

# Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A<sub>2</sub>-dependent synthesis of lipid droplets in human monocytes

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**Abstract** Exposure of human peripheral blood monocytes to free arachidonic acid (AA) results in the rapid induction of lipid droplet (LD) formation by these cells. This effect appears specific for AA in that it is not mimicked by other fatty acids, whether saturated or unsaturated. LDs are formed by two different routes: (i) the direct entry of AA into triacylglycerol and (ii) activation of intracellular signaling, leading to increased triacylglycerol and cholesteryl ester formation utilizing fatty acids coming from the de novo biosynthetic route. Both routes can be dissociated by the arachidonyl-CoA synthetase inhibitor triacsin C, which prevents the former but not the latter. LD formation by AA-induced signaling predominates, accounting for 60–70% of total LD formation, and can be completely inhibited by selective inhibition of the group IVA cytosolic phospholipase A<sub>2</sub>α (cPLA<sub>2</sub>α), pointing out this enzyme as a key regulator of AA-induced signaling. LD formation in AA-treated monocytes can also be blocked by the combined inhibition of the mitogen-activated protein kinase family members p38 and JNK, which correlates with inhibition of cPLA<sub>2</sub>α activation by phosphorylation. Collectively, these results suggest that concomitant activation of p38 and JNK by AA cooperate to activate cPLA<sub>2</sub>α, which is in turn required for LD formation possibly by facilitating biogenesis of this organelle, not by regulating neutral lipid synthesis.—Guijas, C., G. Pérez-Chacón, A. M. Astudillo, J. M. Rubio, L. Gil-de-Gómez, M. A. Balboa, and J. Balsinde. **Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A<sub>2</sub>-dependent synthesis of lipid droplets in human monocytes.** *J. Lipid Res.* 2012. 53: 2343–2354.

**Supplementary key words** arachidonic acid • lipid mediators • monocytes/macrophages • inflammation • phospholipase A<sub>2</sub>

Lipid droplets (LD) are cytosolic inclusions present in most eukaryotic cells that contain a core rich in neutral lipids such as triacylglycerol (TAG) and cholesteryl esters (CE) and are surrounded by a phospholipid monolayer decorated with a variety of proteins, such as PAT family proteins (perilipin, adipose differentiation related protein, and tail-interacting protein of 47 kDa [TIP-47]) and caveolins (1–6). Initially regarded as inert neutral lipid-storage compartments, the interest for LD has increased recently because of their association with inflammatory and metabolic disorders involving an excess lipid storage, including diabetes, obesity, and cardiovascular disease (7–10).

LDs are generated by cells under different environmental conditions, suggesting a distinct pathophysiological significance for each of these conditions. Cells generate lipid droplets from exogenous lipid sources, especially free fatty acids and cholesterol from serum lipoproteins (11–15), probably with an energy-storage purpose; however, when cells are under different stress signals, LD biogenesis occurs in the absence of external lipid via rearrangement of membrane phospholipids and fatty acids into newly formed TAG molecules (16, 17).

The leukocytes, cells typically associated with inflammatory reactions can induce the rapid formation of LDs when exposed to proinflammatory stimuli (18–21). Moreover, it is becoming increasingly recognized that LDs are specialized intracellular sites for the biosynthesis and amplification of the eicosanoid biosynthetic response during inflammation

Abbreviations: AA, arachidonic acid; CE, cholesteryl esters; cPLA<sub>2</sub>α, group IVA cytosolic phospholipase A<sub>2</sub>α; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; LD, lipid droplets; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; TAG, triacylglycerol

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(10, 20–22). Decided amounts of arachidonic acid (AA) are present in the phospholipid monolayer surrounding the LD, and a variety of enzymes involved in AA metabolism have been demonstrated to localize in LDs (23–26). One of these enzymes is the group IVA phospholipase A<sub>2</sub>, also known as cytosolic phospholipase A<sub>2</sub>α (cPLA<sub>2</sub>α), a central enzyme for the release of AA from phospholipids (27–31). cPLA<sub>2</sub>α is phosphorylated and activated by members of the mitogen-activated protein kinase family of enzymes (i.e., the extracellular-regulated kinases [ERK] p42/p44, p38, and c-Jun N-terminal kinase [JNK]), although the specific form involved appears to depend on cell type and stimulus (30).

In this work we have examined the pathways for LD biosynthesis in human monocytes exposed to free AA and have identified the signaling cascade and intracellular events leading to LD formation in human monocytes. On one hand, AA may just serve as a lipid source for TAG biosynthesis and subsequent LD formation; on the other hand, AA concomitantly activates the MAP kinases p38 and JNK, both of which promote LD formation in a manner that depends on a biologically active cPLA<sub>2</sub>α enzyme.

## MATERIALS AND METHODS

### Reagents

Cell culture medium and BODIPY® 493/503 were obtained from Molecular Probes-Invitrogen (Carlsbad, CA). Chloroform and methanol (HPLC grade) were from Fisher Scientific (Hampton, NH). [5,6,8,9,11,12,14,15-<sup>3</sup>H]AA (sp. act. 211 Ci/mmol) was purchased from GE Healthcare (Buckinghamshire, UK). [1,2-<sup>14</sup>C]acetic acid (sp. act. 54.3 mCi/mmol) was from Perkin Elmer (Waltham, MA). Silicagel thin layer chromatography plates were from Macherey-Nagel (Düren, Germany). The p38 MAP kinase inhibitor SB 203580 was from Calbiochem/Merck KGaA (Darmstadt, Germany). Triacsin C was purchased from Enzo Life Sciences (Farmingdale, NY). Paraformaldehyde was from Electron Microscopy Sciences (Hartfield, PA). Antibodies against p-cPLA<sub>2</sub>(Ser505), p-p38(Thr180/Tyr182) and p-JNK(Thr183/Tyr185) were purchased from Cell Signaling (Danvers, MA). The cPLA<sub>2</sub>α inhibitor pyrrophenone was synthesized and generously provided by Dr. Amadeu Llebaria (Institute for Chemical and Environmental Research, Barcelona, Spain) (32). All other reagents were from Sigma-Aldrich.

### Cell isolation and culture conditions

Human monocytes were isolated from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Written informed consent was obtained from each donor. Briefly, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 *g* for 30 min. The mononuclear cellular layer was recovered and washed three times with PBS, resuspended in RPMI 1640 medium supplemented with 40 µg/ml gentamicin, and allowed to adhere in sterile dishes for 2 h at 37°C in a humidified atmosphere of CO<sub>2</sub>/air (1:19). Nonadherent cells were removed by washing extensively with PBS, and the remaining attached monocytes were used the following day. Human macrophages were obtained by incubating plastic-adhered monocytes in RPMI with heat-inactivated 5% human serum for 2 weeks in the absence of exogenous cytokine mixtures.

