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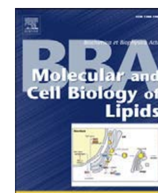


# Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells

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ABSTRACT

The availability of free arachidonic acid (AA) constitutes a limiting step in the synthesis of biologically active eicosanoids. Free AA levels in cells are regulated by a deacylation/reacylation cycle of membrane phospholipids, the so-called Lands cycle, as well as by further remodeling reactions catalyzed by CoA-independent transacylase. In this work, we have comparatively investigated the process of AA incorporation into and remodeling between the various phospholipid classes of human monocytes and monocyte-like U937 cells. AA incorporation into phospholipids was similar in both cell types, but a marked difference in the rate of remodeling was appreciated. U937 cells remodeled AA at a much faster rate than human monocytes. This difference was found not to be related to the differentiation state of the U937 cells, but rather to the low levels of esterified arachidonate found in U937 cells compared to human monocytes. Incubating the U937 cells in AA-rich media increased the cellular content of this fatty acid and led to a substantial decrease of the rate of phospholipid AA remodeling, which was due to reduced CoA-independent transacylase activity. Collectively, these findings provide the first evidence that cellular AA levels determine the amount of CoA-independent transacylase activity expressed by cells and provide support to the notion that CoA-IT is a major regulator of AA metabolism in human monocytes.

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## 1. Introduction

Arachidonic acid (AA) is an ω-6 essential polyunsaturated fatty acid with key roles in inflammation due to it being the precursor of a variety of compounds with potent pro- and anti-inflammatory actions, the so-called eicosanoids [1,2]. Since the bulk of AA in cells is found esterified in the sn-2 position of membrane phospholipids, availability of free AA is a limiting factor for eicosanoid synthesis [3,4].

Control of free AA levels in cells is exquisitely regulated by two competing reactions, namely, phospholipid deacylation by phospholipase $A_2$ enzymes, and reacylation into various phospholipid pools by acyltransferases [4]. Depending on the activation state of the cell, one reaction dominates over the other, i.e. in resting cells, reacylation dominates, and hence, the bulk of cellular AA is found in the esterified form. In stimulated cells, the dominant reaction is the phospholipase $A_2$-mediated deacylation, which results in a dramatic increase of free AA that is now available for eicosanoid synthesis [4–6].

The process of acylation/deacylation in membrane phospholipids is not the only mechanism that regulates free AA levels in cells, as transacylation reactions between phospholipids are also required to achieve the proper distribution of AA among membrane phospholipid pools [3,7]. These reactions are catalyzed by CoA-independent transacylase (CoA-IT), an enzyme that transfers AA primarily from diacyl PC species to ether-linked species, in particular the PE plasmalogens [3,7]. This remodeling of AA within the various phospholipid species appears to be crucial for the generation of eicosanoids during cellular stimulation, inasmuch as ether-linked phospholipids are thought to provide the bulk of free AA to be converted to eicosanoids, and the rate of remodeling is accelerated several-fold [8–11].

In addition to cell activation, other factors may exist that modify the rate of remodeling of phospholipids with AA. For example, a striking difference between proliferating and non-proliferating cells has repeatedly been observed [12–15], and blockade of the CoA-IT-dependent remodeling of proliferating cells promotes apoptotic cell death [16–19]. Despite numerous studies on CoA-IT [3,20–25], purification and/or cloning of the enzyme remains elusive, which has made it difficult to study in depth the cellular regulation of this enzyme.

The objective of this study was to understand better the biochemical features of AA incorporation into, and remodeling within,

*Abbreviations:* AA, arachidonic acid; CoA-IT, coenzyme A-independent transacylase; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol; PMA, phorbol myristate acetate; TAG, triacylglycerol

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phospholipid species of human monocytes and monocyte-like U937 cells, in particular with regard to the state of cellular differentiation and intracellular content of esterified AA. The results presented here suggest that the rate of phospholipid AA remodeling is strikingly dependent on cellular content of this fatty acid.

## 2. Materials and methods

### *2.1. Reagents*

RPMI 1640 medium was from Invitrogen Life Technologies (San Diego, CA). [5,6,8,9,11,12,14,15−$^3$H]AA (specific activity 211 Ci/mmol) and radioactive substrates for enzyme assays were from GE Healthcare (Alcobendas, Madrid, Spain). Thin-layer chromatography plates were from Scharlab (Barcelona, Spain). All other reagents were from Sigma.

