Signaling Role for LPCAT3 in Receptor-regulated Arachidonic Acid Reacylation Reactions in Human Monocytes*

Gema Pérez-Chacón, Alma M. Astudillo, Violeta Ruipérez, María A. Balboa, and Jesús Balsinde[1]

Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas (CSIC), 47003 Valladolid, Spain

and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), 08036 Barcelona, Spain

RUNNING TITLE: AA Reacylation in Human Monocytes

KEY WORDS – Lipid Mediators; Monocytes/Macrophages; Inflammation

Abstract

Cellular availability of free arachidonic acid (AA) is an important step in the production of pro- and anti-inflammatory eicosanoids. Control of free AA levels in cells is carried out by the action of phospholipase A_2s and lysophospholipid acyltransferases, which are responsible for the reactions of deacylation and incorporation of AA from and into the sn-2 position of phospholipids, respectively. In this work we have examined the pathways for AA incorporation into phospholipids in human monocytes stimulated by zymosan. Our data show that stimulated cells exhibit an enhanced incorporation of AA into phospholipids that is not secondary to an increased availability of lysophospholipid acceptors due to phospholipase A_2 activation, but rather reflects the receptor-regulated nature of the AA reacylation pathway. In vitro activity measurements indicate that the receptor-sensitive step of the AA reacylation pathway is the acyltransferase using lysophosphatidylcholine as acceptor, and inhibition of the enzyme lysophosphaticylcholine acyltransferase 3 (LPCAT3) by specific siRNA results in inhibition of the stimulated incorporation of AA into phospholipids. Collectively these results define LPCAT3 as a novel-signal regulated enzyme that is centrally implicated in limiting free AA levels in activated cells.

Arachidonic acid $(AA)^2$ is the common precursor of the eicosanoids, a family of biologically active lipid mediators which play key roles in inflammatory processes and that includes the prostaglandins, thromboxane, leukotrienes, hydroxyeicosatetraenoic acids and lipoxins [1]. AA is an intermediate of a deacylation/reacylation cycle of membrane phospholipids (PL), the so called Lands cycle, in which AA liberated by phospholipases A_2 (PLA_2) is converted to arachidonoyl-coenzyme A (AA-CoA) at the expense of ATP by arachidonoyl-CoA synthetases (ACSL), and immediately incorporated into PL by CoA-dependent acyltransferases [2-5]. In resting cells, AA is predominantly found esterified in membrane phospholipids and is unavailable for eicosanoid biosynthesis. Under these conditions, Ca^{2+}-independent PLA_2 ($iPLA_2$) is thought to account for most of the basal PLA_2 activity of the cells [6-10]. $iPLA_2$ may, therefore, be a major contributor to the low levels of fatty acid liberated during the continuous recycling of membrane phospholipids that occurs under resting conditions [11-14]. Since the rate of fatty acid release by constitutive $iPLA_2$ is lesser than the rate of its reacylation back into phospholipids, no net accumulation of free fatty acid occurs.

Cell stimulation by a variety of agonist receptors leads to the activation of another PLA_2 form, the group IVA cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$), which then becomes the dominant PLA_2 involved in AA release [15-19]. Under stimulation 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 eicosanoids. Despite that, in stimulated cells the AA deacylation reactions dominate, AA reacylation reactions are still very significant, as manifested by the fact that only a minor fraction of the free AA released by $cPLA_2\alpha$ is converted into eicosanoids, the remainder being effectively incorporated back into phospholipids. [2-5]. Once the AA has been incorporated into phospholipids, a remodeling process carried out by CoA-independent

transacylase transfers AA from choline glycerophospholipids (PC) to ethanolamine glycerophospholipids (PE), in a process that takes several hours to take place in primary cells but is strikingly rapid in tumor cell lines [4, 20-22].

Depending on the concentration of free AA, there are two different pathways for the initial incorporation of this fatty acid into phospholipids, namely a low capacity / high-affinity pathway, and a high capacity / low affinity pathway [4]. By means of the former, low concentrations of free AA incorporate into phospholipids via direct acylation of preexisting PLA_2-derived lysophospholipids. This pathway is believed to constitute the major pathway for AA incorporation into phospholipids in a variety of cells under physiological conditions; hence, the availability of lysophospholipid acceptors, particularly lysophosphatidylcholine (lysoPC), is a limiting factor [13, 14, 23-25]. The high capacity / low affinity pathway incorporates free AA via the *de novo* route, which ultimately results in the accumulation of AA into triacylglycerol and diarachidonoyl phospholipids [4, 26]. This latter pathway is thought to primarily function when the high-affinity deacylation/reacylation pathway has been saturated by exposure to high AA concentrations [4], which mostly occurs under pathological conditions.

In the current work we have studied the AA incorporation pathways in human monocytes stimulated with zymosan. Our results indicate that stimulated cells exhibit an enhanced incorporation of AA into phospholipids that is not secondary to an increased lysophospholipid availability due to $cPLA_2\alpha$ activation, but rather reflects a true receptor-regulated nature of the AA reacylation pathway. Our studies indicate that the receptor-sensitive step of the AA reacylation pathway is at the lysoPC:AA-CoA acyltransferase (LPCAT) level, and define the enzyme LPCAT3 as a signal-regulated enzyme.

Materials and Methods

Reagents – RPMI 1640 medium was from Invitrogen Life Technologies (San Diego, CA). 1-O-octadeyl-sn-glycero-3-phosphorylcholine was from Biomol (Plymouth Meeting, PA). [5,6,8,9,11,12,14,15-^{3}H]AA (specific activity 211 Ci/mmol) was from GE Healthcare (Buckinghamshire, UK). [^{14}C(U)]glycerol (specific activity 140 mCi/mmol) was from American Radiolabeled Chemical Inc. (St. Louis, MO), 1-O-[^{3}H]octadecyl-2-lyso-sn-glycero-3-phosphocholine (specific activity 185 Ci/mmol) from GE Healthcare, and [^{3}H]oleyl-L-α-lysophosphatidic acid (specific activity 54.3 Ci/mmol) was from Perkin Elmer (Waltham, MA). TLC plates were from were from Scharlab (Barcelona, Spain). Bromoenol lactone (BEL) was from Cayman Chemical Co. (Ann Arbor, MI). All other reagents were from Sigma.

