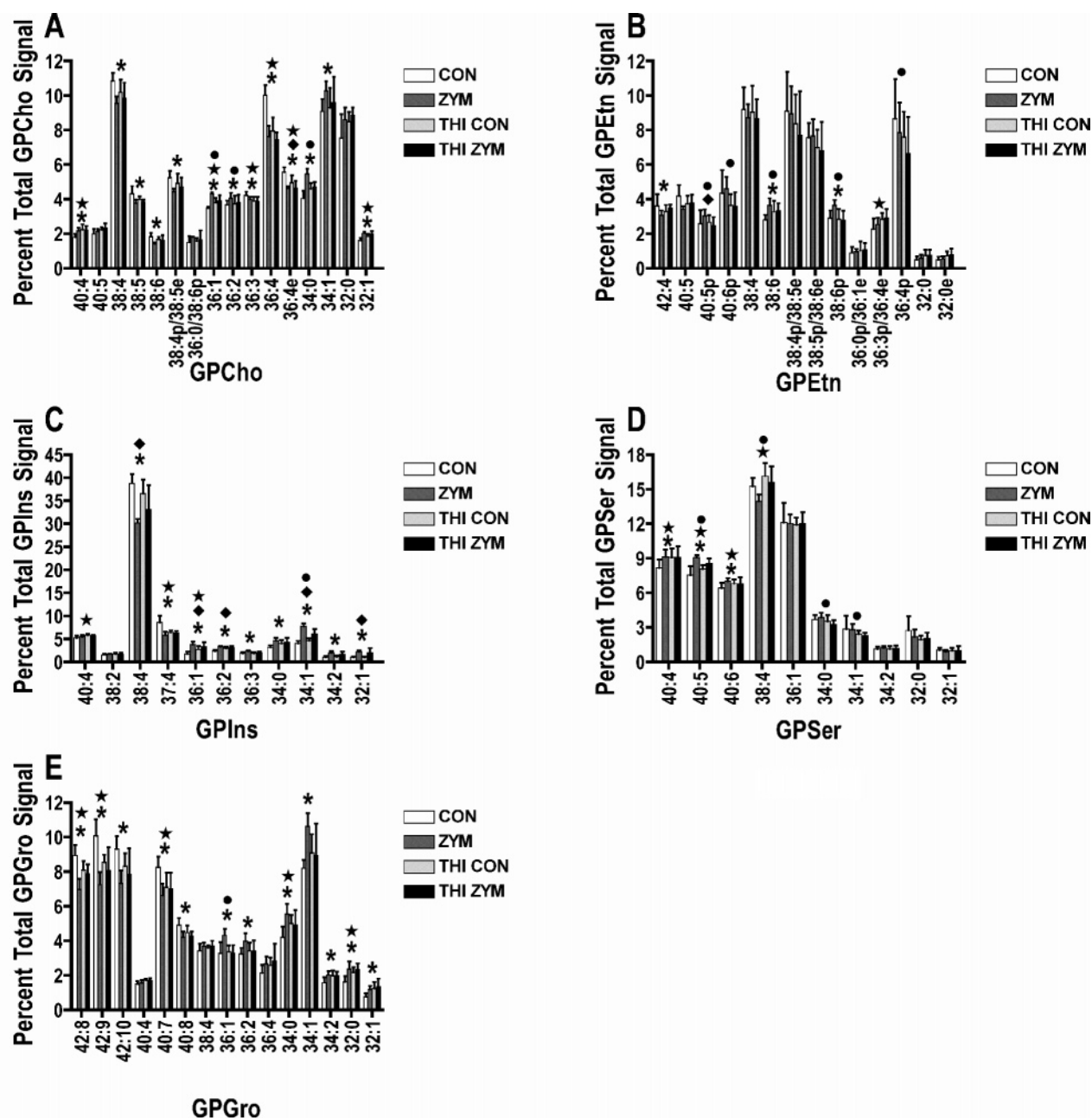


FIGURE 9: Effect of thimerosal on glycerophospholipid remodeling in response to zymosan. RPMs that had been isolated and incubated overnight (35 mm dishes) were transferred to fresh α -MEM/FCS with or without 100 ng/mL LPS. Cells were incubated for 5 h, washed, and overlaid with serum-free medium with or without 5 μ M thimerosal (dissolved directly into medium). After a 5 min incubation, zymosan (160 μ g) was added as indicated. Cells were incubated for 2 h and then harvested for global lipid analysis by direct infusion ESI-MS. Data were analyzed by the class peak ratio analysis method. Results are the mean \pm standard deviation from four separate experiments in which triplicate determinations were made for cells incubated in the absence of thimerosal or zymosan (CON), cells incubated with thimerosal but not zymosan (THI CON), cells incubated with zymosan in the absence of thimerosal (ZYM), and cells incubated with zymosan in the presence of thimerosal (THI ZYM). Species labeled with an asterisk showed significant differences between CON and ZYM cells. Species labeled with a diamond showed significant differences between THI CON and THI ZYM cells. Species labeled with a star showed significant differences between CON and CON-THI cells. Species labeled with a circle showed significant differences between ZYM and THI ZYM cells.

intracellular calcium spikes in some cells and has been shown to stimulate leukotriene and prostaglandin biosynthesis in leukocyte populations, including RPMs (31–36). To determine the degree to which 20:4 reincorporation contributes to glycerophospholipid remodeling in zymosan-stimulated RPMs, we pretreated cells for 5 h with LPS and then challenged them with zymosan for 2 h in the presence or absence of 5 μ M thimerosal. The results (Figure 9) showed

that in the absence of zymosan, thimerosal caused significant decreases in the levels of 20:4-containing 36:4 and 36:4e GPCCho, 37:4 GPIs, and 42:8 and 42:9 GPGro. Decreases were also observed in other 20:4-containing species that did not reach statistical significance. In contrast, thimerosal had relatively little effect on the distribution of lipids in zymosan-stimulated cells, with the notable exception of some 20:4-containing plasmenyl GPEtn species (38:6p and 36:4p),