### *2.2. Cell isolation and culture*

U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 mg/ml). For experiments, the cells were incubated at 37 °C in a humidified atmosphere of $CO_2/O_2$ (1:19) at a cell density of $0.5–1\times10^6$ cells/ml in 12-well plastic culture dishes (Costar). Cell differentiation was induced by adding PMA to a final concentration of 35 ng/ml for 24 h [26,27]. U937 cell differentiation was confirmed morphologically by light microscopy and by conversion of cells to an adherent cell population. For preparation of cells loaded with unlabeled AA, the cells were placed in serum-free medium at a density of $0.5\times10^6$ cells/ml and exposed to 100 μM AA complexed with bovine serum albumin (ratio 5:1) for 20 h, washed 3–4 times with medium containing 0.5 mg/ml BSA, and placed in serum-free medium for subsequent experiments.

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 $750\times g$ 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 and 40 mg/ml gentamicin and allowed to adhere to plastic 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. For all experiments, monocytes 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% $CO_2$ atmosphere.

### *2.3. Measurement of [$^3$H]AA incorporation into cellular phospholipids*

The cells were placed in serum-free medium for 60 min before exposure to exogenous [$^3$H]AA (1 μM; 0.25 μCi/ml). At the indicated times, supernatants were removed and total cellular lipids were extracted according to Bligh and Dyer [28], and separated by thin-layer chromatography with n-hexane/diethyl ether/acetic acid (70:30:1, by vol.). For separation of phospholipid classes, a mobile phase consisting of chloroform/methanol/acetic acid/water (25:20:3:0.3, by vol.) was used.

### *2.4. Measurement of phospholipid [$^3$H]AA remodeling*

For these experiments, the cells were pulse-labeled with [$^3$H]AA (1 μM; 0.25 μCi/ml) for 30 min at 37 °C. The cells were then washed four times with medium containing 0.5 mg/ml bovine serum albumin to remove the unincorporated label. Afterward the cells were placed in serum-free medium and incubated at 37 °C for the indicated periods of time [14]. The lipids were extracted and separated as described earlier.

### *2.5. Gas chromatography/mass spectrometry analysis of fatty acid methyl esters*

$5–10\times10^6$ cells were taken, washed once with PBS, and lipids extracted according to Bligh and Dyer [28]. The resulting extract was transmethylated with 500 μl of 0.5 M KOH in MeOH for 30 min at 37 °C. One volume of 0.5 M HCl was added to neutralize and fatty acid methyl esters were extracted twice with 2 volumes of n-hexane. Analysis of fatty acid methyl esters was carried out in an Agilent 6890 N gas chromatograph coupled to an Agilent 5975 mass-selective detector (MSD) operated in electron impact mode (EI, 70 eV), equipped with an Agilent DB23 column (60 m × 0.25 mm I.D. × 0.15 μm film thickness). 1 μl of sample was injected in splitless mode. Inlet temperature was maintained at 250 °C. Oven temperature was held at 50 °C for 1 min, then increased to 175 °C at intervals of 25 °C per min, and to 230 °C at intervals of 2.75 °C per min. The final temperature was maintained for 5 min, and the run time was 33 min. The mass spectrometry transfer line was maintained at 250 °C and the mass spectrometer quadrupole and source at 150 °C and 230 °C, respectively. Helium was used as a carrier gas at a constant pressure of 180 kPa. Data acquisition was carried out both in scan and selected ion monitoring (SIM) mode. Scan mode was used for compound identification, comparing with authentical FAME standards and the NIST (National Institute of Standards and Technology) MS library spectra. SIM mode was used for quantitation, using 74 and 87 fragments for saturated, 83 for monounsaturated, 67 and 81 for diunsaturated and 79 and 91 for polyunsaturated fatty acid methyl esters. A 37-component mixture from Supelco was used for calibration curves, and nonadecanoic acid was used as an internal standard. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

### *2.6. Analysis of AA-containing phospholipids by liquid chromatography/mass spectrometry*

For lipid separation by high-performance liquid chromatography, a binary pump Hitachi LaChrom Elite® L-2130 was used, together with a Hitachi Autosampler L-2200 (Merck). The liquid chromatography system was coupled on-line to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). In all cases the effluent was split and 0.2 ml/min entered the electrospray interface of the mass spectrometer. Nebulizer was set to 30 psi, dry gas to 8 l/min and dry temperature to 350 °C. Analysis of AA-containing PI, PC and PE species was carried out exactly as described by Balgoma et al. [11,29].