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 x g during 30 min. The mononuclear cellular layer was then recovered and washed three times with PBS, resuspended in RPMI supplemented with 2 mM L-glutamine, 40 mg/ml of gentamicin and allowed to adhere to plastic in sterile dishes for 2 h. Non-adherent cells were then removed by extensively washing with PBS, and the remaining attached monocytes were used on the next day. U937 monocyte-like cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). For all experiments, the cells 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 37ºC in a humidified 5% CO_2 atmosphere.

Preparation of zymosan – Zymosan was prepared as described elsewhere [27, 28]. Briefly, zymosan particles were suspended in phosphate-buffered saline, boiled for 60 min, and washed three times. The final pellet was resuspended in phosphate-buffered saline at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No PLA_2 activity was detected in the zymosan batches used in this study, as assessed by *in vitro* activity assay [29-32].

Measurement of [^{3}H]AA and [^{14}C]glycerol incorporation – Monocytes were untreated or treated with 1 mg/ml zymosan in the presence of exogenous [^{3}H]AA (0.25 μCi/ml; 1 nM) or [^{14}C]glycerol (0.1 μCi/ml; 0.7 μM). [^{14}C]glycerol was added 5 minutes before stimulation. At different times, the reactions were stopped by replacing the incubation medium with ice-cold 0.1% Triton X-100 and total lipids were then extracted according to the method of Bligh and Dyer [33], and separated by thin-layer chromatography. Neutral lipids were separated with hexane/ether/acetic acid (70:30:1, v/v/v) as a mobile phase; and the various phospholipid classes were separated by using choloform/methanol/28% ammonia (65:25:5, v/v/v) as a mobile phase. TLC spots were cut out and analyzed for radioactivity by liquid scintillation counting.

Measurement of [^{3}H]AA release - Monocytes were incubated for 20 h with 0.25 μCi/ml [^{3}H]AA. Afterward, supernatants were removed and cell monolayers were washed several times with serum-free medium containing 0.5 mg/ml bovine serum albumin to remove unincorporated [^{3}H]AA. When needed, the cells were preincubated with inhibitors (10 μM BEL, 1 μM pyrrophenone, or 200 μM propranolol for 30 min). After this time, the cells were treated without or with 1 mg/ml zymosan for the indicated times. Subsequently, supernatants were collected, centrifuged to eliminate debris and

detached cells, and measured for radioactivity by liquid scintillation counting.

Determination of arachidonoyl-CoA synthetase activity – Arachidonoyl-CoA synthetase activity was measured exactly as described by Wilson *et al*. [34] in a total volume of 150 µl. Monocytes were incubated in the absence or presence of 1 mg/ml zymosan for 30 min. Afterward, the cells were homogenized, and 50 µg of cell extract was mixed with 20 mM $MgCl_2$, 10 mM ATP, 1 mM CoA, 1 mM 2-mercaptoethanol, 100 mM Tris-HCl (pH 8) and [^{3}H]AA (25-150 µM), and incubated at 37ºC for 10 min. Reactions were stopped by adding 2.25 ml of 2-propanol/heptane/2 M sulfuric acid (40:10:1, v/v/v). After the addition of 1.5 ml of heptane and 1 ml of water, mixture was vortexed and centrifuged at 1000 x g for 5 min. The aqueous phase was collected, extracted twice with 2 ml of heptane containing 4 mg/ml linoleic acid and finally analyzed for radioactivity by liquid scintillation counting.

Determination of lysophospholipid:arachidonoyl-CoA acyltransferase activities – This was determined as described by Lands *et al*. [35]. Monocytes, treated without or with 1 mg/ml zymosan for 30 min were homogenized, and 50 µg of cell extract was mixed with 50 mM Tris-HCl (pH 7.5), 1 mM CoA, 10 mM ATP, 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 µM [^{3}H]AA, and 5-50 µM lysophospholipid (lysoPA, lysoPC, lysoPE, or lysoPI) in a final volume of 150 µl. After a 20-min incubation at 37 ºC, the reactions were stopped by adding chloroform, and the lipids were extracted according to Bligh and Dyer [33]. For separation of phosphatidic acid from lysophosphatidic acid, a system consisting of chloroform/methanol/28% ammonia/water (50:40:8:2, v/v/v/v) was used as a mobile phase, and plates previously sprayed with 1% potassium oxalate were utilized. For separation of PC, PI and PE from their respective lyso counterparts, a

system of choloform/methanol/28% ammonia (65:25:5, v/v/v) was used as a mobile phase.

Small interference RNA (siRNA) inhibition assays – Control siRNA, fluorescein amidite-labeled control siRNA, and siRNA directed against LPCAT2 (5'-3'GCAUGAAGAGAGUACCUCA) and LPCAT3 (5'-3'CCAUUGCCUCAUUCAACAU) were from Ambion (Austin, TX). Monocytes were transfected in antibiotic-free OPTIMEM® medium with 200 nM siRNA in the presence of 2.5 μg/ml Lipofectamine 2000 (Invitrogen Life Technologies), following the manufacturer's instructions. After 24 h, medium was replaced by serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine and 40 μg/ml gentamicin and monocytes were maintained 24 h under these conditions. mRNA expression for LPCAT2 and LPCAT3 was measured by quantitative PCR and, the cells, either unstimulated or stimulated with 1 mg/ml zymosan for 30 min, were assayed for [^{3}H]AA incorporation. To assess the efficiency of transfection, the cells were transfected with a fluorescein amidite-labeled control siRNA under the same conditions. The number of cells was counted by microscopy in at least 4 different fields, and the efficiency of transfection was calculated as the percentage of cells exhibiting green fluorescence with respect to the total number of cells.

Quantitative RT-PCR methods – Total RNA was extracted with the TRI reagent solution (Ambion) and 1 μg of RNA was reverse-transcribed using 0.3 ng random primers (Ambion) and 50 units of M-MLV reverse transcriptase (Ambion). TaqMan® real-time PCR technology (Applied Biosystems, Foster City, CA) was used to assess the percentage of inhibition of LPCAT2 and LPCAT3 mRNA levels with specific siRNAs.

