

Signaling Role for Lysophosphatidylcholine Acyltransferase 3 in Receptor-Regulated Arachidonic Acid Reacylation Reactions in Human Monocytes

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

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 A₂s 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 A₂ 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 (lysoPC) as acceptor, and inhibition of the enzyme lysoPC acyltransferase 3 by specific small interfering RNA results in inhibition of the stimulated incorporation of AA into phospholipids. Collectively, these results define lysoPC acyltransferase 3 as a novel-signal-regulated enzyme that is centrally implicated in limiting free AA levels in activated cells. *The Journal of Immunology*, 2010, 184: 1071–1078.

Arachidonic acid (AA) 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 PGs, thromboxane, leukotrienes, hydroxyeicosatetraenoic acids, and lipoxins (1). AA is an intermediate of a deacylation/reacylation cycle of membrane phospholipids (PLs), the so called Lands cycle, in which AA liberated by phospholipase A₂ (PLA₂) is converted to arachidonoyl-CoA at the expense of ATP by arachidonoyl-CoA synthetases (ACSLs) and immediately incorporated into PL by CoA-dependent acyltransferases (2–5). In resting cells, AA is predominantly found esterified in membrane PLs and is unavailable for eicosanoid biosynthesis. Under these conditions, Ca²⁺-independent PLA₂ (iPLA₂) is thought to account for most of the basal PLA₂ activity of the cells (6–10). iPLA₂ may, therefore, be a major contributor to the low levels of fatty acid liberated during the continuous recycling of membrane PLs that occurs under resting conditions (11–14). Because the rate of fatty

acid release by constitutive iPLA₂ is lesser than the rate of its reacylation back into PLs, no net accumulation of free fatty acid occurs.

Cell stimulation by a variety of agonist receptors leads to the activation of another PLA₂ form, the group IVA, calcium-dependent cytosolic PLA₂α (cPLA₂α), which then becomes the dominant PLA₂ involved in AA release (15–19). Under stimulation conditions, the rate of AA release clearly exceeds that of reincorporation into PLs, 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 cPLA₂α is converted into eicosanoids, the remainder being effectively incorporated back into PLs (2–5). Once the AA has been incorporated into PLs, a remodeling process carried out by CoA-independent transacylase transfers AA from choline glycerophospholipids (PCs) to ethanolamine glycerophospholipids, 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 PLs, 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 PLs via direct acylation of preexisting PLA₂-derived lysophospholipids. This pathway is believed to constitute the major pathway for AA incorporation into PLs 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 PLs (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.

Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, Valladolid; and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Barcelona, Spain

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Address correspondence and reprint requests to Dr. Jesús Balsinde, Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, 47003 Valladolid, Spain. E-mail address: jbalinde@ibgm.uva.es

Abbreviations used in this paper: AA, arachidonic acid; ACSL, arachidonoyl-CoA synthetase; BEL, bromoenol lactone; cPLA₂α, calcium-dependent cytosolic phospholipase A₂ α (group IVA); iPLA₂, independent phospholipase A₂; LPCAT, lysoPC:arachidonoyl-CoA acyltransferase; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPI, lysophosphatidylinositol; PA, phosphatidic acid; PC, choline glycerophospholipid; PI, phosphatidylinositol; PLA₂, phospholipase A₂; siRNA, small interfering RNA.

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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 PLs that is not secondary to an increased lysophospholipid availability due to cPLA₂α 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:arachidonoyl-CoA acyltransferase (LPCAT) level and defines 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*-octadecyl-sn-glycero-3-phosphorylcholine was obtained from BIOMOL (Plymouth Meeting, PA). [5,6,8,9,11,12,14,15-³H]AA (sp. act. 211 Ci/mmol) was purchased from GE Healthcare (Buckinghamshire, UK). [¹⁴C(U)]glycerol (sp. act. 140 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), 1-*O*-[³H]octadecyl-2-lyso-sn-glycero-3-phosphocholine (sp. act. 185 Ci/mmol) from GE Healthcare, and [³H]oleyl-L-α-lysophosphatidic acid (lysoPA) (sp. act. 54.3 Ci/mmol) was from PerkinElmer (Waltham, MA). TLC plates were from Scharlab (Barcelona, Spain). Bromoenol lactone (BEL) was from Cayman Chemical (Ann Arbor, MI). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell isolation and culture