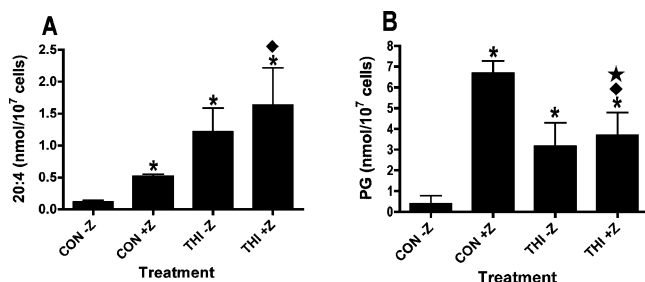


FIGURE 10: Effect of thimerosal on 20:4 levels and PG synthesis in RPMs. Experimental conditions were exactly as described in the legend of Figure 9, except that the cells were incubated for 1 h after zymosan addition. Cells were harvested for analysis of 20:4 content, and medium was harvested for analysis of PGs. Results are the mean \pm standard deviation from three separate experiments in which triplicate determinations were made for cells incubated in the absence of thimerosal or zymosan (CON -Z), cells incubated with thimerosal but not zymosan (THI -Z), cells incubated with zymosan in the absence of thimerosal (CON+Z), and cells incubated with zymosan in the presence of thimerosal (THI +Z). Bars labeled with an asterisk showed significant differences from CON -Z cells. Bars labeled with a diamond showed significant differences from CON +Z cells. Bars labeled with a star showed significant differences from THI -Z cells.

which exhibited statistically significant decreases in RPMs treated with zymosan in the presence as opposed to the absence of thimerosal.

Figure 10 shows the effects of thimerosal on PG synthesis and 20:4 levels in zymosan-treated and control cells. Thimerosal alone caused an 11-fold increase in 20:4 levels, as compared to a 4.3-fold increase observed with zymosan alone. Addition of zymosan to thimerosal-treated cells further increased 20:4 levels, though due to the high variance between experiments, this increase was not significant. Thimerosal alone caused a stimulation of PG formation, although levels reached were only 47% as high as those observed in zymosan-treated cells, even though thimerosal treatment effected higher levels of 20:4 than did zymosan alone. Addition of zymosan to thimerosal-treated cells resulted in a small (1.2-fold) but statistically significant increase in the level of PG formation.