This method related the amount of LPCAT2 and LPCAT3 mRNA present to level of β-actin, controlling for the amount of RNA present. Specific human LPCAT2 and LPCAT3 primers and probe were obtained from Applied Biosystems. Quantitative PCR was carried out using the Chromo 4^{TM} Detector (Bio-Rad Laboratories) according to previously described methods, with each reaction containing 5 ng of reverse transcribed RNA in 20 µl of TaqMan® One-Step RT-PCR Master Mix Reagents. Within-assay variation of PCR measurements was calculated from duplicates. Data analyses were performed with the Opticon Monitor 3.1 software (Bio-Rad Laboratories). The relative expression of each mRNA was calculated as the ΔCt (value obtained by subtracting the Ct number of target sample from that of control sample, β-actin). The amount of target mRNA relative to β-actin mRNA was thus expressed as $2^{-(\Delta Ct)}$. Values are given as the ratio of the target mRNA to β-actin mRNA.

Data analysis – All experiments were performed in duplicate. Data are shown as means ± SD from three different experiments. SPSS v.14 software for Windows (SPSS, Chicago, IL) was used for data analysis. Data were compared using the paired Student's *t*-test and differences were regarded as significant when $p < 0.05$.

Results

[^{3}H]AA release and re-incorporation into cellular phospholipids in zymosan-stimulated monocytes– In keeping with previous estimates [36, 37], treatment of [^{3}H]AA-labeled human monocytes with zymosan resulted in abundant release of radioactivity to the extracellular medium (Fig. 1). Such a response was almost completely abrogated by 1 μM pyrrophenone, but not by 10 μM BEL, demonstrating that it is cPLA$_2$α, not iPLA$_2$, that is responsible for receptor-mediated AA release. In vitro activity assays demonstrated that at the concentrations employed in this study, cPLA$_2$α and iPLA$_2$ activities were quantitatively inhibited by pyrrophenone and BEL, respectively. In addition, at 1 μM no effect of pyrrophenone was detected on cellular iPLA$_2$ activity, and 10 μM BEL did not have any effect on cPLA$_2$α activity (data not shown).

Since AA mobilization in response to stimuli represents a balance between what is released from phospholipids by phospholipases minus what is reincorporated back into phospholipids by acyltransferases, we wished to explore the AA reacylation pathway in zymosan-stimulated monocytes. To this end, unlabeled cells were exposed to zymosan in the presence of 1 nM [^{3}H]AA and, at different times, the incorporation of radiolabel into the different cellular phospholipids was studied. Note that at this very low concentration, exogenous [^{3}H]AA exerts no stimulatory effects on its own, and thus the effects observed are those due to zymosan interacting with its surface receptor. Fig. 2 shows that treating the monocytes with zymosan results in a rapid stimulation of the incorporation of [^{3}H]AA into glycerophospholipids, particularly into PC, with lesser amounts being found in PE and PI. Significant amounts of [^{3}H]AA were also found in triacylglycerol (15% of total AA in lipids at 60 min; see below).

AA incorporation into phospholipids in activated cells occurs primarily via

deacylation/reacylation reactions - Two routes for AA incorporation exist in mammalian cells, the Lands cycle of deacylation/reacylation and the Kennedy pathway for *de novo* biosynthesis of phospholipids [4]. While at low AA concentrations such as those used in this study the Lands cycle is thought to account for practically all of the incorporation in unstimulated cells [4], we wished to investigate whether this was also true in activated cells. We stimulated cells with zymosan in the presence of both [^{3}H]AA and [^{14}C]glycerol, the latter to selectively label the lipids synthesized *de novo*. We found that the amount of ^{14}C-radioactivity in both phospholipids and triacylglycerol linearly accumulated with time in activated cells (Fig. 3A), demonstrating activation of the *de novo* biosynthetic pathway. [^{3}H]AA accumulated in phospholipids, but also in triacylglycerol (Fig. 3*B*), raising the possibility that AA might also significantly incorporate through the *de novo* route under the activation conditions. However, analysis of the phospholipid/triacylglycerol ratio for both isotopes indicated a factor of 2 for ^{14}C and of 6 for ^{3}H. This difference suggests that the bulk of ^{3}H-radioactivity accumulating in phospholipids comes from a pathway distinct from the *de novo* pathway (which is the one through which the ^{14}C-radioactivity incorporates).

To obtain further evidence for the above observation, AA incorporation experiments were carried out in the presence of propranolol, a phosphatidate phosphatase-1 inhibitor that blunts fatty acid incorporation via *de novo* but not via direct deacylation/reacylation reactions [38-42]. A strong inhibition of AA incorporation into triacylglycerol was observed in stimulated monocytes treated with propranolol (Fig. 4); however, AA incorporation into phospholipids was not inhibited. Control experiments had indicated that at the propranolol concentrations used in these experiments (200 μM), phosphatidate phosphatase-1 activity was quantitatively inhibited, as judged by activity assay (data not shown) [38, 39]. These data confirm that, although in activated cells the

de novo route for phospholipid biosynthesis becomes activated, its contribution to the increased incorporation of AA into phospholipids is minor.

Stimulated incorporation of AA into phospholipids of activated cells is not a consequence of cPLA$_2\alpha$ activation – Given that zymosan stimulation of monocytes results in cPLA$_2\alpha$ activation (Fig. 1), the increased AA reacylation observed under these conditions could be merely triggered by the increased availability of lysophospholipid acceptors that occurs in activated cells. To investigate this possibility, [^{3}H]AA incorporation experiments were carried out in the presence of 1 μM pyrrophenone, which, as shown above, results in the complete inhibition of cPLA$_2\alpha$-mediated phospholipid hydrolysis. Fig. 5*A* shows that zymosan-stimulated [^{3}H]AA incorporation into phospholipids proceeded the same whether the cells were treated or not with pyrrophenone. These results clearly indicate that zymosan-stimulated AA incorporation is not secondary to cPLA$_2\alpha$-mediated lysophospholipid rises, suggesting that the lysophospholipid level already present in the resting cells must be sufficient to support the zymosan-stimulated phospholipid AA reacylation.