Human monocytes were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Briefly, blood cells were diluted 1/1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 × *g* during 30 min. The mononuclear cellular layer was then recovered and washed three times with PBS, resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and 40 μg/ml gentamicin and allowed to adhere to plastic in sterile dishes for 2 h. Nonadherent 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, 100 U/ml penicillin, and 100 μg/ml streptomycin. 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 37°C in a humidified 5% CO₂ atmosphere.

Preparation of zymosan

Zymosan was prepared as described elsewhere (27, 28). Briefly, zymosan particles were suspended in PBS, boiled for 60 min, and washed three times. The final pellet was resuspended in PBS at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No PLA₂ activity was detected in the zymosan batches used in this study, as assessed by *in vitro* activity assay (29–32).

Measurement of [³H]AA and [¹⁴C]glycerol incorporation

Monocytes were untreated or treated with 1 mg/ml zymosan in the presence of exogenous [³H]AA (0.25 μCi/ml; 1 nM) or [¹⁴C]glycerol (0.1 μCi/ml; 0.7 μM). [¹⁴C]Glycerol was added 5 min 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 TLC. Neutral lipids were separated with hexane/ether/acetic acid (70:30:1, v/v/v) as a mobile phase; and the various PL classes were separated by using chloroform/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 [³H]AA release

Monocytes were incubated for 20 h with 0.25 μCi/ml [³H]AA. Afterward, supernatants were removed, and cell monolayers were washed several times with serum-free medium containing 0.5 mg/ml BSA to remove unincorporated [³H]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 with or without 1 mg/ml zymosan for the indicated times. Subsequently, supernatants were col-

lected, centrifuged to eliminate debris and detached cells, and measured for radioactivity by liquid scintillation counting.

Determination of ACSL activity

ACSL 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 cell extract was mixed with 20 mM MgCl₂, 10 mM ATP, 1 mM CoA, 1 mM 2-ME, 100 mM Tris-HCl (pH 8), and [³H]AA (25–150 μM) and incubated at 37°C for 10 min. Reactions were stopped by adding 2.25 ml 2-propanol/heptane/2 M sulfuric acid (40:10:1, v/v/v). After the addition of 1.5 ml heptane and 1 ml water, mixture was vortexed and centrifuged at 1000 × *g* for 5 min. The aqueous phase was collected, extracted twice with 2 ml 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 with or without 1 mg/ml zymosan for 30 min, were homogenized, and 50 μg cell extract was mixed with 50 mM Tris-HCl (pH 7.5), 1 mM CoA, 10 mM ATP, 20 mM MgCl₂, 1 mM 2-ME, 50 μM [³H]AA, and 5–50 μM lysophospholipid (lysoPA, lysoPC, ethanolamine lysoglycerophospholipid, or lysophosphatidylinositol [lysoPI]) in a final volume of 150 μl. After a 20-min incubation at 37°C, the reactions were stopped by adding chloroform, and the lipids were extracted according to Bligh and Dyer (33). For separation of phosphatidic acid from lysoPA, 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 used. For separation of PC, phosphatidylinositol (PI), and ethanolamine glycerophospholipid from their respective lyso counterparts, a system of chloroform/methanol/28% ammonia (65:25:5, v/v/v) was used as a mobile phase.