### *2.7. Assay for CoA-independent transacylase activity*

CoA-independent transacylase activity was measured following the procedure originally described by Venable et al. [23], as modified by Balsinde et al. [30]. Briefly, the assay mixture was composed of 120 mM NaCl, 2 mM EGTA, 100 mM Tris-HCl (pH 7.5), cell homogenate (up to 100 μg of protein) and various concentrations of 1-0-[$^3$H]hexadecyl-2-lyso-sn-glycero-3-phosphocholine (lyso platelet-activating factor, lyso-PAF) as a substrate, in a final volume of 0.2 ml. In this assay system, the lysophospholipid acceptor for the acylation reaction (lysoPAF) is added to the assay mixture and the phospholipid donor is provided by the homogenate. After incubation at 37 °C for 5 min, the reaction was stopped by the addition of 0.75 ml of chloroform/methanol (1:2). Chloroform (0.25 ml) and water (0.25 ml) were added and the mixture was vortexed vigorously before centrifugation at $1000\times g$ for 5 min. The organic phase was evaporated and chromatographed on Silica gel G plates with chloroform/methanol/acetic acid/water (50:25:8:4 by vol.)

as the developing solvent. PC and lysoPAF were cut out of the plate and assayed for radioactivity by liquid scintillation counting.

### 2.8. Other assays

$Ca^{2+}$-dependent phospholipase $A_2$ activity was measured by using a modification of the mammalian membrane assay described by Diez et al. [31]. Briefly, cell homogenates were incubated for 1–2 h at 37 °C in 100 mM HEPES (pH 7.5) containing 1.3 mM $CaCl_2$ and 100,000 dpm of [$^3$H]AA-labeled membrane, used as substrate, in a final volume of 0.15 ml. Prior to assay, the cell membrane substrate was heated at 57 °C for 5 min, in order to inactivate coenzyme A-independent transacylase activity [9]. The assay contained 25 μM bromoenol lactone to completely inhibit endogenous $Ca^{2+}$-independent phospholipase $A_2$ activity [30]. After lipid extraction, free [$^3$H]AA was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. For $Ca^{2+}$-independent $PLA_2$ 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 μM labeled phospholipid substrate (1-palmitoyl-2-[$^3$H]palmitoyl-glycero-3-phosphocholine, sp. act. 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) in a final volume of 150 μl. 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 [$^3$H]palmitic acid was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. All of these assay conditions have been validated previously with regard to time, homogenate protein and substrate concentration [14,32–39].

Arachidonoyl-CoA synthetase activity was measured according to the procedure described by Wilson et al. [40], as modified by Pérez-Chacón et al. [41]. 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 37 °C for 10 min. Reactions were stopped by adding 2.25 ml of 2-propanol/heptane/2 M sulfuric acid (40:10:1, by vol.). After the addition of 1.5 ml of heptane and 1 ml of water, mixture was vortexed and centrifuged at 1000 × 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.

LysoPC:arachidonoyl-CoA acyltransferase activity was determined according to the procedure described by Lands et al. [42], as modified by Pérez-Chacón et al. [41]. The cells 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 50 μM lysoPC 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 [28]. For separation of PC from lysoPC, a system of chloroform/methanol/28% ammonia (65:25:5, by vol.) was used as a mobile phase.

## 3. Results

When resting U937 promonocytic cells were exposed to exogenous AA (1 μM) the fatty acid was rapidly incorporated into phospholipids but not into neutral lipids (Fig. 1A). Analysis of the distribution of the AA into phospholipid species revealed that PC and PE were the major species incorporating AA and that, after only a few minutes, the amount of AA in PC decreased significantly in parallel to an increase in PE (Fig. 1B), likely reflecting the remodeling action of CoA-IT. From these data, it can be noted that at approx. 0.25 h, the amount of AA in PE equaled that in PC. In order to make direct comparisons between different conditions, we have defined the time at which the amount of AA in PC is the same as that in PE as the *remodeling time* (rt). On the other hand, levels of AA in PI remained fairly constant along the time course of the experiment.

Comparative studies of AA incorporation and remodeling were carried out in human monocytes under identical conditions as those depicted in Fig. 1. While incorporation of exogenous AA into monocytes was generally similar to that in U937 cells (Fig. 2A), a striking difference was immediately noticed when the distribution of AA in phospholipid classes was studied (Fig. 2B). In monocytes, the remodeling time was much higher than in U937 cells, i.e. approx. 15 h. Thus, AA remodeling in human U937 cells is extremely faster than in monocytes, despite the process occurring under conditions where both types of cells incorporate similar amounts of exogenous AA into phospholipids.