Our previous work has indicated that iPLA$_2$ is a significant contributor to the steady-state lysophospholipid level in resting phagocytic cells [23-25, 43]. Hence, inhibition of iPLA$_2$ results in reduced levels of lysophospholipid acceptors, which in turn leads to a decreased incorporation of AA into phospholipids under unstimulated conditions [14, 23-25]. When the effect of zymosan on AA incorporation was assayed in monocytes treated with BEL, and hence exhibiting diminished lysophospholipid levels [44], the response was slightly but significantly reduced (Fig. 5*B*). Importantly however, the response was also similarly reduced in the resting cells, resulting in a ratio of AA incorporation in stimulated *versus* resting cells that was the same as that

observed in cells not deficient in $iPLA_2$ activity (Fig. 5*B*). Therefore, the increased AA incorporation into phospholipids in zymosan-stimulated cells is not limited by cellular lysophospholipid levels, but may rather reflect a previously unrecognized receptor-regulated nature of the AA reacylation pathway.

Increased activity of LPCAT in zymosan-stimulated monocytes - We considered next the possibility that some of the enzymes of the reacylation pathway were receptor-regulated and therefore, that their activity increased in the activated cells. To explore this point, homogenates from resting and zymosan-stimulated monocytes were prepared (1 mg/ml stimulus; 30 min), and *in vitro* activity assays were performed. The activities measured were: arachidonoyl-CoA synthetase, lysoPC:arachidonoyl-CoA acyltransferase (LPCAT), lysoPE:arachidonoyl-CoA acyltransferase (LPEAT), lysoPI:arachidonoyl-CoA acyltransferase (LPIAT), and lysoPA:arachidonoyl-CoA acyltransferase (LPAAT). Of all these activities, only LPCAT was found to be increased in homogenates from zymosan-treated cells versus resting cell homogenates (Figs. 6 and 7). These data suggest that LPCAT is a signal-regulated activity underlying the increased AA incorporation into phospholipids of activated monocytes.

LPCAT3 regulates phospholipid AA incorporation in activated cells – Four isoforms of LPCAT exist in mammalian cells, termed LPCAT1, LPCAT2, LPCAT3, and LPCAT4 [45-51] but only two of them, LPCAT2 and LPCAT3, have been documented to participate in AA reacylation reactions [49-53]. Human peripheral blood monocytes express both LPCAT2 and LPCAT3 (data not shown). To study the involvement of these enzymes in zymosan-stimulated AA incorporation, we sought to block their expression by siRNA. Only partial inhibition of LPCAT2 and LPCAT3 could be

achieved (25 ± 5%, inhibition for both genes, as assessed by quantitative RT-PCR; mean ± SD, n=3), which was not unexpected given that monocytes, as primary cells, are known to be hard to transfect. By using fluorescein amidite-labeled siRNAs we estimated an efficiency of transfection of 28 ± 6% (mean ± SD, n=3). Despite these low levels, we were still able to detect significant inhibition of the AA incorporation into PC in response to zymosan (Fig. 8A). Interestingly, no significant inhibition of the AA incorporation response in cells deficient in LPCAT2 was observed (Fig. 8A). Similar studies were also conducted with the monocyte-like cell line U937 and the results, as shown in Fig. 8B, also indicated that inhibition of LPCAT3, but not of LPCAT2, significantly blunted the zymosan-stimulated AA incorporation. Collectively, these findings suggest that LPCAT3 is the key enzyme responsible for the increase in AA incorporation into phospholipids in stimulated cells. Further evidence was obtained

Discussion

The metabolism of AA reflects a carefully balanced series of biochemical pathways. The Lands cycle is a mechanism for the deacylation/reacylation of membrane phospholipids by which polyunsaturated fatty acids such as AA are incorporated into different species [2-4]. While many studies have been conducted in resting cells, much less is known on the regulatory features of phospholipid AA incorporation in activated cells, where the sustained activation of cPLA$_2\alpha$ results in a rate of AA release that exceeds that of reacylation back into phospholipids. Hence, in activated cells a net accumulation of free AA occurs that is followed by its conversion into different oxygenated compounds, collectively called the eicosanoids. It has traditionally been assumed that phospholipid AA incorporation in activated cells may be secondary to the PLA$_2$ hydrolytic step because AA incorporates preferentially into the sn-2 position of phospholipids and thus, for an enhanced AA reacylation to occur, 2-lysophospholipid acceptors, produced only by PLA$_2$, should be provided. That lysophospholipid availability may limit AA reacylation in activated cells is also inferred from the finding that the specific activities of the enzymes of the reacylation pathway, arachidonoyl-CoA synthetase and the CoA-dependent acyltransferases, are several-fold higher that that of PLA$_2$ in homogenates from resting and activated cells [23, 54-58].

In this study, we demonstrate that zymosan-stimulation of human monocytes results in the mobilization of AA that is dependent upon cPLA$_2\alpha$ activation, as judged by complete inhibition of the response by pyrrophenone but not by BEL. We also document that the zymosan-stimulated cells exhibit an increased incorporation of AA into all major classes of phospholipids predominantly via a lysophospholid reacylation pathway and not via *de novo*. Strikingly, lack of sensitivity of AA incorporation to pyrrophenone clearly suggests that phospholipid AA incorporation is not merely

triggered by the increased abundance of lysophospholipid acceptors produced by receptor-activated cPLA$_2\alpha$, but may actually represent a receptor-regulated pathway on its own. Direct evidence to this proposal was obtained by directly measuring the activities of the enzymes of the reacylation pathway, namely acyl-CoA synthetases and lysophospholipid acyltransferases.

Five different acyl-CoA synthetase forms have been described, termed ACSL-1, -3, -4, -5, and -6 [59-61]. Of these, ACSL-4 and ACSL-6 have been shown to exhibit some selectivity for AA, and the latter also for docosahexaenoic acid in intact cells [62, 63]. However, we failed to detect any enhancement of acyl-CoA synthetase activity in homogenates from zymosan-activated cells utilizing AA as substrate, suggesting that this activity may not be regulated by extracellular signals. Therefore, we moved to the next step of the reacylation pathway, i.e. the lysophospholipid acyltransferase. We measured this activity utilizing various lysophospholipid acceptors, namely lysoPC, lysoPE, lysoPI, and lysoPA. The first three lysophospholipids are utilized in the Lands cycle, and the latter one is an acceptor of the *de novo* phospholipid biosynthetic pathway. Our data clearly show the selective activation of LPCAT upon zymosan-stimulation of the monocytes, indicating that the reacylation route is indeed regulated at the acyltransferase level. This is to the best of our knowledge the first study demonstrating receptor-regulated, stable changes in LPCAT activity. Acyltransferase activity changes using lysoPE, lysoPI or lysoPA were not detected. The absence of an increased activity towards PA was not unexpected, given our data showing that zymosan-induced phospholipid AA incorporation does not proceed via the *de novo* biosynthetic pathway. However, our inability to detect increased acyltransferase activity using either lysoPE or lysoPI is difficult to explain in view of our own data showing that AA incorporates not only into PC, but also into PE and PI during zymosan

stimulation of the cells, and that acyl transferase enzymes showing clear preference for lysoPE and lysoPI have been described [48, 64, 65]. It is possible that a significant portion of the AA reincorporated into these phospholipids in activated cells, particularly PE, enters indirectly via transacylation reactions utilizing AA-containing PC as an AA donor. Such a route has been demonstrated to exist in human neutrophils and to significantly contribute to phospholipid fatty acid remodeling [66].