Small interfering RNA inhibition assays

Control small interfering RNA (siRNA), fluorescein amidite-labeled control siRNA, and siRNA directed against LPCAT2 (5'-3'GCAUGAAGAGA-GUACCUCA) and LPCAT3 (5'-3'CCAUGCCUCAUUCACAAU) 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 for 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 [³H]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 four 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 U of Moloney murine leukemia virus 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 Detector (Bio-Rad, Hercules, CA) according to previously described methods, with each reaction containing 5 ng reverse-transcribed RNA in 20 μl 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). 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^{-ΔCt}. 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 \pm SD from three different experiments. SPSS version 14 software for Windows (SPSS, Chicago, IL) was used for data analysis. Data were compared using the paired Student *t* test and differences were regarded as significant when *p* < 0.05.

Results

$[^3\text{H}]$ AA release and reincorporation into cellular PLs in zymosan-stimulated monocytes

In keeping with previous estimates (36, 37), treatment of $[^3\text{H}]$ 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 cPLA₂ α , not iPLA₂, that is responsible for receptor-mediated AA release. In vitro activity assays demonstrated that at the concentrations used in this study, cPLA₂ α and iPLA₂ activities were quantitatively inhibited by pyrrophenone and BEL, respectively. In addition, at 1 μM , no effect of pyrrophenone was detected on cellular iPLA₂ activity, and 10 μM BEL did not have any effect on cPLA₂ α activity (data not shown).

Because AA mobilization in response to stimuli represents a balance between what is released from PLs by phospholipases minus what is reincorporated back into PLs 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 $[^3\text{H}]$ AA, and at different times, the incorporation of radiolabel into the different cellular PLs was studied. Note that at this very low concentration, exogenous $[^3\text{H}]$ AA exerts no stimulatory effects on its own, and thus, the effects observed are those owing 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 $[^3\text{H}]$ AA into glycerophospholipids, particularly into PC, with lesser amounts being found in ethanolamine glycerophospholipids and PI. Significant amounts of $[^3\text{H}]$ AA were also found in triacylglycerol (15% of total AA in lipids at 60 min; see below).

AA incorporation into PLs in activated cells occurs primarily via deacylation/reacylation reactions

Two routes for AA incorporation into phospholipids exist in mammalian cells: the Lands cycle of deacylation/reacylation and the Kennedy pathway for de novo biosynthesis of PLs (4). Although at

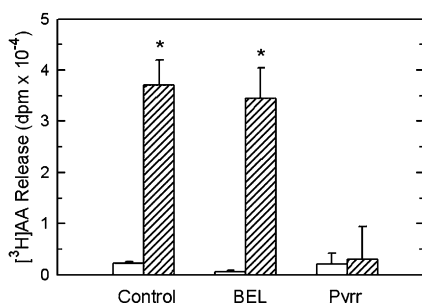


FIGURE 1. Zymosan-induced $[^3\text{H}]$ AA release in human monocytes. $[^3\text{H}]$ AA-labeled human monocytes were treated without (\square) or with (striped bars) 1 mg/ml zymosan, and in the absence (control) or presence of 10 μM BEL or 1 μM pyrrophenone (pyrr). After 60 min, supernatants were collected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