Fatty acids were dissolved in ethanol, and an appropriate aliquot was diluted in the incubation medium to obtain the desired

concentration. When radioactive fatty acids were used, they were spiked into an ethanol solution containing cold fatty acids to generate the required specific radioactivity before adding them to the incubation media. Ethanol concentrations in the incubation media were always below 0.1%, and the appropriate controls were run to ensure that ethanol had no effect on its own on cells. When inhibitors were used, they were added to the incubation media 30 min before treating the cells with AA. For all experiments, the cells were incubated in media consisting of serum-free RPMI 1640 medium (supplemented with 2 mM L-glutamine) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### GC-MS analysis of fatty acid methyl esters

After incubations, the cells were washed twice with PBS, and a cell extract corresponding to 10<sup>7</sup> cells was scraped in ice-cold water and sonicated in a tip homogenizer twice for 15 s. Before extraction and separation of lipid classes, internal standards were added. For total phospholipids, 10 nmol of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine was added; for TAG, 10 nmol of 1,2,3-triheptadecanoylglycerol was added; and for CE, 30 nmol of cholesteryl tridecanoate was added. Total lipids were extracted according to Bligh and Dyer [33], and the resulting lipid extract was separated by thin-layer chromatography using *n*-hexane/diethyl ether/acetic acid (70:30:1, by vol.) as the mobile phase. Spots corresponding to the various lipid classes were scraped, and phospholipids were extracted from the silica with 800 µl methanol followed by 800 µl chloroform/methanol (1:2, v/v) and 500 µl chloroform/methanol (2:1, v/v). TAG and CE were extracted with 1 ml chloroform/methanol (1:1, v/v) followed by 1 ml of chloroform/methanol (2:1, v/v). Glycerolipids were transmethylated with 500 µl of 0.5 M KOH in methanol for 30 min at 37°C. To neutralize, 500 µl of 0.5 M HCl was added. Cholesteryl esters were transmethylated as follows. Each fraction was resuspended in 400 µl of methyl propionate, and 600 µl of 0.84 M KOH in methanol was added for 1 h at 37°C. Afterward, 50 µl and 1 ml of acetic acid and water, respectively, were added to neutralize. Extraction of fatty acid methyl esters was carried out with 1 ml *n*-hexane twice.

Analysis of fatty acid methyl esters was carried out in a Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (70 eV) equipped with an Agilent 7693 autosampler and an Agilent DB23 column (60 m length × 250 µm internal diameter × 0.15 µm film thickness) under the conditions described previously (34, 35) with a slight modification of the procedure to improve separation of fatty acid methyl esters. Briefly, oven temperature was held at 50°C for 1 min, increased to 175°C at a rate of 25°C/min, increased to 215°C at a rate of 1.5°C/min, with the final ramp being reached at 235°C at a rate of 10°C/min. The final temperature was maintained for 5 min, and the run time was 39.67 min. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

### Measurement of fatty acid incorporation into TAG

Monocytes preincubated with various concentrations of triacsin C were exposed to 3 nM [<sup>3</sup>H]AA (0.25 µCi/ml) or 7 nM [<sup>3</sup>H]palmitic acid (0.25 µCi/ml) for 30 min. Afterward, the cells were washed four times with PBS containing 0.5% albumin to remove the fatty acid that had not been incorporated. Cells were scraped twice with 0.1% Triton X-100 in PBS, and total lipids were extracted according to the method of Bligh and Dyer (33), reconstituted in chloroform/methanol (2:1, v/v), and separated by thin-layer chromatography with *n*-hexane/ether/acetic acid (70:30:1, v/v/v). The spots corresponding to TAG were cut out, and the plate and analyzed for radioactivity by liquid scintillation counting (36–38).

## Measurement of fatty acid synthesis

For these experiments, [<sup>14</sup>C]acetic acid (0.1 μCi/ml) was added to the cells at the time they were treated or not with 10 μM AA plus 3 μM triacsin C for 2 h. Afterward, the reactions were stopped, and the cell monolayers were scraped twice with 0.1% Triton X-100 in PBS. Lipids were extracted according to the method of Bligh and Dyer (33). The total lipid fraction was subjected to alkaline hydrolysis and, after re-extraction, total <sup>14</sup>C-radioactivity levels in the organic phase were determined by scintillation counting.

## Viability assays

For viability assays, monocytes were cultured in 96-well microtiter plates. At the end of the different treatments, viability was measured by using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (Promega Biotech Iberica, Madrid, Spain). This is a colorimetric method for determining the number of viable cells in culture. The solution contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) that, when reduced, produces a colored formazan product that absorbs maximally at 490 nm. The compound is reduced due to the mitochondrial activity in the cell. The amount of formazan that is produced is directly proportionally to the number of viable cells.

## PCR

RNA was extracted using the TRIzol reagent method (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was obtained by using the Moloney murine leukemia virus reverse transcriptase from 1 μg of RNA. PCR was then performed using specific primers for long-chain acyl-CoA synthetases as follows: 5'-ccagaaggcttcaagactg-3' (forward) and 5'-gccttctgtgctgtgcaac-3' (reverse) for ACSL-1, 5'-catcgccatctctgtgaga-3' (forward) and 5'-gggtgcttccatcaacagt-3' (reverse) for ACSL-3, 5'-ccgacctaa-gggagtgatga-3' (forward) and 5'-cctgcagccataggtaaagc-3' (reverse) for ACSL-4, and 5'-accagtggctgtctctaccag-5' (forward) and 5'-gctgatgtccgctgtattga-3' (reverse) for ACSL-6. Cycling conditions were as follows: 1 cycle at 95°C for 10 min, 35 cycles at 95°C for 30 s, 58°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

Quantitative PCR was carried out with an ABI 7500 machine (Applied Biosystems, Carlsbad, CA) using the Brilliant III Ultra-Fast SYBR<sup>®</sup>Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA) and specific primers for each gene as follows: acetyl-CoA carboxylase, 5'-tcacacctgaagacctaaagcc-3' (forward) and 5'-agcccactgctgtactg-3' (reverse); fatty acid synthase, 5'-acagcgggaatgggtact-3' (forward) and 5'-gactggtacaacgagcggat-3' (reverse); stearoyl-CoA desaturase, 5'-ttctactctcaagtctacac-3' (forward) and 5'-ccgagcttgaagagcgg-3' (reverse); very long-chain fatty acid elongase-6, 5'-aacgagcaaaagttgaactgagg-3' (forward) and 5'-tcgaagagcaccgaataactga-3' (reverse). Cycling conditions were: 1 cycle at 95°C for 3 min and 40 cycles at 95°C for 12 s, 60°C for 15 s and 72°C for 28 s. The relative mRNA abundance for a given gene was calculated using the algorithm  $2^{-\Delta\Delta Ct}$ , with β-actin and cyclophilin A as internal standards (39).

## Cellular staining and fluorescence microscopy

For these experiments, the cells were plated on coverslips on the bottom of 6-well dishes in a volume of 2 ml. The cells were fixed with 1 ml of 4% paraformaldehyde in PBS containing 3% sucrose for 20 min. Afterward, paraformaldehyde was removed by washing the cells thrice with PBS, and BODIPY493/503 and DAPI stainings were carried out by treating cells with these dyes at concentrations of 2 μg/ml and 1 μg/ml, respectively, in PBS for 10 min. Coverslips were mounted on microscopy slides with 25 μl of a polyvinyl alcohol solution until analysis by fluorescence

microscopy. Fluorescence was monitored by microscopy using a NIKON Eclipse 90i device equipped with a CCD camera (model DS-Ri1; Nikon, Tokyo, Japan). A mercury HBO excitation lamp (Osram, Munich, Germany) was used, and the fluorescence from DAPI and BODIPY493/503 was recovered using the combination of a UV-2A (Ex 330-380; DM 400; BA 420) and a B-2A (Ex 450-490; DM 505; BA 520) filter, respectively. Images were analyzed with the software NIS – Elements (Nikon). Green and blue channels were merged with the Image-J software (<http://rsb.info.nih.gov/ij/>).