To date, four enzymes with LPCAT activity have been described in humans [45], LPCAT1, LPCAT2, and LPCAT4, members of the AGPAT family, and LPCAT3, belonging to the MBOAT family. In mouse, LPCAT2 [53] and LPCAT3 [49] have been suggested to show a preference for AA. In human cells, it is LPCAT3 the form that appears to exhibit preference for AA, although also by linoleic acid [50, 52]. Shindou *et al.* [53] have recently reported the increase of lyso-platelet-activating factor acetyltransferase activity in RAW364.7 cells transfected with the mouse lyso-platelet-activating factor acetyl transferase/LPCAT2 gene, and stimulated with Toll-like receptor agonists. However, no enhanced lysoPC acyltransferase activity was observed in these experiments, suggesting that LPCAT2 participated in platelet-activating factor metabolism rather than in a more general fatty acid remodeling role. On the other hand, endogenous LPCAT activity in murine peritoneal macrophages was found to increase in response to bacterial lipopolysaccharide, although the basal expression of lyso-platelet-activating factor acetyltransferase/LPCAT2 was almost undetectable, suggesting that the LPCAT activity measured was due to other forms [53]. In this regard, using siRNA technology, our studies reported here suggest that the LPCAT form involved in AA reacylation in activated cells is LPCAT3, and that LPCAT2 appears to have, if any, only a minor role.

In summary, results shown in this work have provided clues to understand the

regulation of AA incorporation into the phospholipids of stimulated human monocytes. Specifically, evidence has been provided to indicate that this process is not secondary to the activation of intracellular PLA_2s and the subsequent rise in lysophospholipid levels. Rather our studies have suggested that phospholipid AA incorporation as a receptor-regulated pathway and identified LPCAT3 as a novel lipid-signaling enzyme that is centrally involved in this pathway. Clearly, further studies will be necessary to establish the factors that control the availability of other lysophospholipid class such as lysoPE and lysoPI during the activation process as well as to ascertain the involvement of other acyltransferases.

Acknowledgments

We thank Montse Duque and Yolanda Sáez for expert technical assistance. We also thank the personnel from Centro de Hemoterapia y Hemodonación de Castilla y León for supplying buffy coats from healthy volunteers. CIBERDEM is an initiative of Instituto de Salud Carlos III.

References

1. Funk, C. D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871-1875.
2. Lands, W. E. 2000. Stories about acyl chains. Biochim. Biophys. Acta 1483, 1-14.
3. MacDonald, J. I., and H. Sprecher.1991. Phospholipid fatty acid remodeling in mammalian cells. Biochim. Biophys. Acta 1084, 105-121
4. Chilton, F. H., A. N. Fonteh, M. E. Surette, M. Triggiani, and J. D. Winkler. 1996. Control of arachidonate levels within inflammatory cells. Biochim. Biophys. Acta 1299, 1-15.
5. Pérez-Chacón, G., A. M. Astudillo, D. Balgoma, M. A. Balboa, and J. Balsinde. 2009. Control of Free Arachidonic Acid Levels by Phospholipases A_2 and Lysophospholipid Acyltransferases. Biochim. Biophys. Acta 1791, 1103-1113
6. Balsinde, J., and E. A. Dennis. 1997. Function and inhibition of intracellular calcium-independent phospholipase A_2. J. Biol. Chem. 272, 16069-16072
7. Winstead, M. V., J. Balsinde, and E. A. Dennis. 2000. Calcium-independent phospholipase A_2: structure and function. Biochim. Biophys. Acta 1488, 28-39
8. Balsinde, J., and M. A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells. Cell Signal 17, 1052-1062.
9. Balsinde, J., R. Pérez, and M. A. Balboa. 2006. Calcium-independent phospholipase A_2 and apoptosis. Biochim. Biophys. Acta 1761, 1344-1350
10. Hooks, S.B, and B. S. Cummings. 2008. Role of Ca^{2+}-independent phospholipase A_2 in cell growth and signaling. Biochem. Pharmacol. 76, 1059-67

11. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase A_2 enzymes present in $P388D_1$ macrophages. J. Biol. Chem. 271, 6758-6765
12. Balboa, M. A., and J. Balsinde. 2002. Involvement of calcium-independent phospholipase A_2 in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. J. Biol. Chem. 277, 40384-40389
13. Balsinde, J. 2002. Roles of various phospholipases A_2 in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. Biochem. J. 364, 695-702
14. Pérez, R., R. Melero, M. A. Balboa, and J. Balsinde. 2004. Role of group VIA calcium-independent phospholipase A_2 in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. J. Biol. Chem. 279, 40385-40391
15. Gijón, M. A, and C. C. Leslie. 1999. Regulation of arachidonic acid release and cytosolic phospholipase A_2 activation. J. Leukoc. Biol. 65, 330-336
16. J. Balsinde, M. A. Balboa, P. A. Insel, and E. A. Dennis. 1999. Regulation and inhibition of phospholipase A_2. Annu. Rev. Pharmacol. Toxicol. 39, 175-189
17. Balsinde, J., M. V. Winstead, and E. A. Dennis. 2002. Phospholipase A_2 regulation of arachidonic acid mobilization. FEBS Lett. 531, 2-6
18. Hirabayashi, T., T. Murayama, and T. Shimizu. 2004. Regulatory mechanism and physiological role of cytosolic phospholipase A_2. Biol. Pharm. Bull. 27, 1168-1173
19. Balboa, M. A., and J. Balsinde. 2006. Oxidative stress and arachidonic acid mobilization. Biochim. Biophys. Acta 1761, 385-391
20. Balsinde, J., S. E. Barbour, I. D. Bianco, and E. A. Dennis. 1994. Arachidonic acid mobilization in $P388D_1$ macrophages is controlled by two distinct Ca^{2+}-dependent