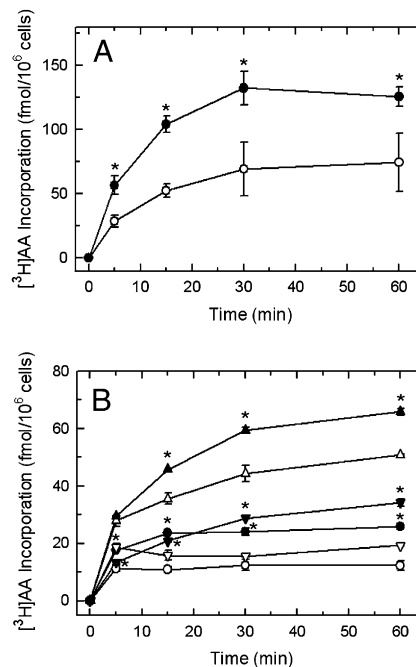


FIGURE 2. Zymosan-induced $[^3\text{H}]$ AA incorporation into monocyte cell PLs. Human monocytes were either untreated (open symbols) or treated (closed symbols) with 1 mg/ml zymosan in the presence of 1 nM $[^3\text{H}]$ AA (0.25 $\mu\text{Ci}/\text{ml}$) for the indicated times. Lipids were then extracted, and $[^3\text{H}]$ AA incorporation was measured in total PLs (A) or PLs classes (B), PI (\circ , \bullet), PC (\triangle , \blacktriangle), and PE (∇ , \blacktriangledown). Data are shown as means \pm SD from three different determinations carried out in duplicate. **p* < 0.05, significance of nonstimulated cells versus zymosan-stimulated cells at each condition.

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 $[^3\text{H}]$ AA and $[^{14}\text{C}]$ glycerol, the latter to selectively label the lipids synthesized de novo. We found that the amount of ^{14}C -radioactivity in both PLs and triacylglycerol linearly accumulated with time in activated cells (Fig. 3A), demonstrating activation of the de novo biosynthetic pathway. $[^3\text{H}]$ AA accumulated in PLs, but also in triacylglycerol (Fig. 3B), raising the possibility that AA might also significantly incorporate through the de novo route under the activation conditions. However, analysis of the PL/triacylglycerol ratio for both isotopes indicated a factor of 2 for ^{14}C and of 6 for ^3H . This difference suggests that the bulk of ^3H -radioactivity accumulating in PLs 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 PLs 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 PL biosynthesis becomes activated, its contribution to the increased incorporation of AA into PLs is minor.

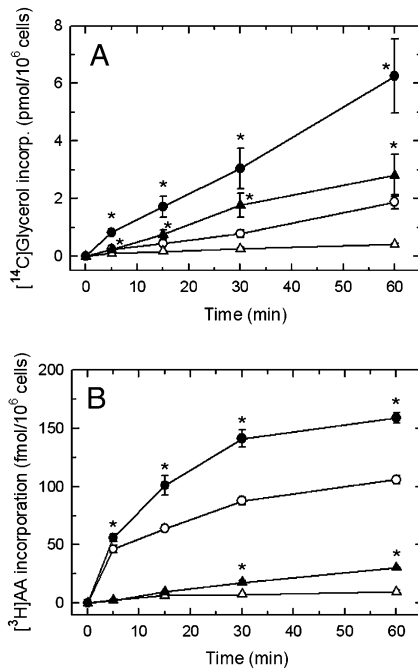


FIGURE 3. Time course of the effect of zymosan on the incorporation of [^{14}C]glycerol (A) or [^3H]AA (B) into the lipids of human monocytes. The cells were either untreated (open symbols) or treated (closed symbols) with 1 mg/ml zymosan in the presence of 1 nM [^3H]AA (0.25 $\mu\text{Ci/ml}$) or 0.7 μM [^{14}C]glycerol (0.1 $\mu\text{Ci/ml}$) for the indicated times. Afterward, lipids were extracted, and [^{14}C]glycerol (A) or [^3H]AA (B) incorporation was measured in total PLs (\circ , \bullet) and triacylglycerol (\triangle , \blacktriangle). Data are shown as means \pm SD from three different determinations carried out in duplicate. * $p < 0.05$, significance of cells not treated with zymosan-stimulated cells at each condition.