## PLA<sub>2</sub> activity assays

Ca<sup>2+</sup>-dependent PLA<sub>2</sub> activity was measured using a modification of the mammalian membrane assay described by Diez et al. [40]. Briefly, monocyte homogenates were incubated for 1–2 h at 37°C in 100 mM HEPES (pH 7.5) containing 1.3 mM CaCl<sub>2</sub> and 100,000 dpm [<sup>3</sup>H]AA-labeled membrane, used as a substrate, in a final volume of 0.15 ml. Before assay, the cell membrane substrate was heated at 57°C for 5 min to inactivate CoA-independent transacylase activity (41). The assay contained 25 μM bromoenol lactone to completely inhibit endogenous Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity [36]. After lipid extraction, free [<sup>3</sup>H]AA was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. For Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity, the cell homogenates were incubated for 2 h at 37°C in 100 mM HEPES (pH 7.5) containing 5 mM EDTA and 100 mM labeled phospholipid substrate (1-palmitoyl-2-[<sup>3</sup>H]palmitoyl-glycero-3-phosphocholine, sp. act. 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) in a final volume of 150 ml. The phospholipid substrate was used in the form of sonicated vesicles in buffer. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1:2). After lipid extraction, free [<sup>3</sup>H]palmitic acid was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. In some experiments, Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity was also measured using a mixed-micelle substrate or the natural membrane assay. For the mixed micelle assay, Triton X-100 was added to the dried lipid substrate at a molar ratio of 4:1. Buffer was added, and the mixed micelles were made by a combination of heating above 40°C, vortexing, and water bath sonication until the solution clarified. The natural membrane assay was carried out exactly as described above, except that CaCl<sub>2</sub> was omitted and 5 mM EDTA was added instead. All of these assay conditions have been validated previously regarding time, homogenate protein, and substrate concentration (42–48).

## Immunoblot analyses

After the different treatments, the cells were lysed for 30 min in ice-cold buffer containing 20 nM Tris-HCl (pH 7.4), 150 nM NaCl, 0.5% Triton X-100, 100 nM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitor cocktail (Sigma). Total protein (10–50 μg) was resolved on 10–12% SDS-PAGE gels and transferred to PVDF membrane. After transfer, nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 at room temperature for 2 h. The membranes were then probed with the corresponding antibodies followed by HRP-conjugated secondary antibodies in blocking solution. β-Actin was used as a load control. The immunoblots were visualized using enhanced luminescence. Densitometry was performed on scanned images using Quantity One<sup>®</sup> software (Bio-Rad Laboratories), and values were normalized for the corresponding controls of each experiment.

## Statistical analysis

All experiments were carried out at least three times with incubations in duplicate or triplicate. Statistical analysis was carried out by the Student's *t*-test, with *p* values < 0.05 taken as statistically significant.

## RESULTS

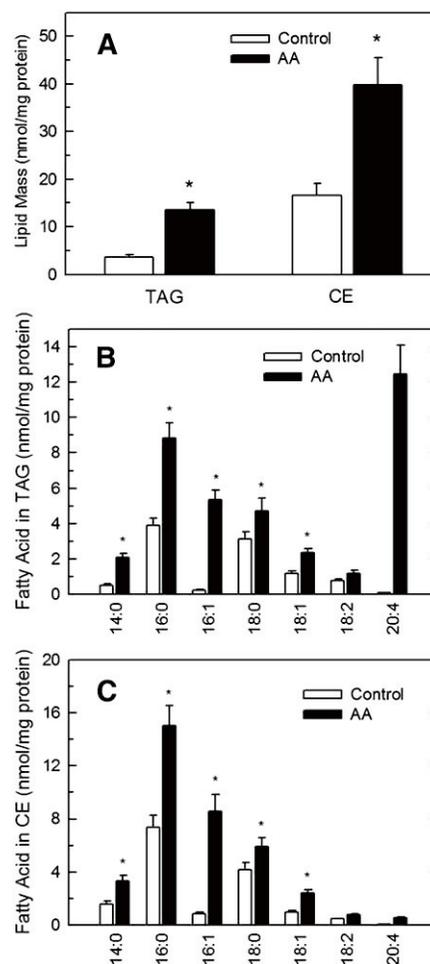
### AA promotes TAG and CE synthesis and increases formation of lipid droplets in human monocytes

Exposure of human peripheral blood monocytes to low micromolar doses of exogenous AA (10  $\mu$ M) for 2 h significantly increased the intracellular amount of TAG and CE (Fig. 1A). Analysis by GC-MS of the fatty acid composition of TAG in control versus AA-treated cells indicated that not only AA increased in this fraction but also several other fatty acids, especially palmitic acid (16:0) and palmitoleic acid (16:1) (Fig. 1B). Thus, these results indicate that increased TAG synthesis by exogenous AA does not merely reflect the “passive,” direct incorporation of the fatty acid into this lipid class but also involves the recruitment of other fatty acids. This “active” signaling component manifested even better when the fatty acid composition of CE was analyzed (Fig. 1C). Because no AA was found in this fraction, all the increases in CE in the AA-treated cells were due to the mobilization of other fatty acids to this lipid class. Palmitic acid and palmitoleic acid were, again, the major fatty acids incorporated into CE (Fig. 1C).

To characterize further the mechanism underlying this “active” signaling component of exogenous AA on monocytes, it was necessary to dissociate it from the “passive” component leading to incorporation of AA into TAG. This was achieved by using triacsin C, an inhibitor of certain long-chain acyl-CoA synthetase forms (49). Mammalian cells contain five long-chain acyl-CoA synthetases, termed ACSL-1, -3, -4, -5, and -6, and all five were found to be expressed in human monocytes, as judged by PCR (Fig. 2A). The triacsin C-sensitive forms are ACSL-1, -3, and -4, and it is known that these are the ones involved in the incorporation of AA into cellular lipids (30). Triacsin C concentrations as low as 3  $\mu$ M quantitatively inhibited incorporation of AA into TAG (Fig. 2B), yet incorporation of palmitic acid was only partially inhibited, to about 60–70%, reflecting the participation of triacsin C-sensitive and -insensitive routes in palmitic acid incorporation (Fig. 2C). Fig. 2D shows the effect of triacsin C on TAG fatty acid distribution in AA-treated human monocytes. In the presence of triacsin C, there still was a greatly increased formation of TAG because the recruitment of fatty acids distinct from AA was not significantly affected. The impairment of AA incorporation reduced the total amount of TAG produced by 25–30% with respect to that produced in the absence of triacsin C (Fig. 2E). Collectively, these data indicate that the majority of TAG produced after exposure of the monocytes to AA occurs through AA-initiated signaling, not as a consequence of merely increased availability of lipid.

Based on the data presented above, 3  $\mu$ M triacsin C was routinely added to the incubations to specifically study the intracellular signaling actions of AA. Viability studies using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay demonstrated that triacsin C was not toxic to the cells alone or in combination with the fatty acids at the concentrations indicated (not shown).