phospholipase A_2 enzymes. Proc. Natl. Acad. Sci. U.S.A. 91, 11060-11064

21. Surette, M. E., A. N. Fonteh, C. Bernatchez, and F. H. Chilton. 1999. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. Carcinogenesis 20, 757-763
22. Trimboli, A. J., B. M. Waite, G. Atsumi, A. N. Fonteh, A. M. Namen, C. E. Clay, T. E. Kute, K. P. High, M. C. Willingham, and F. H. Chilton. 1999. Influence of coenzyme A-independent transacylase and cyclooxygenase inhibitors on the proliferation of breast cancer cells. Cancer Res. 59, 6171-6177
23. Balsinde, J., I. D. Bianco, E. J. Ackermann, K. Conde-Frieboes, and E. A. Dennis. 1995. Inhibition of calcium-independent phospholipase A_2 prevents arachidonic acid incorporation and phospholipid remodeling in $P388D_1$ macrophages. Proc. Natl. Acad. Sci. U.S.A. 92, 8527-8531
24. Balsinde, J., M. A. Balboa, and E. A. Dennis. 1997. Antisense inhibition of group VI Ca^{2+}-independent phospholipase A_2 blocks phospholipid fatty acid remodeling in murine $P388D_1$ macrophages. J. Biol. Chem. 272, 29317-29321
25. Pérez, R., X. Matabosch, A. Llebaria, M. A. Balboa, and J. Balsinde. 2006. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. J. Lipid Res. 47, 484-491
26. Chilton, F. H., and R. C. Murphy. 1987. Stimulated production and natural occurrence of 1,2-diarachidonoylglycerophosphocholine in human neutrophils. Biochem. Biophys. Res. Commun. 145, 1126-1133
27. Balsinde, J., B. Fernández, J. A. Solís-Herruzo, and E. Diez. 1992. Pathways for arachidonic acid mobilization in zymosan-stimulated mouse peritoneal macrophages. Biochim. Biophys. Acta 1136, 75-82.

28. Balsinde, J., M. A. Balboa, and E. A. Dennis. 2000. Identification of a third pathway for arachidonic acid mobilization and prostaglandin production in activated P388D_1 macrophage-like cells. J. Biol. Chem. 275, 22544-22549

29. Balboa, M. A., Y. Sáez, and J. Balsinde. 2003. Calcium-independent phospholipase A_2 is required for lysozyme secretion in U937 promonocytes. J. Immunol. 170, 5276-5280

30. Balboa, M. A., R. Pérez, and J. Balsinde. 2008. Calcium-independent phospholipase A_2 mediates proliferation of human promonocytic U937 cells. FEBS J. 275, 1915-1924

31. Ruipérez, V., J. Casas, M. A. Balboa, and J. Balsinde. 2007. Group V phospholipase A_2-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. J. Immunol. 179, 631-638

32. Ruipérez, V., A. M. Astudillo, M. A. Balboa, and J. Balsinde. 2009. Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A_2s. J. Immunol. 182, 3877-3883

33. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917

34. Wilson, D. B., S. M. Prescott, and P. W. Majerus. 1982. Discovery of an arachidonoyl coenzyme A synthetase in human platelets. J. Biol. Chem. 257, 3510-3515.

35. Lands, W. E., M. Inoue, Y. Sugiura, and H. Okuyama, H. 1982. Selective incorporation of polyunsaturated fatty acids into phosphatidylcholine by rat liver microsomes. J. Biol. Chem. 257, 14968-14972

36. Pawlowski, N. A., G. Kaplan, A. L. Hamill, Z. A. Cohn., and W. A. Scott. 1983. Arachidonic acid metabolism by human monocytes. Studies with platelet-depleted

cultures. J. Exp. Med. 158, 393-412

37. Hoffman, T., E. F. Lizzio, J. Suissa, D. Rotrosen, J. A. Sullivan, G. L. Mandell, and E. Bonvini. 1988. Dual stimulation of phospholipase activity in human monocytes. Role of calcium-dependent and calcium-independent pathways in arachidonic acid release and eicosanoid formation. J. Immunol. 140, 3912-3918
38. Balsinde, J., and E. A. Dennis. 1996. Bromoenol lactone inhibits magnesium-dependent phosphatidate phosphohydrolase and blocks triacylglycerol biosynthesis in mouse P388D_1 macrophages. J. Biol. Chem. 271, 31937-31941
39. Fuentes, L., R. Pérez, M. L. Nieto, J. Balsinde, and Balboa, M. A. 2003. Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A_2. J. Biol. Chem. 278, 44683-44690
40. Balboa, M. A., J. Balsinde, and E. A. Dennis. 1998. Involvement of phosphatidate phosphohydrolase in arachidonic acid mobilization in human amnionic WISH cells. J. Biol. Chem. 273, 7684-7690
41. Balsinde, J., and E. A. Dennis. 1996. The incorporation of arachidonic acid into triacylglycerol in P388D_1 macrophage-like cells. Eur. J. Biochem. 235, 480-485
42. Balsinde, J., M. A. Balboa, P. A. Insel, and E. A. Dennis. 1997. Differential regulation of phospholipase D and phospholipase A_2 by protein kinase C in P388D_1 macrophages. Biochem. J. 321, 805-809
43. Pérez, R., M. A. Balboa, and J. Balsinde. 2006. Involvement of group VIA calcium-independent phospholipase A_2 in macrophage engulfment of hydrogen peroxide-treated U937 cells. J. Immunol. 176: 2555-2561
44. Balgoma, D., O. Montero, M. A. Balboa, and J. Balsinde. 2008. Calcium-independent phospholipase A_2-mediated formation of 1,2-diarachidonoyl-

glycerophosphoinositol in monocytes. FEBS J. 275: 6180-6191

45. Shindou, H., D. Hishikawa, T. Harayama, K. Yuki, and T. Shimizu. 2009. Recent progress on acyl CoA:lysophospholipid acyltransferase research. J. Lipid Res. 50, S46-S51