Stimulated incorporation of AA into PLs 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, [^3H]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 PL hydrolysis. Fig. 5A shows that zymosan-stimulated [^3H]AA incorporation into PLs 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 PL 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 PLs 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. 5B). 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. 5B). Therefore, the increased AA incorporation into PLs in zymosan-stimulated cells is not limited

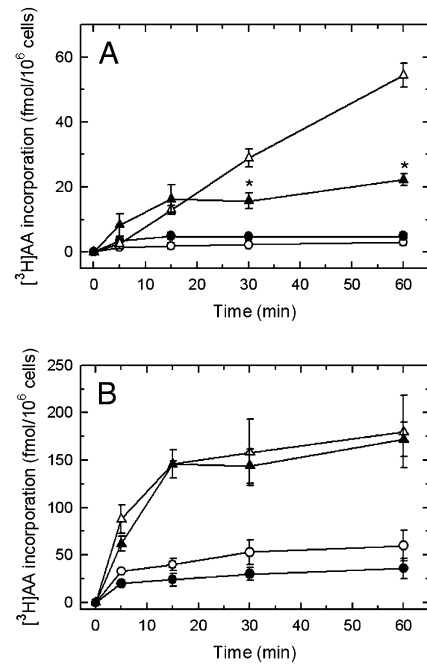


FIGURE 4. Inhibition of [^3H]AA incorporation into triacylglycerol by the phosphatidate phosphatase-1 inhibitor propranolol. Human monocytes were untreated (open symbols) or treated (closed symbols) with 200 μM propranolol for 30 min. Afterward, the cells were exposed to 1 nM [^3H]AA (0.25 $\mu\text{Ci/ml}$) in the absence (\circ , \bullet) or presence (\triangle , \blacktriangle) of 1 mg/ml zymosan for the indicated times, and [^3H]AA incorporation was measured in triacylglycerol (A) or total PLs (B). Data are shown as means \pm SD from three different determinations carried out in duplicate. * $p < 0.05$, significance of cells not treated with propranolol versus propranolol-treated cells at the indicated conditions (A).

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 as follows: ACSL, LPCAT ethanolanine lysoglycerophospholipid:arachidonoyl-CoA acyltransferase, lysoPI:arachidonoyl-CoA acyltransferase, and lysoPA:arachidonoyl-CoA acyltransferase. Of all these activities, only LPCAT was found to be increased in homogenates from zymosan-treated cells versus resting cell homogenates (Figs. 6, 7). These data suggest that LPCAT is a signal-regulated activity underlying the increased AA incorporation into PLs of activated monocytes.

LPCAT3 regulates PL 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 ($25 \pm 5\%$, inhibition for both genes, as assessed by quantitative RT-PCR; mean \pm SD, $n = 3$), which was not unexpected given that monocytes, as primary cells, are known to

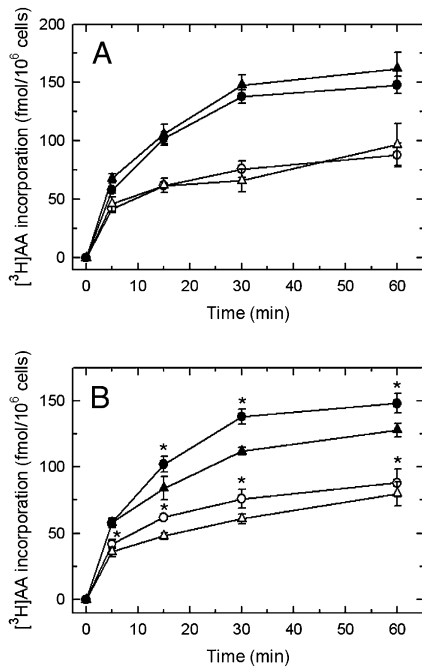


FIGURE 5. Effect of PLA₂ inhibitors on [³H]AA incorporation in zymosan-stimulated monocytes. Human monocytes were preincubated without (○, ●) or with (△, ▲) 1 μM pyrrophenone (A), or without (○, ●) or with (△, ▲) 10 μM BEL (B) for 30 min. Afterward, the cells were either untreated (open symbols) or treated (closed symbols) with 1 mg/ml zymosan in the presence of 1 nM [³H]AA and (0.25 μCi/ml), and [³H]AA incorporation was measured in total PLs for the indicated times. Data are shown as means ± SD from three different determinations carried out in duplicate. **p* < 0.05, significance of cells not treated with BEL versus BEL-treated cells at the indicated conditions (B).