Zymosan-Dependent PG Synthesis in RAW264.7 Cells. We have previously shown that RAW264.7 cells contain lower levels of 20:4 than RPMs do and that this is reflected in a markedly different distribution of species among phospholipid classes. Furthermore, RAW264.7 cells failed to demonstrate significant changes in the distribution of glycerophospholipid species when exposed to LPS, as observed in RPMs (13). To determine if these apparent differences between RAW264.7 cells and RPMs would extend to the zymosan response, we investigated PG biosynthesis and glycerophospholipid remodeling in zymosan-stimulated RAW264.7 cells. In the absence of any pretreatment, RAW264.7 cells exposed to a maximal zymosan stimulus produced 73 ± 16 pmol of PGs/ 10^7 cells over a 2 h period, representing 1.1% of the level produced by RPMs under the same conditions. One obvious reason for the quantitative differences in PG formation between RAW264.7 cells and RPMs lies in the fact that RPMs constitutively express large quantities of COX-1 whereas RAW264.7 cells do not (37). Under conditions in which cells are not pretreated to induce the expression of COX-2, its levels are very low, and PG formation is essentially COX-1-dependent. Clearly, RAW264.7

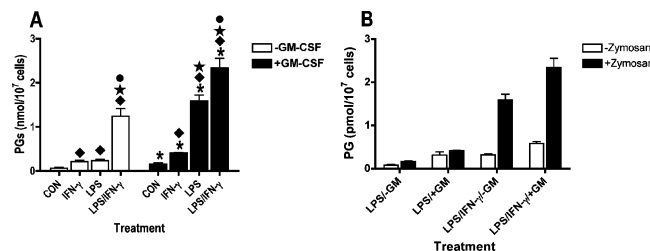


FIGURE 11: Effect of GM-CSF, LPS, and IFN- γ on PG synthesis in RAW264.7 cells. (A) RAW264.7 cells (35 mm dishes) were preincubated for 22 h in the presence (+GM-CSF) or absence (-GM-CSF) of 20 ng/mL GM-CSF. The cells were then transferred to fresh DMEM/FCS containing no additional stimulus (CON), 10 ng/mL IFN- γ (IFN- γ), 100 ng/mL LPS (LPS), or LPS with IFN- γ (LPS/IFN- γ). The medium also contained GM-CSF in the case of cells preincubated with that cytokine. Following a 5 h incubation, the cells were washed and transferred to fresh, serum-free DMEM. Zymosan (160 μ g) was then added, and the cells were incubated for 2 h prior to the medium being harvested for PG analysis. (B) Cells were preincubated for 22 h with (+GM) or without (-GM) GM-CSF and then for 5 h with LPS alone or with LPS and IFN- γ . The cells were then washed and placed in fresh, serum-free DMEM with or without zymosan as indicated. Following a 2 h incubation, the medium was harvested for PG analysis. All data are the mean \pm standard deviation from a representative of three experiments in which triplicate determinations were made. In panel A, bars labeled with an asterisk indicate that PG synthesis in GM-CSF-treated cells was significantly different from that in cells receiving the same treatment, but in the absence of GM-CSF pretreatment. Bars labeled with a diamond showed significant differences from CON cells exposed to the same GM-CSF pretreatment. Bars labeled with a star showed significant differences from IFN- γ cells exposed to the same GM-CSF pretreatment. Bars labeled with a circle showed significant differences from LPS cells exposed to the same GM-CSF pretreatment.

cells require pretreatment with an agent such as LPS that induces expression of COX-2 prior to zymosan addition for observation of significant PG formation. This is in contrast to RPMs, which produce large quantities of PGs regardless of prior LPS exposure. Furthermore, zymosan-dependent 20:4 mobilization and PG formation require interaction with the dectin-1 receptor, which is poorly expressed on RAW264.7 cells (38, 39). GM-CSF has been shown to induce an increased level of expression of dectin-1 in murine macrophages (40). In addition, we have shown that pretreatment of RAW264.7 cells with GM-CSF and cotreatment with IFN- γ augment PG synthesis in response to LPS (13). Consequently, we explored the effects of these cytokines and LPS pretreatment on zymosan-dependent PG synthesis in RAW264.7 cells.