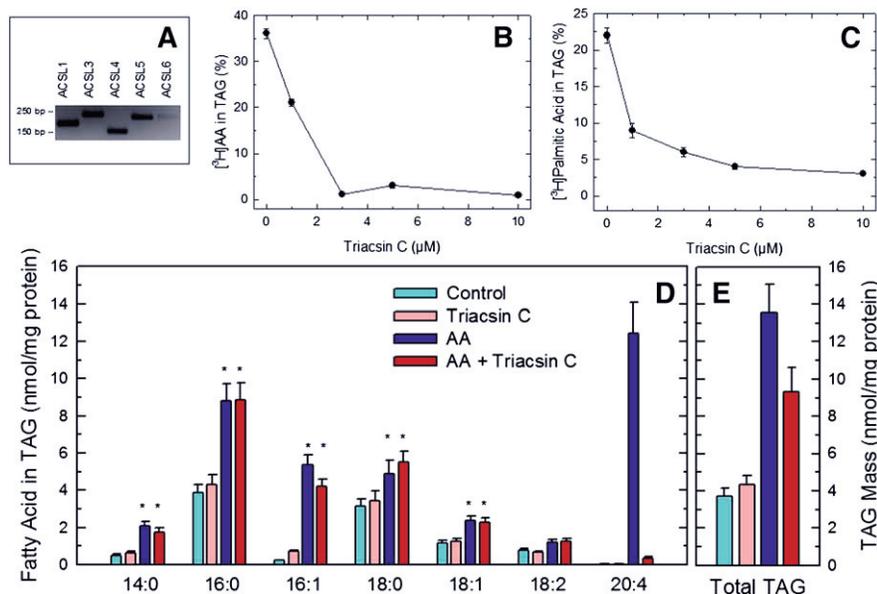
To study whether the enhanced production of TAG and CE induced by AA in human monocytes results in the



**Fig. 1.** AA-induced TAG and CE formation in human monocytes. The cells were treated with 10  $\mu$ M AA for 2 h. Afterward, fatty acids in TAG and CE were analyzed by GC-MS after converting the fatty acid glycerol and cholesteryl esters into fatty acid methyl esters. A: Total cellular TAG values in control versus AA-treated cells and the result of adding the masses of all fatty acids under each condition and dividing by 3. Total CE values in control versus AA-treated cells are also shown, as is the result of adding the masses of all fatty acids under each condition. B: Fatty acid profile of TAG in control versus AA-treated cells. C: Fatty acid profile of CE in control versus AA-treated cells. Data are given as means  $\pm$  SE of three independent experiments. \*Significantly different ( $p < 0.05$ ) from their respective controls.

formation of LD, microscopy experiments were performed to visualize these cytoplasmic organelles (Fig. 3). Unlike human macrophages (26), resting human monocytes contain very few LD. However, incubation of the cells with 10  $\mu$ M AA for 2 h induced a very significant production of LD, which was also readily observable in cells pretreated with triacsin C (Fig. 3). LD staining in the AA-treated cells appeared to be more punctate in the absence than in the presence of triacsin C (Fig. 3). It is possible that this difference could be related to the different polyunsaturated fatty acid content of these LD (see Fig. 2).

The effect of other fatty acids on LD formation in monocytes was investigated for comparison. Palmitic acid (16:0), a saturated fatty acid, induced no significant LD formation (Fig. 3). Other saturated fatty acids tested (i.e., myristic acid



**Fig. 2.** Effect of triacsin C on the incorporation of fatty acids into TAG in human monocytes. A: PCR analysis of ACSL-1, -3, -4, -5, and -6 mRNA expression in human monocytes. B and C: Monocytes treated with the indicated concentrations of triacsin C for 30 min were incubated with 3 nM [<sup>3</sup>H]AA (B) or 7 nM [<sup>3</sup>H]palmitic acid (C) for 30 min. Afterward, lipids were extracted, and [<sup>3</sup>H]AA and [<sup>3</sup>H]palmitic acid incorporation were measured in TAG as described under Materials and Methods. The <sup>3</sup>H radioactivity incorporated into TAG is expressed as a percentage of the radioactivity originally added to the cells. D: Effect of triacsin C on the distribution of fatty acids in TAG after treatment of the monocytes with 10 μM AA for 2 h. The analysis of TAG fatty acids was carried out by GC-MS after converting the fatty acid glyceryl esters into fatty acid methyl esters. E: Total cellular TAG values and the result of adding the masses of all fatty acids under each condition and dividing by 3. Data are given as means ± SE of three independent experiments. \*Significantly different (*p* < 0.05) from their respective controls.

[14:0] or stearic acid [18:0]) also failed to induce LD formation (not shown). Unsaturated fatty acids, such as oleic (18:1), linoleic (18:2), or  $\gamma$ -linolenic ( $\gamma$ 18:3), induced LD formation, albeit to a much lower extent than AA, and in a triacsin C-dependent manner (Fig. 3), suggesting that, unlike AA, these fatty acids induce LD formation by serving primarily as lipid fuel, not by initiating intracellular signaling. In support of this suggestion, the low LD formation induced by oleic, linoleic, and  $\gamma$ -linolenic in the absence of triacsin C was not affected by preincubation of the cells with inhibitors of kinases and of cPLA<sub>2</sub>α (not shown).

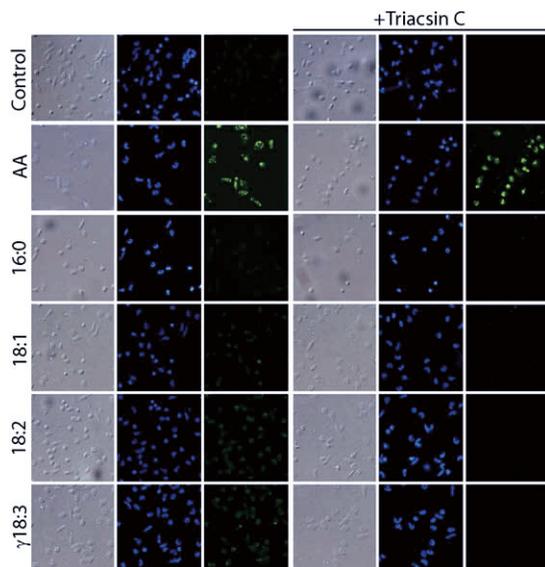
#### AA induces de novo fatty acid synthesis in monocytes

Fatty acid moieties incorporated into newly synthesized LD that do not derive from exogenous sources are known to originate from stimulation of the de novo biosynthetic pathway or from membrane phospholipid rearrangements regulated by phospholipases (16). The data in Figs. 1 and 2 show that palmitoleic acid was, together with palmitic acid, the fatty acid that showed the most significant increases in LD from AA-treated cells, this occurring in the TAG and in the CE fractions. Because palmitoleic acid is thought to constitute a marker for de novo fatty acid biosynthesis in cells and tissues (50), the increases in this fatty acid in human monocyte LD are highly suggestive of a de novo origin. To explore this possibility, the total cellular content of esterified fatty acid was studied by GC-MS in human monocytes

treated or not with exogenous AA for 2 h. Resting monocytes were found to contain 224 ± 12 nmol of esterified fatty acids per mg protein (mean ± SEM; *n* = 3). Treatment of the cells with AA for 2 h increased the amount of endogenous fatty acids by 1.3-fold, to 295 ± 17 nmol per mg protein (mean ± SEM; *n* = 3), clearly indicating that de novo synthesis of fatty acid had occurred. Because these experiments were conducted in the presence of triacsin C, the increase in the esterified fatty acid mass seen in the AA-treated cells could not be due to incorporation of exogenous AA into various lipids (Fig. 4) nor did it proceed at the expense of the pre-existing free fatty acid pool because that one is exceedingly low, typically comprising 2–5% of total cellular fatty acid (i.e., <10 nmol per mg protein).

To directly confirm that AA induces fatty acid synthesis in monocytes, the cells were exposed to [<sup>14</sup>C]acetic acid, and the incorporation of <sup>14</sup>C-radioactivity into cellular fatty acids was determined in cells treated or not with 10 μM AA for 2 h. The AA-treated cells incorporated twice as much radioactivity into fatty acids as did cells not treated with AA, thus demonstrating activation of fatty acid synthesis by AA.