46. Nakanishi, H., H. Shindou, D. Hishikawa, T. Harayama, R. Ogasawara, A. Suwabe, R. Taguchi, and T. Shimizu. 2006. Cloning and characterization of mouse lung-type acyl-CoA:lysophosphatidylcholine acyltransferase 1 (LPCAT1). Expression in alveolar type II cells and possible involvement in surfactant production. J Biol Chem 281, 20140-20147

47. Chen, X., B. A. Hyatt, M. L. Mucenski, R. J. Mason, and J. M. Shannon, J. M. 2006. Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells. Proc. Natl. Acad. Sci. U.S.A. 103, 11724-11729

48. Cao, J., D. Shan, T. Revett, D. Li, L. Wu, W. Liu, J. F. Tobin, and R. E. Gimeno. 2008. Molecular identification of a novel mammalian brain isoform of acyl-CoA:lysophospholipid acyltransferase with prominent ethanolamine lysophospholipid acylating activity, LPEAT2. J. Biol. Chem. 283, 19049-19057

49. Hishikawa, D., H. Shindou, S. Kobayashi, H. Nakanishi, R. Taguchi, and T. Shimizu. 2008. Discovery of a lysophospholipid acyltransferase family essential for membrane asymmetry and diversity. Proc. Natl. Acad. Sci. U.S.A. 105, 2830-2835

50. Zhao, Y., Y. Q. Chen, T. M. Bonacci, D. S. Bredt, S. Li, W. R. Bensch, D. E. Moller, M. Kowala, R. J. Konrad, and G. Cao. 2008. Identification and characterization of a major liver lysophosphatidylcholine acyltransferase. J. Biol. Chem. 283, 8258-8265

51. Matsuda, S., T. Inoue, H. C. Lee, N. Kono, F. Tanaka, K. Gengyo-Ando, S. Mitani, and H. Arai. 2008. Member of the membrane-bound O-acyltransferase (MBOAT)

family encodes a lysophospholipid acyltransferase with broad substrate specificity. Genes Cells 13. 879-888

52. Kazachkov, M., Q. Chen, L. Wang, and J. Zou. 2008. Substrate preferences of a lysophosphatidylcholine acyltransferase highlight its role in phospholipid remodeling. Lipids 43, 895-902
53. Shindou, H., D. Hishikawa, H. Nakanishi, T. Harayama, S. Ishii, R. Taguchi, and T. Shimizu. 2007. A single enzyme catalyzes both platelet-activating factor production and membrane biogenesis of inflammatory cells. Cloning and characterization of acetyl-CoA:Lyso-PAF acetyltransferase. J. Biol. Chem. 282, 6532-6539
54. Tou, J . S. 1989. Platelet-activating factor regulates phospholipid metabolism in human neutrophils. Lipids 24, 812-817
55. Balsinde, J., B. Fernández, and J. A. Solís-Herruzo. 1994. Increased incorporation of arachidonic acid into phospholipids in zymosan-stimulated mouse peritoneal macrophages. Eur. J. Biochem. 221, 1013-1018.
56. Reinhold, S. L., G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1989. Phospholipid remodeling in human neutrophils. Parallel activation of a deacylation/reacylation cycle and platelet-activating factor synthesis. J. Biol. Chem. 264, 21652-21659.
57. Flesch, I., T. Schonhardt, and E. Ferber. 1989. Phospholipases and acyltransferases in macrophages. Klin. Wochenschr. 67, 119-122
58. Yamashita, A. T. Sugiura, and K. Waku. 1997. Acyltransferases and transacylases involved in fatty acid remodeling of phospholipids and metabolism of bioactive lipids in mammalian cells. J. Biochem. (Tokyo) 122, 1-16
59. Mashek, D. G., K. E. Bornfeldt, R. A. Coleman, J. Berger, D. A. Bernlohr, P. Black, C. C. Dirusso, S. A. Farber, W. Guo, N. Hashimoto, V. Khodiyar, F. A. Kuypers, L.

J. Maltais, D. W. Nebert, A. Renieri, J. E. Schaffer, A. Stahl, P. A. Watkins, V. Vasiliou, and T. T. Yamamoto. 2004. Revised nomenclature for the mammalian long-chain acyl-CoA synthetase gene family. J. Lipid Res. 45, 1958-1961

60. Kim, J. H., T. M. Lewin, and R. A. Coleman. 2001. Expression and characterization of recombinant rat Acyl-CoA synthetases 1, 4, and 5. Selective inhibition by triacsin C and thiazolidinediones. J. Biol. Chem. 276, 24667-24673
61. Van Horn, C. G., J. M. Caviglia, L. O. Li, S. Wang, D. A. Granger, and R. A. Coleman. 2005. Characterization of recombinant long-chain rat acyl-CoA synthetase isoforms 3 and 6: identification of a novel variant of isoform 6. Biochemistry 44, 1635-1642
62. Kang, M. J., T. Fujino, H. Sasano, H. Minekura, N. Yabuki, H. Nagura, H. Iijima, and T. T. Yamamoto. 1997. A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. Proc. Natl. Acad. Sci. U.S.A. 94, 2880-2884
63. Marszalek, J. R., C. Kitidis, C. C. Dirusso, and H. F. Lodish. 2005. Long-chain acyl-CoA synthetase 6 preferentially promotes DHA metabolism. J. Biol. Chem. 280, 10817-10826
64. Lee, H. C., T. Inoue, R. Imae, N. Kono, S. Shirae, S. Matsuda, K. Gengyo-Ando, S. Mitani, and H. Arai. 2008. Caenorhabditis elegans mboa-7, a member of the MBOAT family, is required for selective incorporation of polyunsaturated fatty acids into phosphatidylinositol. Mol. Biol. Cell 19, 1174-1184.
65. Gijón, M. A., W. R. Riekhof, S. Zarina, R. C. Murphy, and D. R. Voelker. 2009. Lysophospholipid acyltransferases and arachidonate recycling in human neutrophils. J. Biol. Chem. 283, 30235-30245

66. Nieto, M. L., M. E. Venable, S. A. Bauldry, D. G. Greene, M. Kennedy, D. A. Bass, and R. L. Wykle. 1991. Evidence that hydrolysis of ethanolamine plasmalogens triggers synthesis of platelet-activating factor via a transacylation reaction. J. Biol. Chem. 266, 18699-18706

Footnotes to the text

*This work was supported by the Spanish Ministry of Science and Innovation (Grants BFU2007-67154 and SAF2007-60055), and the Regional Government of Castile & León (Grant CSI09-A08). A.M.A. was supported by a predoctoral fellowship from the Regional Government of Castile & León.