RAW264.7 cells were preincubated for 22 h with or without GM-CSF and then for an additional 5 h with or without LPS and/or IFN- γ . The cells were then washed, transferred to fresh, serum-free medium, and exposed to zymosan for 2 h. Figure 11A shows that LPS pretreatment (5 h) augmented the formation of PGs in response to subsequent zymosan phagocytosis and that substantially greater increases were obtained with the combination of LPS and IFN- γ . Pretreatment of the cells with GM-CSF prior to LPS and IFN- γ elicited a greater zymosan-dependent PG synthetic response under all conditions. When cells were treated with LPS for 5 h, they exhibited a significant ongoing level of PG synthesis even in the absence of zymosan exposure. These background levels of PG synthesis were higher if the pretreatment regimen included IFN- γ and GM-

CSF (Figure 11B). Thus, the levels of PGs formed in response to zymosan must be interpreted against this background level of synthesis that results from the pretreatment regimen.

During the course of these studies, we noted that the relative effects of the various pretreatments remained consistent but that the quantities of PGs formed in response to zymosan varied widely, depending on the length of time that the RAW264.7 cells had been maintained in culture. For example, under maximal synthetic conditions (pretreatment with GM-CSF, LPS, and IFN- γ), cells that had been maintained in culture for 2 days produced 3700 ± 100 pmol of PGs/ 10^7 cells, whereas cells that had been maintained in culture for 30 days produced 1000 ± 100 pmol of PGs/ 10^7 cells (mean \pm the standard deviation; $n = 3$). Consequently, all remaining studies were carried out using cells that had been maintained in culture for fewer than 10 days (four to five passages following thawing). Under these conditions, maximally stimulated RAW264.7 cells produced 3800 ± 700 pmol of PGs/ 10^7 cells in response to a 2 h zymosan exposure, as compared to similarly pretreated cells incubated in the absence of zymosan, which produced 800 ± 150 pmol of PGs/ 10^7 cells (mean \pm standard deviation from four separate experiments in which triplicate determinations were made).

20:4 Mobilization and Glycerophospholipid Remodeling in Zymosan-Stimulated RAW264.7 Cells. To maximize the response to zymosan, RAW264.7 cells were pretreated for 22 h with GM-CSF followed by 5 h with LPS and IFN- γ . The cells were then washed, transferred to fresh serum-free medium, and exposed to zymosan for varying time periods. The cells were harvested for global lipid profiling. Figures 1–5 (panels E and F) provide results for GPCho, GPEtn, GPIs, GPSer, and GPGro, respectively, which comprise 46, 22, 6.8, 6.2, and 6.3% of the phospholipids of RAW264.7 cells, respectively (41). Zymosan stimulation effected essentially no significant changes in 20:4-containing glycerophospholipids in RAW264.7 cells with the notable exception of a decrease in the amount of 38:4p GPEtn. The lack of major changes in 20:4-containing glycerophospholipid species correlated with the small decrease in glycerophospholipid 20:4 content in zymosan-stimulated RAW cells (from 7.4 ± 1.2 to 5.5 ± 1.3 mol %) as compared to RPMs (from 23 ± 1 to 16 ± 1 mol %) as determined by fatty acid analysis.

DISCUSSION

Mass Spectrometry-Based Global Lipid Profiling. The results presented here demonstrate that ESI-MS-based global lipid profiling with class peak ratio analysis offers a reliable method for characterizing the distribution of the vast array of glycerophospholipid species within a cell and for identifying species that change in response to cellular perturbations. Comparison of results obtained by class peak ratio analysis with those obtained by the internal standard ratio method demonstrates that the majority of changes detected by ratio analysis correlate with changes in absolute quantities of lipid species. However, the small variance in data obtained with ratio analysis makes this method more sensitive for the identification of changes in lipid species in discovery phase experiments. Although this approach does not offer absolute quantification of individual species, it provides a highly

sensitive and rapid method for monitoring changes in the cellular lipidome to target key species for subsequent quantitative analysis.

Zymosan-Dependent Glycerophospholipid Remodeling in RPM. Application of this global lipid profiling methodology in identifying changes in glycerophospholipids of RPMs during zymosan phagocytosis indicates decreases in the levels of all major 20:4-containing GPCho species, as well as some GPIs and GPGro species. Pretreatment of the cells with LPS leads to some differences in the time course and magnitude of the zymosan-dependent glycerophospholipid changes, but the overall pattern is very similar to that observed in cells not previously exposed to LPS. This is consistent with the relatively modest effect of LPS pretreatment on PG synthesis in these cells.