The profile of major fatty acids of control versus AA-treated monocytes is shown in Fig. 4. Significant increases were detected in the content of the saturated fatty acids myristic (14:0) and palmitic acid (16:0), but especially palmitoleic acid (16:1), the levels of which increased by 5-fold. This increase in palmitoleic acid levels was completely

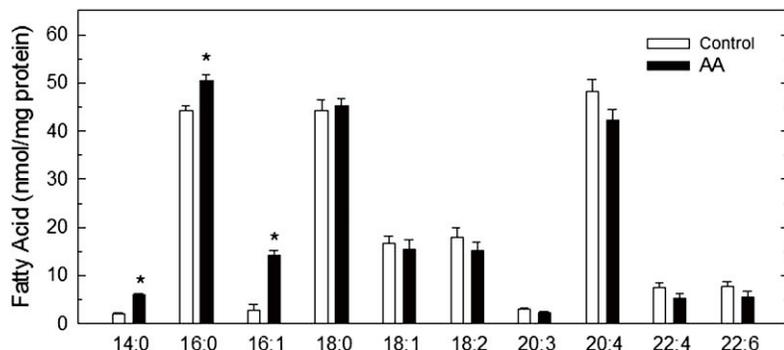


**Fig. 3.** AA-induced LD formation in human monocytes. Monocytes, treated without (left column) or with (right column) 3  $\mu$ M triacsin C for 30 min, were exposed to AA, palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), or  $\gamma$ -linolenic acid ( $\gamma$ 18:3) for 2 h as indicated. All fatty acids were added at a concentration of 10  $\mu$ M. After fixation and permeabilization, cells were stained with BODIPY493/503 (2  $\mu$ g/ml) to visualize LD (green; right panels) and DAPI (1  $\mu$ g/ml) to mark the nuclei (blue; central panels). Left panels show the Nomarski images.

prevented by pretreating the cells with the selective stearoyl-CoA desaturase inhibitor Cay10566 (10  $\mu$ M) (51). A tendency was found for some polyunsaturated fatty acids, particularly AA but also linoleic (18:2) and adrenic (22:4) acids, to decrease in the AA-treated monocytes, but it did not reach statistical significance (Fig. 4). This might be a reflection of cPLA<sub>2</sub> $\alpha$  activation under these conditions (see below). Collectively, these results suggest that exposure of human monocytes to AA increases de novo fatty acid synthesis, which channels primarily saturated fatty acids and palmitoleic acid to neutral lipids and ultimately results in increased LD biogenesis.

#### AA up-regulates the expression of genes involved in lipogenesis in human monocytes

Fatty acid synthesis in mammalian cells is known to involve a series of enzymes acting sequentially, namely acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase, and



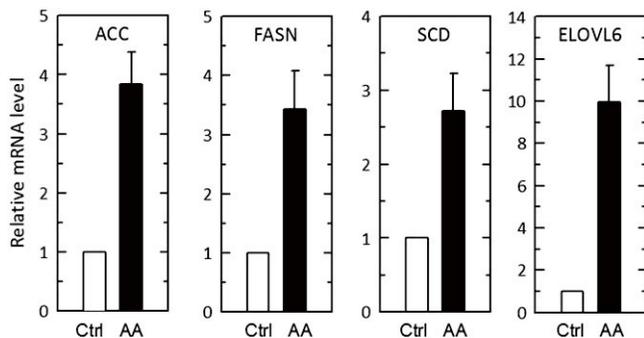
**Fig. 4.** Cellular fatty acid content in control and AA-treated cells. The profile of major fatty acids in control (open bars) or cells treated for 2 h with 10  $\mu$ M AA in the presence of 3  $\mu$ M triacsin C (closed bars) was determined by GC-MS after converting the fatty acid glycerol and cholesteryl esters into fatty acid methyl esters. Data are expressed as means  $\pm$  SE of three independent determinations. \*Significantly different ( $p < 0.05$ ) from their respective controls.

long-chain fatty acid elongase. The entire pathway is controlled by the transcription factor sterol regulatory element-binding protein-1c (52, 53). By using quantitative PCR, we found that treating the monocytes with AA for 2 h led to significant increases in the mRNA levels of acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase, and very long-chain fatty acid elongase-6 (Fig. 5). These results suggest that the lipid biosynthetic response of monocytes to AA involves sterol regulatory element-binding protein-dependent transcription.

#### AA-mediated signaling leading to LD formation involves p38/JNK-activated PLA<sub>2</sub> $\alpha$

Cellular responses to various stimuli are known to frequently involve the activation of lipid signaling mediated by cPLA<sub>2</sub> $\alpha$  (27–31). This enzyme can be potently inhibited by the indole derivative pyrrophenone, a compound that exhibits more than 1,000-fold selectivity for inhibition of cPLA<sub>2</sub> $\alpha$  versus other intracellular PLA<sub>2</sub>s (54). Fig. 6 shows that preincubation of human monocytes with pyrrophenone concentrations as low as 1  $\mu$ M led to a strong inhibition of LD biogenesis in response to AA. In the experiments shown in Fig. 6, triacsin C was included to specifically focus on the signaling component of the AA effect. Thus, the data point to an indispensable role for cPLA<sub>2</sub> $\alpha$  in AA-induced LD formation and suggest that this enzyme is an intracellular target for AA in human monocytes. To verify that AA signals to cPLA<sub>2</sub> $\alpha$  activation in human monocytes, cell homogenates, either untreated or treated with 10  $\mu$ M AA, were prepared, and assays were conducted to assess PLA<sub>2</sub> activity. Significant increases in the Ca<sup>2+</sup>-dependent activity of the homogenates from AA-treated cells versus untreated cells was detected (Fig. 7A). Conversely, no significant changes in the Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity of homogenates was found, thus highlighting the specificity of the Ca<sup>2+</sup>-dependent increase. The Ca<sup>2+</sup>-dependent activity measured corresponded to cPLA<sub>2</sub> $\alpha$  because inclusion of pyrrophenone completely abolished it. In contrast, inclusion of 5  $\mu$ M scapularial, a selective inhibitor of secreted PLA<sub>2</sub>s (55, 56), showed no significant effect (data not shown).

Activation of cPLA<sub>2</sub> $\alpha$  is associated to its phosphorylation on Ser<sup>505</sup> (30). Thus, the levels of phosphorylation of the enzyme on this residue were measured by immunoblot after exposing the cells to 10  $\mu$ M AA. An anti-phospho-cPLA<sub>2</sub> $\alpha$  antibody was used that specifically recognizes the phosphorylated Ser<sup>505</sup> residue. Thus, any increase in cPLA<sub>2</sub> $\alpha$