[1]Corresponding Author. Phone: 34-983-423-062; FAX, 34-983-184-800; E-mail, jbalsinde@ibgm.uva.es

[2]The abbreviations used are: AA, arachidonic acid; ACSL, arachidonoyl-CoA synthetase; BEL, bromoenol lactone; PLA_2, phospholipase A_2; $cPLA_2\alpha$, calcium-dependent cytosolic phospholipase $A_2\alpha$ (group IVA); $iPLA_2$, calcium-independent phospholipase A_2; PA, phosphatidic acid; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol; LPAAT, LysoPA:acyl-CoA acyltransferase; LPCAT, lysoPC;acyl-CoA acyltransferase; LPEAT, lysoPE:acyl-CoA acyltransferase; LPIAT, lysoPI:acyl-CoA acyltransferase

Figure Legends

Figure 1. Zymosan-induced [^{3}H]AA release in human monocytes. [^{3}H]AA-labeled human monocytes were treated without (open bars) or with (striped bars) 1 mg/ml zymosan, and in the absence (Control) or presence of 10 μM BEL or 1 μM pyrrophenone (pyrr). After 60 min, supernatants were collected and assayed for radioactivity. Data are shown as means ± SD from three different determinations carried out in duplicate. Asterisks denotes significance (p <0.05) of non-stimulated cells versus zymosan-stimulated cells at each condition.

Figure 2. Zymosan-induced [^{3}H]AA incorporation into monocyte cell phospholipids. Human monocytes were either untreated (open symbols) or treated (closed symbols) with 1 mg /ml zymosan in the presence of 1 nM [^{3}H]AA (0.25 μCi/ml) for the indicated times. Lipids were then extracted and [^{3}H]AA incorporation was measured in (A) total phospholipids or (B) phospholipids classes, PI (○, ●), PC (△, ▲) and PE (▽, ▼). Data are shown as means ± SD from three different determinations carried out in duplicate. Asterisks denotes significance (p <0.05) of non-stimulated cells versus zymosan-stimulated cells at each condition.

Figure 3. Time-course of the effect of zymosan on the incorporation of [^{14}C]glycerol (A) or [^{3}H]AA (B) into the lipids of human monocytes. The cells were either untreated (open symbols) or treated (closed symbols) with 1 mg/ml zymosan in the presence of 1 nM [^{3}H]AA (0.25 μCi/ml) or 0.7 μM [^{14}C]glycerol (0.1 μCi/ml) for the indicated times. Afterwards lipids were extracted and [^{14}C]glycerol (A) or [^{3}H]AA (B) incorporation was measured in total phospholipids (○, ●) and triacylglycerol (△,▲). Data are shown

as means ± SD from three different determinations carried out in duplicate. Asterisks denotes significance (p <0.05) of non-stimulated cells versus zymosan-stimulated cells at each condition.

Figure 4. Inhibition of [^{3}H]AA incorporation into triacylglycerol by the phosphatidate phosphatase-1 inhibitor propranolol. Human monocytes were untreated (open symbols) or treated (closed symbols) with 200 μM propranolol for 30 min. Afterward, the cells were exposed to 1 nM [^{3}H]AA (0.25 μCi/ml) in the absence (○, ●) or presence (△, ▲) of 1 mg/ml zymosan for the indicated times, and [^{3}H]AA incorporation was measured in triacylglycerol (A) or total phospholipids (B). Data are shown as means ± SD from three different determinations carried out in duplicate. Asterisks in panel A denote significance (p <0.05) of cells not treated with propranolol versus propranolol-treated cells at the indicated conditions.

Figure 5. Effect of PLA_2 inhibitors on [^{3}H]AA incorporation in zymosan-stimulated monocytes. Human monocytes were preincubated without (○, ●) or with (△, ▲) 1 μM pyrrophenone (panel A), or without (○, ●) or with (△, ▲) 10 μM BEL (panel B) for 30 min. Afterward, the cells were either untreated (open symbols) or treated (closed symbols) with 1 mg /ml zymosan in the presence of 1 nM [^{3}H]AA (0.25 μCi/ml), and [^{3}H]AA incorporation was measured in total phospholipids for the indicated times. Data are shown as means ± SD from three different determinations carried out in duplicate. Asterisks in panel B denote significance (p <0.05) of cells not treated with BEL versus BEL-treated cells at the indicated conditions.

Figure 6. AA-CoA synthetase activity in homogenates from human monocytes. Homogenates were prepared from unstimulated monocytes (○) or monocytes treated with 1 mg/ml zymosan (●), and AA-CoA synthetase activity was determined at 30 min as described in Experimental Procedures. Data are shown as means ± SD from three different determinations carried out in duplicate.

Figure 7. Lysophospholipid:acyl-CoA acyltransferase activity in homogenates from human monocytes. Homogenates were prepared from unstimulated monocytes (○) or monocytes treated with 1 mg/ml zymosan (●), and acyltransferase activity was determined at 30 min as described in Experimental Procedures using lysoPA (A), lysoPI (B), lysoPE (C) or lysoPC (D) as acceptors. Data are shown as means ± SD from three different determinations carried out in duplicate.

Figure 8. siRNA inhibition of LPCAT3 blocks [^{3}H]AA incorporation into PC. Monocytes (A) or U937 monocyte-like cells (B) were treated either with a negative siRNA control, or siRNA for LPCAT2, or siRNA for LPCAT3, as indicated, for 48 h. After this time, the cells were stimulated with 1 mg/ml zymosan in the presence of 1 nM [^{3}H]AA (0.25 μCi/ml), and after a 30-min incubation, [^{3}H]AA incorporation into PC was determined. Data are shown as means ± SD from three different determinations carried out in duplicate. Asterisks denote significance (p <0.05) of control cells versus LPCAT3-deficient cells at the indicated conditions.