be hard to transfect. By using fluorescein amidite-labeled siRNAs, we estimated an efficiency of transfection of $28 \pm 6\%$ (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 PLs in stimulated cells. Further evidence was obtained

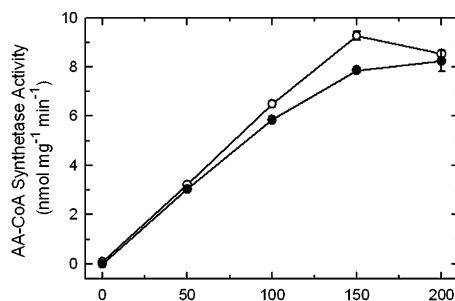


FIGURE 6. ACSL activity in homogenates from human monocytes. Homogenates were prepared from unstimulated monocytes (○) or monocytes treated with 1 mg/ml zymosan (●), and ACSL activity was determined at 30 min as described in *Materials and Methods*. Data are shown as means ± SD from three different determinations carried out in duplicate.

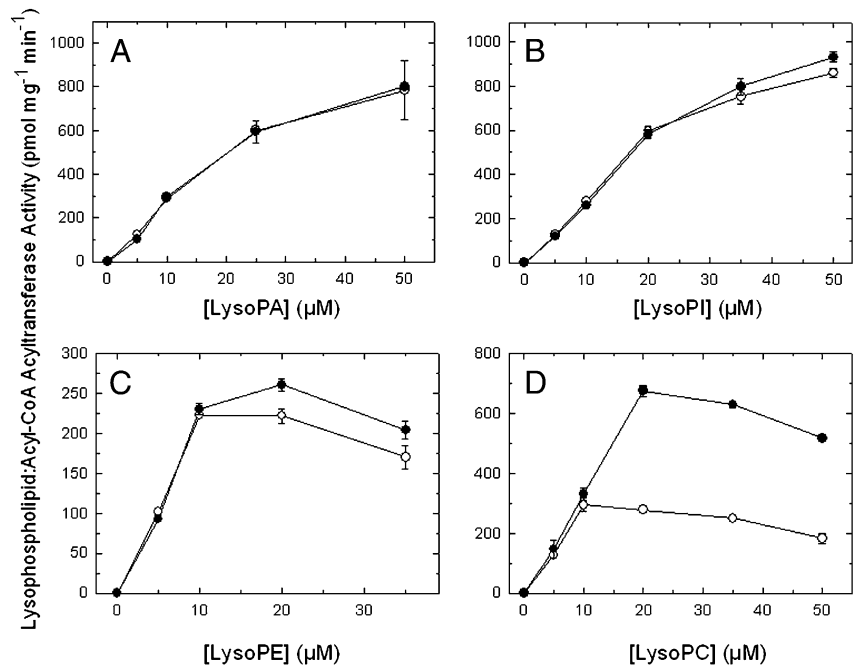
Discussion

The metabolism of AA reflects a carefully balanced series of biochemical pathways. The Lands cycle is a mechanism for the deacylation/reacylation of membrane PLs by which polyunsaturated fatty acids, such as AA, are incorporated into different species (2–4). Although many studies have been conducted in resting cells, much less is known on the regulatory features of PL AA incorporation in activated cells, where the sustained activation of cPLA₂α results in a rate of AA release that exceeds that of reacylation back into PLs. 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 PL AA incorporation in activated cells may be secondary to the PLA₂ hydrolytic step because AA incorporates preferentially into the sn-2 position of PLs, and thus, for an enhanced AA reacylation to occur, 2-lysophospholipid acceptors, produced only by PLA₂, 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, ACSL and the CoA-dependent acyltransferases, are severalfold higher than that of PLA₂ 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 on cPLA₂α 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 PLs predominantly via a lysophospholipid reacylation pathway and not via de novo. Strikingly, lack of sensitivity of AA incorporation to pyrrophenone clearly suggests that PL AA incorporation is not merely triggered by the increased abundance of lysophospholipid acceptors produced by receptor-activated cPLA₂α 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 using 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 using various lysophospholipid acceptors, namely lysoPC, ethanolamine lysoglycerophospholipid, lysoPI, and lysoPA. The first three lysophospholipids are used in the Lands cycle, and the latter one is an acceptor of the de novo PL 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 ethanolamine lysoglycerophospholipid, lysoPI, or lysoPA were not detected. The absence of an increased activity toward phosphatidic acid (PA) was not unexpected, given our data showing that zymosan-induced PL AA incorporation does not proceed via the de novo biosynthetic pathway. However, our inability to detect increased acyltransferase activity using either ethanolamine lysoglycerophospholipid or lysoPI is difficult to explain in view of our