The decreases in the levels of 20:4-containing lipids resulting from zymosan stimulation are accompanied by increases in species containing 16- and 18-carbon fatty acids as assessed by class peak ratio analysis. These increases are generally smaller in magnitude than the decreases observed in 20:4-containing species and are not uniformly detected by the internal standard ratio method. Clearly, cPLA₂-dependent glycerophospholipid hydrolysis generates lysophospholipids that are either further metabolized or reacylated. Since 20:4 is being consumed for eicosanoid biosynthesis, reacylation must be achieved with other available fatty acids. Similarly, de novo synthesis of lipids to replace those metabolized will require incorporation of fatty acids other than 20:4, at least in the short term. The major fatty acids in RPMs are 20:4, 18:0, and 16:0, so it is not surprising that levels of lipids containing 16- and 18-carbon fatty acids would increase during zymosan stimulation. It should be noted, however, that under these serum-free conditions, there is no exogenous source of fatty acid to replace the 20:4 used for eicosanoid biosynthesis. Consequently, it is likely that a net reduction in the total glycerophospholipid content of the cells results from zymosan treatment.

Role of PG Biosynthesis in Glycerophospholipid Remodeling. When RPMs were stimulated with zymosan in the presence of indomethacin to block PG synthesis, the decreases in the levels of 20:4-containing diacyl GPCho, GPIs, and GPGro species were partially inhibited. This result confirms that at least some of the glycerophospholipid remodeling that occurs during zymosan phagocytosis is directly related to PG biosynthesis, as would be expected since PG formation irreversibly removes 20:4 from the glycerophospholipid pool. A likely explanation for the effect of indomethacin is that 20:4 released by cPLA₂ in response to zymosan is reincorporated into diacyl glycerophospholipid species if it is not used for eicosanoid biosynthesis. If this is correct, then the lack of an effect of indomethacin on zymosan-dependent decreases in alkyl acyl GPCho species may be due to the utilization of these species for platelet activator biosynthesis (42), or to a slower rate of reacylation of these species. Furthermore, it is possible that the effects of indomethacin observed in this experiment underestimate the contribution of PG biosynthesis to glycerophospholipid remodeling, since some 20:4 not used for PG biosynthesis may be shunted to the lipoxygenase pathway rather than to reacylation (43). Alternatively, it should be noted that we cannot rule out the possibility that PG synthesis inhibition

in some way interferes with mobilization of 20:4 from the diacyl species or that indomethacin directly inhibits this process.

Role of Reacylation in Glycerophospholipid Remodeling. In the absence of more specific inhibitors, thimerosal has been used to evaluate the role of reacylation in glycerophospholipid remodeling, particularly with regard to eicosanoid biosynthesis. Thimerosal treatment of RPMs in the absence of a stimulus results in a decrease in the levels of some 20:4-containing GPCho and GPGro species. Since the level of any species depends on the balance in the rates of synthesis versus catabolism, the decreases in these lipids in the presence of a synthesis inhibitor suggest a rapid baseline rate of turnover. It should be noted, however, that thimerosal has been shown to increase intracellular calcium levels in some cells, which may lead to activation of cPLA₂ or other calcium-dependent lipases. If this has occurred, then the decrease in the levels of these species in the presence of thimerosal may overestimate their rate of turnover under the true steady state condition. Nevertheless, the fact that some of the most metabolically active species include 20:4-containing GPGro's and that these same species change dramatically during zymosan stimulation suggests a metabolic activity that has not been previously recognized for this lipid class. Thimerosal treatment did not have a significant effect on the magnitude of the changes in 20:4-containing species that decrease in response to zymosan. This latter finding suggests that thimerosal-inhibitable processes do not play a major role in reacylation of these species during the zymosan response. Clearly, in zymosan-stimulated cells, the diversion of mobilized 20:4 into eicosanoid biosynthesis prevents its reacylation into glycerophospholipids. The degree to which other processes may act to prevent reacylation is a subject for further study.