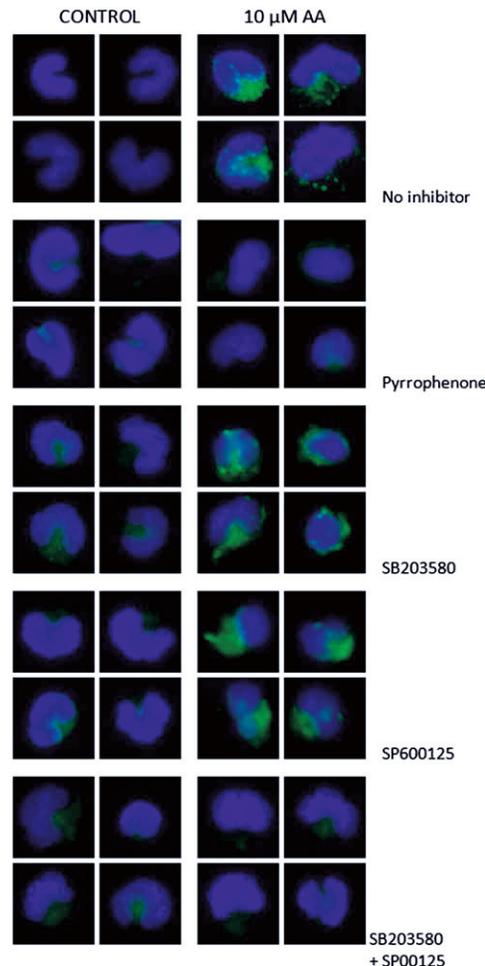


**Fig. 5.** Effect of AA on the expression of genes involved in de novo fatty acid synthesis in human monocytes. The relative expression of genes in control (open bars) or cells treated for 2 h with 10  $\mu$ M AA (closed bars) was determined by quantitative PCR as described under Materials and Methods. All incubations received 3  $\mu$ M triacsin C. ACC, acetyl-CoA carboxylase  $\alpha$ ; ELOVL6, very long-chain fatty acid elongase 6; FASN, fatty acid synthase; SCD, stearoyl-CoA desaturase. Data are given as means  $\pm$  SE of three independent experiments.

phosphorylation under these conditions reflects an increase in the phosphorylation of Ser<sup>505</sup> and not of other residues. This approach showed that under resting conditions, cPLA<sub>2</sub> $\alpha$  was already phosphorylated to some extent, but exposure of the cells to AA markedly increased such phosphorylation (Fig. 7A, inset).

To identify the kinase implicated in the phosphorylation of cPLA<sub>2</sub> $\alpha$  on Ser<sup>505</sup>, well established inhibitors of putative cPLA<sub>2</sub> $\alpha$  upstream kinases were used. The methoxyflavone derivative PD98059 was used to selectively inhibit the extracellular-signal regulated kinases p42 and p44 (57), the pyridinyl-imidazole SB203580 was used to selectively inhibit p38 (58), and the anthrapyrazolone inhibitor SP600125 was used to selectively block JNK (59). Initial experiments showed that AA induces an early and sustained activation of p38 and JNK (Fig. 7B, D, E). Conversely, activation of the extracellular-signal regulated kinases p42 and p44 could not be detected under any condition tested (not shown). In keeping with the latter, PD98059 exerted no inhibitory effect on cPLA<sub>2</sub> $\alpha$  phosphorylation (Fig. 7C, D). However, SB203580 and SP600125 reduced cPLA<sub>2</sub> $\alpha$  phosphorylation (Fig. 7C, F). Importantly, when the p38 and JNK inhibitors were added together, cPLA<sub>2</sub> $\alpha$  phosphorylation was decreased to a lower extent than that found under resting conditions (Fig. 7C, F). These data suggest that p38 and JNK act to phosphorylate/activate cPLA<sub>2</sub> $\alpha$  and that the extent of phosphorylation of cPLA<sub>2</sub> $\alpha$  at this residue results from the simultaneous action of p38 and JNK.

Fluorescence microscopy analysis of LD formation in the presence of these inhibitors revealed that, when used separately, SB203580 and SP600125 had no effect on LD formation in response to AA; for inhibition of LD formation to be clearly observed, the presence of both inhibitors at the same time was required (Fig. 6). Analysis of TAG and CE production in cells treated with the inhibitors confirmed that, when added separately, SB203580 and SP600125 had no effect on TAG or CE levels (Fig. 8); however, when added together, a strong inhibition of TAG and CE formation was observed

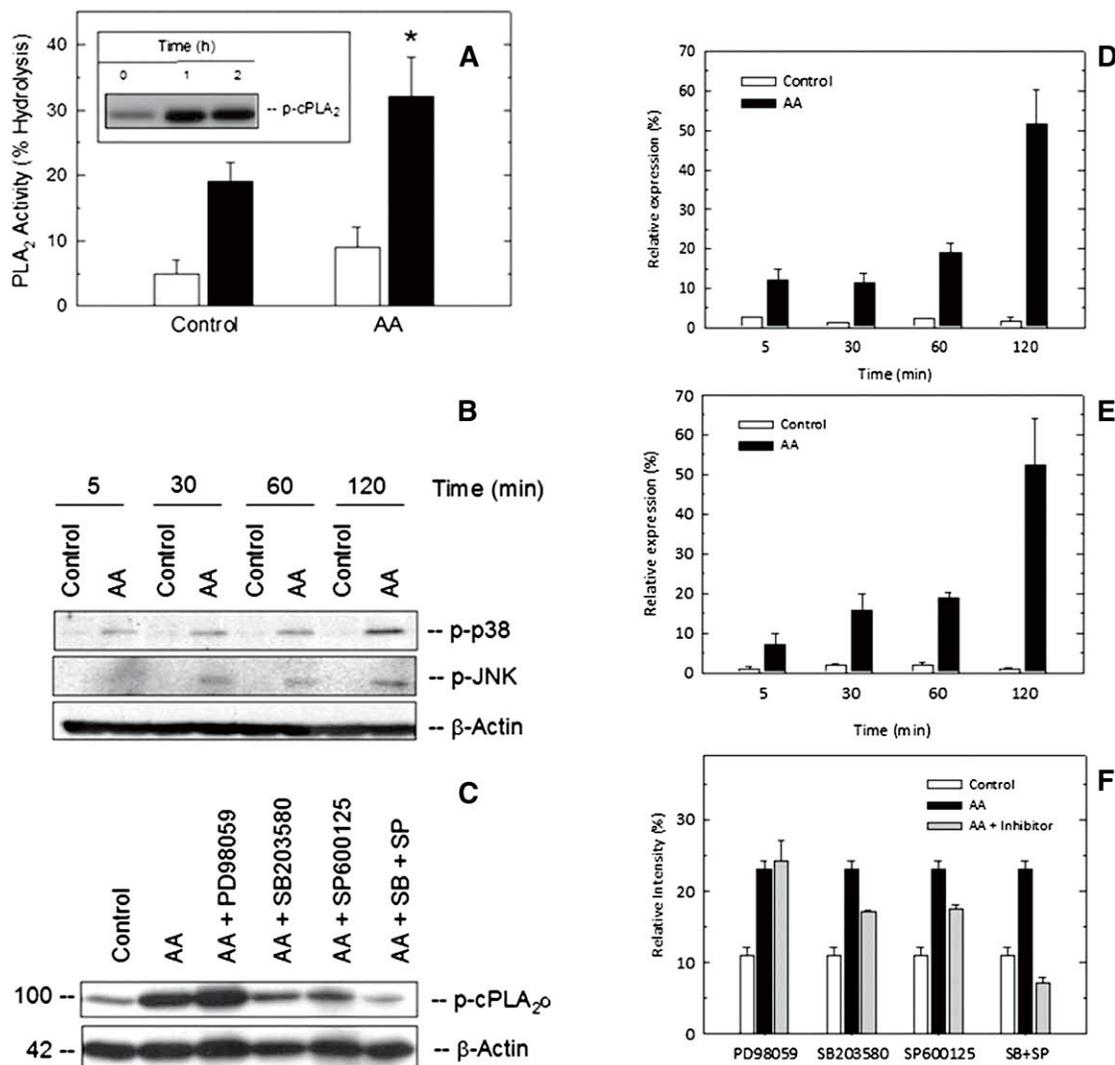


**Fig. 6.** Effect of various inhibitors on AA-induced LD formation. Monocytes, preincubated with 3  $\mu$ M triacsin C for 30 min, were untreated (left column) or treated with 10  $\mu$ M AA for 2 h (right column) in the presence of the indicated inhibitor at the following concentrations: 1  $\mu$ M pyrrophenone, 10  $\mu$ M SB203580, and 10  $\mu$ M SP600125. After fixation and permeabilization, cells were stained with BODIPY493/503 (2  $\mu$ g/ml) to visualize LD and with DAPI (1  $\mu$ g/ml) to mark the nuclei.