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 *Materials and Methods* using lysoPA (A), lysoPI (B), lysoPE (C), or lysoPC (D) as acceptors. Data are shown as means \pm SD from three different determinations carried out in duplicate.



own data showing that AA incorporates not only into PC but also into ethanolamine glycerophospholipid and PI during zymosan stimulation of the cells and that acyl transferase enzymes showing clear preference for ethanolamine lysoglycerophospholipid and lysoPI have been described previously (48, 64, 65). It is possible that a significant portion of the AA reincorporated into these PLs in activated cells, particularly ethanolamine glycerophospholipids, enters indirectly via transacylation reactions using AA-containing

PC as an AA donor. Such a route has been demonstrated to exist in human neutrophils and to significantly contribute to PL fatty acid remodeling (66).

To date, four enzymes with LPCAT activity have been described in humans (45), LPCAT1, LPCAT2, and LPCAT4, members of the 1-acylglycerol-3-phosphate O-acyltransferase family, and LPCAT3, belonging to the membrane-bound O-acyltransferase 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 acyltransferase activity in RAW364.7 cells transfected with the mouse lyso-platelet-activating factor acetyl transferase/LPCAT2 gene and stimulated with TLR 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 LPS, 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 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 provide clues to understanding the regulation of AA incorporation into the PLs of stimulated human monocytes. Specifically, evidence has been provided to indicate that this process is not secondary to the activation of intracellular PLA₂s and the subsequent rise in lysophospholipid levels. Rather, our studies have suggested that PL AA incorporation is 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 classes, such as ethanolamine lysoglycerophospholipid and lysoPI, during the activation process as well as to ascertain the involvement of other acyltransferases.

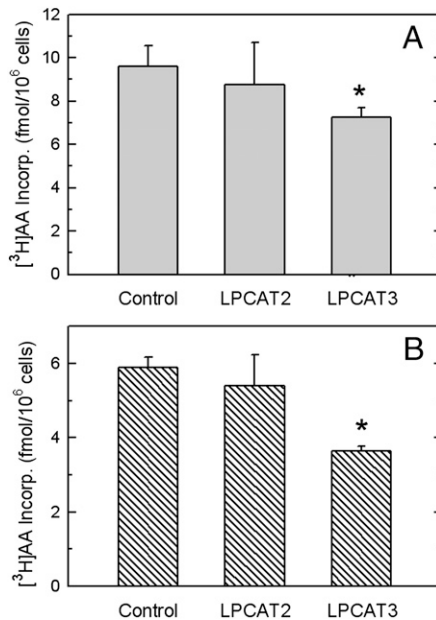


FIGURE 8. siRNA inhibition of LPCAT3 blocks [³H]AA incorporation into PC. Monocytes (A) or U937 monocyte-like cells (B) were treated either with a negative siRNA control, or siRNA for LPCAT2, or siRNA for LPCAT3, as indicated, for 48 h. After this time, the cells were stimulated with 1 mg/ml zymosan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

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Disclosures

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