Earlier investigations of stimulus-dependent glycerophospholipid remodeling in leukocytes have resulted in conflicting results. Experiments employing radiolabeled 20:4 indicate that alkyl acyl GPCho and diacyl GPCho species are the primary source of 20:4 in rabbit alveolar macrophages treated with opsonized zymosan (44). However, when longer pre-labeling periods were used, alkyl acyl GPEtn was detected as the primary source of 20:4 in these cells (45). A similar approach using a long labeling period in human lung macrophages showed depletion of 20:4 in the following order following exposure to ionophore A23187: GPCho ≥ GPIIn/GPSer ≥ GPEtn (46). Experiments utilizing the incorporation of ¹⁸O from H₂¹⁸O indicated transacylation of 20:4, which was most rapid in GPCho and GPIIn, suggesting rapid remodeling in these species in ionophore A23187-stimulated mouse peritoneal macrophages (47). Direct mass measurement of lipids indicated that the major source of 20:4 mobilized in ionophore A23187-stimulated human neutrophils was alkyl acyl GPEtn species (48), while parallel experiments using radiolabeled 20:4 suggested that GPCho was the major species remodeled in these cells. This result suggested that the radiolabel was incorporated into highly labile pools leading to spurious conclusions. Further studies combining radiolabeling and direct mass measurements indicated that the specific activity of leukotriene B₄ produced by the neutrophils most closely matched that of alkyl acyl GPCho, but that release of 20:4 from this pool was followed by reacylation using alkyl acyl GPEtn as a source of 20:4

(49). In contrast, studies using direct mass measurements in ionophore A23187-treated rat peritoneal neutrophils demonstrated that 20:4 depletion occurred in GPCho and GPIIn only (50).

Our observation of substantial zymosan-dependent decreases in the levels of 20:4-containing GPCho species is consistent with some of these prior studies, indicating that 20:4 utilized for eicosanoid biosynthesis is ultimately obtained from these pools. However, the methods applied here do not distinguish between direct hydrolysis of GPCho by cPLA₂ to release 20:4 for eicosanoid synthesis versus hydrolysis of another 20:4-containing species followed by its rapid reacylation using 20:4 from GPCho. Notably, we did not observe significant net decreases in the relative distribution of 20:4-containing GPEtn's in zymosan-stimulated RPMs except in the case of thimerosal-treated cells which demonstrated decreases in the levels of a number of 20:4-containing plasmalogen GPEtn species. It is possible that the alkyl acyl GPEtn's serve as an immediate substrate for 20:4 mobilization but are rapidly reacylated by a thimerosal-inhibitable process. This hypothesis leads to a number of interesting possibilities. One is that 20:4-containing alkyl acyl GPEtn's represent a pool of 20:4 that is tightly coupled to PG biosynthesis. Failure to replenish this pool could explain the fact that the quantities of PGs produced in zymosan-stimulated thimerosal-treated RPMs are smaller even though levels of free 20:4 are actually higher than in cells stimulated with zymosan in the absence of thimerosal. Similarly interesting is our finding that the only 20:4-containing species found to undergo significant decreases in their amounts in response to zymosan in RAW264.7 cells is 36:4p GPEtn. Perhaps these cells utilize this species as a source of 20:4 for PG biosynthesis but lack the pathway needed to rapidly regenerate this pool using 20:4 from other sources, such as 20:4-containing GPCho's. If this is true, it could help to explain the poor PG synthetic response of these cells.

In conclusion, comprehensive lipid profiling of RPMs during zymosan phagocytosis reveals major decreases in the relative quantities of 20:4-containing diacyl and alkylacyl GPCho and selected GPIIn and GPGro species. Considering the high proportion of GPCho in cell membranes in general, and RPM specifically (24), this would appear to be the major ultimate source of 20:4 used for eicosanoid biosynthesis during zymosan phagocytosis in these cells. However, other species, particularly alkyl acyl GPEtn's, may serve as transient sources that are rapidly replenished. These results demonstrate the power of mass spectral lipid profiling for the identification of target species of interest for the further study of precursor–product relationships in lipid mediator biosynthesis. Future work is directed toward obtaining absolute quantification of the changes in the levels of key species identified in these studies.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Full profiles of all lipid species analyzed corresponding to Figures 1–5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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