(Fig. 8). Taken together, these results indicate that conditions that lead to complete blockade of cPLA<sub>2</sub> $\alpha$  phosphorylation activation by AA by the simultaneous inhibition of p38 and JNK (Fig. 7C) result in ablation of the cell's capacity to synthesize neutral lipids and produce LD in response to AA.

#### AA induced neutral lipid synthesis in human macrophages

To extend these results to other cells of the phagocytic lineage, studies were conducted with monocyte-derived macrophages. Fig. 9 shows that incubation of the macrophages with 10  $\mu$ M AA for 2 h also induced neutral lipid synthesis, as manifested by noticeable increases in the intracellular content of TAG and CE. Analysis by GC-MS of the fatty acid composition of TAG in control versus AA-treated cells revealed increases in various saturated and monounsaturated fatty acids, including palmitoleic acid. These results are qualitatively similar to those obtained previously with AA-treated monocytes. However, because resting macrophages contain much more TAG than resting monocytes



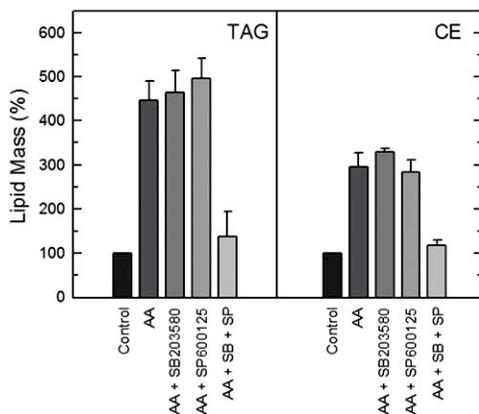
**Fig. 7.** Stimulation of mitogen-activated protein kinases and cPLA $\alpha$  by AA in human monocytes. **A:** PLA $_2$  activity of homogenates from monocytes. Homogenates from untreated cells (Control) or from cells treated with 10  $\mu$ M AA for 2 h were prepared, and PLA $_2$  activity was measured in the absence (*open bars*) or presence (*closed bars*) of 1 mM CaCl $_2$  in the assay mix. Inset shows the detection of cPLA $_2\alpha$ , phosphorylated at Ser $^{505}$  at different times, by immunoblot. **B:** Monocytes were treated without (Control) or with 10  $\mu$ M AA for the indicated times and analyzed for expression of phosphorylated p38 and JNK by immunoblot. **C:** Analysis of the kinases implicated in cPLA $_2\alpha$  phosphorylation. Monocytes were treated with 10  $\mu$ M AA for 2 h as indicated. Some of the samples were preincubated with the following specific kinase inhibitors as indicated: 10  $\mu$ M PD98059, 10  $\mu$ M SB203580, 10  $\mu$ M SP600125, or 10  $\mu$ M SB203580, plus 10  $\mu$ M SP600125. All incubations proceeded in the presence of 3  $\mu$ M triacsin C. Phosphorylation of cPLA $_2\alpha$  at Ser $^{505}$  was analyzed by immunoblot. The Western blots for phosphorylated p38, JNK, and cPLA $_2\alpha$  were quantified from three different experiments (means  $\pm$  SE), and the quantifications are shown in panels D, E, and F, respectively.

(Figs. 1 and 9), the increases in TAG in macrophages after addition of AA were not as dramatic as those observed with monocytes. Thus, the findings in macrophages represent a trend that, although qualitatively similar to those in monocytes, is of less clear significance. Regarding CE, increases in palmitic acid and palmitoleic acid were prominent in the AA-treated macrophages, which is also in agreement with the results with monocytes (Fig. 9).

## DISCUSSION

In this work we have shown that AA rapidly and potently induces LD formation in human monocytes and have delineated the intracellular signaling involved. Our results

indicate that two distinct and separable mechanisms exist for increasing TAG content in human monocytes exposed to exogenous AA, namely (*i*) the direct incorporation of part of the exogenous AA into TAG in LDs, probably for an energy-storage purpose, and (*ii*) the mobilization of other fatty acids from endogenous sources and their incorporation into TAG. Although the first mechanism may work to protect the cell from free fatty acid abundance, the second mechanism is the consequence of AA-triggered intracellular signaling. Both routes can be separated by the arachidonyl-CoA synthetase inhibitor triacsin C, which completely abrogates the former, leaving intact the latter. Use of triacsin C also shows that the pathway involving regulated signaling is responsible for the majority of LD produced after



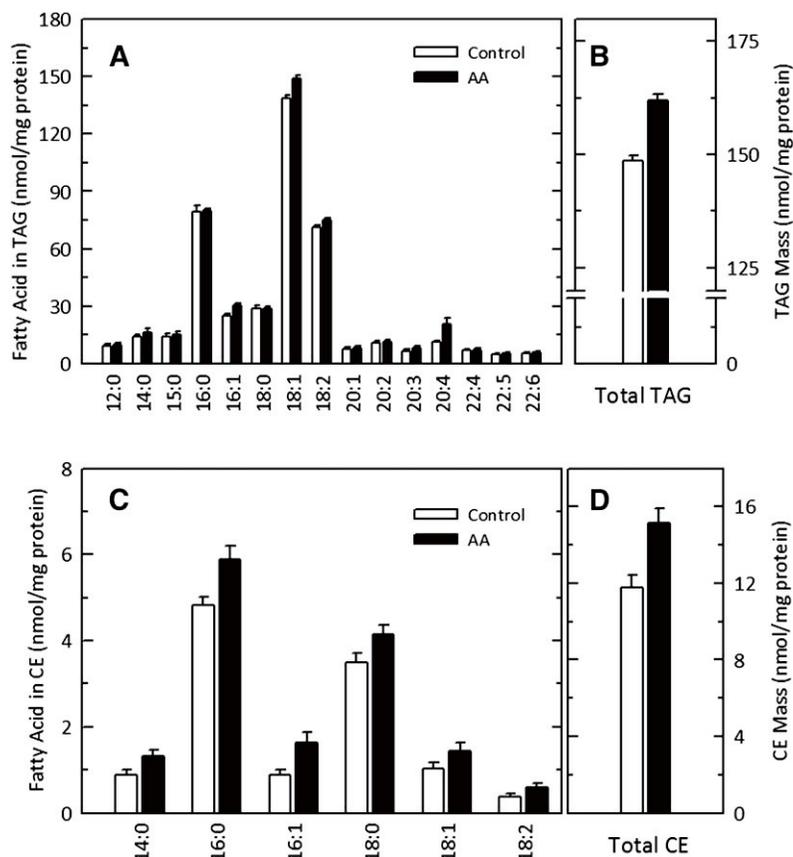
**Fig. 8.** Effect of MAP kinase inhibitors on total TAG and CE mass. Monocytes were untreated (C) or treated with 10  $\mu$ M AA for 2 h in the absence or presence of 10  $\mu$ M PD98059, 10  $\mu$ M SB203580, 10  $\mu$ M SP600125, or 10  $\mu$ M SB203580, plus 10  $\mu$ M SP600125 (SB+SP), as indicated. All incubations received 3  $\mu$ M triacsin C. Afterward, total TAG (open bars) and CE (gray bars) was determined by GC-MS after converting the fatty acid glyceryl and cholesteryl esters into fatty acid methyl esters. Data are expressed as means  $\pm$  SE of three independent determinations.

exposure of the monocytes to AA and, in turn, allows us to specifically study the signaling component of the AA response. By doing so, we have unveiled an indispensable role for cPLA<sub>2</sub> $\alpha$  in LD formation via activation of fatty acid synthesis, leading to increased neutral lipid formation.

Free AA is known to affect several intracellular and intercellular signaling pathways and to induce LD synthesis

in a variety of cell systems (19, 21, 25). The current results add to this view by showing that AA selectively activates p38- and JNK-mediated phosphorylation cascades that ultimately lead to increased transcription of genes involved in fatty acid synthesis that depend on sterol regulatory element-binding protein-1c (52, 53). Whether these effects are mediated by the fatty acid itself or a metabolite is under investigation. Preliminary evidence from our laboratory seems to suggest that an oxygenated metabolite is not involved because inhibitors of cyclooxygenase and lipoxygenase do not block the AA effect. Recently, it has been reported that some of the stimulatory effects of exogenous palmitoleic acid on murine fibroblast-like cell lines might be due not to the fatty acid itself but to the fatty acid accumulating into phosphatidylinositol species (51). In this regard, we have previously found that AA incorporates into 25–30 different phospholipid molecular species in monocytes, of which only three increase in activated cells (60, 61). One of these three species is an ethanolamine phospholipid containing palmitoleic acid in addition to AA, namely 1-palmitoleoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (60). Given that palmitoleic acid increases so markedly in the AA-treated monocytes, it is tempting to speculate with a possible role for this particular phospholipid species in mediating at least some of the AA effects reported in this paper. Experiments are in progress to explore this possibility.

The activating effect of AA on neutral lipid synthesis occurs not only in monocytes but also in macrophages, suggesting that this effect could be a common feature of



**Fig. 9.** Cellular fatty acid content in neutral lipids in control and AA-treated human macrophages. The profile of major fatty acids in control (open bars) or cells treated for 2 h with 10  $\mu$ M AA (closed bars) in the presence of 3  $\mu$ M triacsin C was determined by GC-MS after converting the fatty acid glyceryl and cholesteryl esters into fatty acid methyl esters. A: Fatty acid content in TAG. B: Total cellular TAG mass, obtained from adding the masses of all fatty acids under each condition, and dividing by 3. C: Fatty acid content in CE. D: Total cellular CE mass, obtained from adding the masses of all fatty acids under each condition. Data are expressed as means  $\pm$  SE of three independent determinations.

phagocytic cells. This observation may be relevant from a physiological/pathophysiological perspective because, although monocytes may be exposed to high circulating AA levels as a result of an acute encounter with foreign material, the macrophages are the cells frequently exposed to high concentrations of unesterified free AA, this occurring at tissue sites of inflammation. Free AA at these inflammatory foci may arise as a result of regulated membrane phospholipid hydrolysis or from cells undergoing apoptosis in situ.

The role that cPLA<sub>2</sub>α appears to play as a central regulator of this AA-initiated pathway leading to neutral lipid synthesis and LD formation is intriguing. Given its ability to release fatty acids from phospholipids, it could be envisioned as a role for cPLA<sub>2</sub>α in providing fatty acids that directly or indirectly (i.e., to serve as substrates for formation of longer chain species) participate in the formation of neutral lipid for storage in LD. However, this possibility appears unlikely because, in addition to the well-described preference of cPLA<sub>2</sub>α for AA, our data suggest that the fatty acids used for LD synthesis in the AA-activated cells derive from stimulated de novo synthesis and not from regulated membrane phospholipid hydrolysis. On the other hand, it is remarkable that the preferred product of cPLA<sub>2</sub>α action on phospholipids may act to activate the enzyme intracellularly. However, recent data suggest that cPLA<sub>2</sub>α may play roles in cell physiology that are fundamentally distinct from regulating AA availability, namely the regulation of the structure of the organelles to which the enzyme translocates during cell activation, membrane fusion, and membrane trafficking processes (62). cPLA<sub>2</sub>α has recently been found to regulate the formation of membrane tubules between Golgi cisternae (62). This effect is thought to be due to the remodeling of Golgi phospholipid fatty acid chains by cPLA<sub>2</sub>α, which results in the conversion of cylindrically shaped phospholipids to conically shaped products in local regions that force the membrane to adopt a curved structure, thus facilitating the formation of membrane tubules (62). Likewise, cells depleted of cPLA<sub>2</sub>α by RNA silencing result in the appearance of tubulo-vesicular profiles of the smooth endoplasmic reticulum, compatible with a role of cPLA<sub>2</sub>α in regulating the structure of this organelle (11).

cPLA<sub>2</sub>α has recently been reported to translocate to LD after cell activation (24, 25, 63). Studies on cPLA<sub>2</sub>α targeting to membranes have indicated that, in addition to increased Ca<sup>2+</sup> availability, there are other factors regulating the association of the enzyme to membranes, including the local concentration of phosphoinositides in the membrane (64, 65) and the phosphorylation state of the cPLA<sub>2</sub>α (66). The latter observation is of particular relevance to this work on the basis of our previous results, indicating that phosphorylation of cPLA<sub>2</sub>α on Ser505 by JNK is required for the enzyme to translocate to phagosomal membranes during phagocytosis (67). In analogy with these data, it is possible that the simultaneous phosphorylation of the enzyme by p38 and JNK facilitates the targeting of cPLA<sub>2</sub>α to the LD monolayer. Studies are underway to explore this attractive hypothesis.

The identity of the mitogen-activated protein kinase family member regulating the phosphorylation of cPLA<sub>2</sub>α on Ser<sup>505</sup> appears to be notoriously cell- and species specific. Most of the data available point to p42/p44 ERKs or p38 (30), and in humans, more recent work has also implicated JNK (67, 68). We are not aware of other studies reporting the simultaneous involvement in phagocytic cells of two of these kinases (i.e., p38 and JNK) in regulating cPLA<sub>2</sub>α phosphorylation/activation and attendant cellular responses. Because giving the p38 and JNK inhibitors separately does not inhibit cPLA<sub>2</sub>α phosphorylation or neutral lipid synthesis and LD formation but adding the inhibitors in combination completely blocks these responses, we can infer that p38 and JNK act cooperatively to mediate the AA-dependent cPLA<sub>2</sub>α activation and LD biogenesis. Moreover, because both kinases regulate the phosphorylation of cPLA<sub>2</sub>α at the same site (i.e., Ser<sup>505</sup>), it is tempting to speculate that these effects could be due to the phosphorylation of a downstream enzyme by both JNK and p38, which then activates cPLA<sub>2</sub>α and leads to full LD synthesis. This situation would be analogous to the one described by Aimand et al. (69) in cardiomyocytes, where it was found that inhibition of mitogen and stress-activated kinase-1, a kinase that is phosphorylated/activated by both p38 and ERK p42/p44, results in inhibition of cPLA<sub>2</sub>α activation and cPLA<sub>2</sub>α-mediated cellular responses.

In conclusion, we demonstrate that AA is a potent inducer of neutral lipid synthesis in human phagocytes at pathophysiological relevant concentrations. We further elucidate the intracellular pathways leading to LD production. The cascade involves concomitant activation of cPLA<sub>2</sub>α by p38 and JNK and activation of de novo fatty acid synthesis. Although further in vitro and in vivo studies are necessary to elucidate the complex actions of AA on innate immune cells, the dissection of signaling pathways triggered by extracellular AA could offer opportunities of therapeutic intervention to ameliorate the inflammatory response. ■

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