To further characterize the earlier mentioned findings, we used ion-trap mass spectrometry to determine the molecular phospholipid species to which exogenous AA initially incorporated, i.e. a short time after addition of the fatty acid (30 min). For these experiments, [$^2$H]AA, not [$^3$H]AA, was used. From the data in Figs. 1 and 2, detection of exogenous AA into various molecular species of all PI, PC and PE classes would be expected in the U937 cells, the PE species arising primarily from transacylation from PC species. In the case of human monocytes, appearance of exogenous AA into PI and PC species would be expected, but little or no PE molecular species would be expected. The results shown in Fig. 3 indicate that this is exactly what happened. Thus, in U937 cells some of the initial PC species for AA incorporation have lost the fatty acid, which was likely transferred to various PE molecular species at 30 min, whereas such an amount of time was not long enough to permit significant transfer from PC species to PE species in human monocytes. From the data in Fig. 3, it is tempting to speculate that PC(16:0/[$^2$H]AA) and PC(18:2/[$^2$H]AA) are two preferred donors of AA to PE species, since they are present in human monocytes at 30 min but not in U937 cells. On the other hand, PC

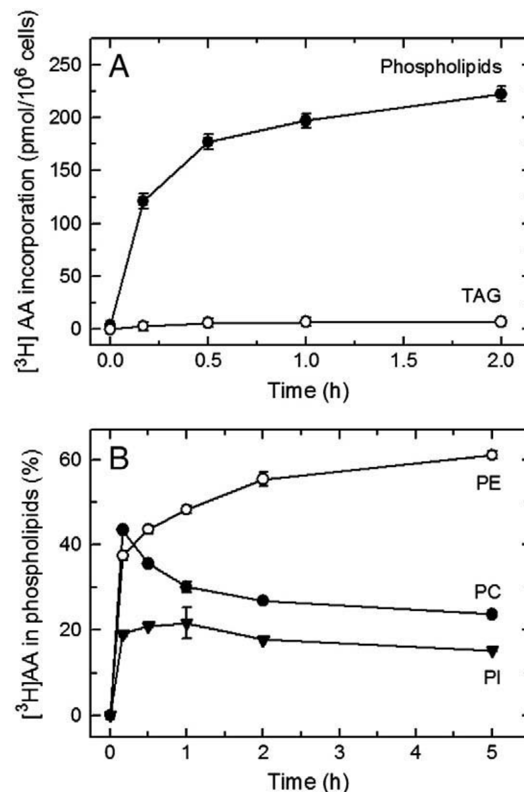


**Fig. 1.** [$^3$H]AA incorporation and remodeling into U937 cells. Cells were incubated with 1 μM [$^3$H]AA (0.25 μCi/ml) for the indicated times. Afterward total lipids were extracted and [$^3$H]AA incorporation in phospholipids (●) and TAG (○) (A) or remodeling in PI (▼), PC (●), and PE (○) (B) was measured. Data are given as means ± SEM of at least three independent determinations.

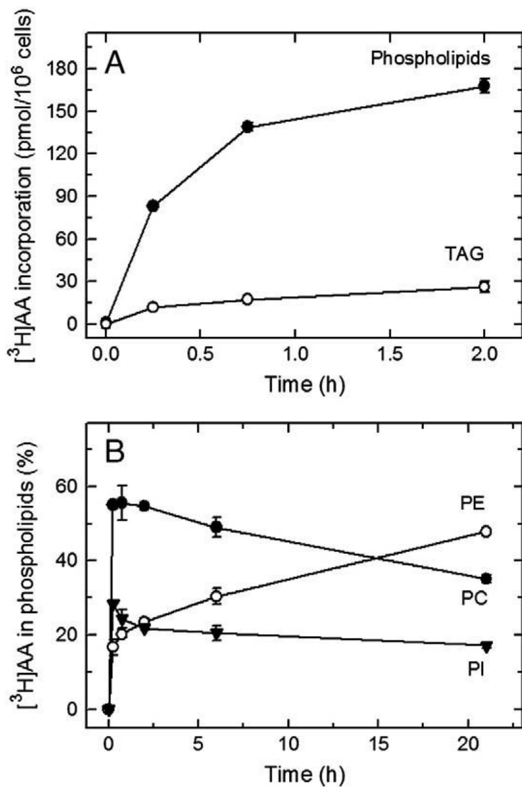


**Fig. 2.** [$^3$H]AA incorporation and remodeling into human monocytes. For [$^3$H]AA incorporation assay (A), cells were incubated with 1 μM [$^3$H]AA (0.25 μCi/ml) for the indicated times. Afterward total lipids were extracted and [$^3$H]AA incorporation in phospholipids (●) and TAG (○) was measured. For phospholipid AA remodeling (B), the cells were exposed to 1 μM [$^3$H]AA (0.25 μCi/ml) for 30 min, washed with medium containing BSA and incubated in fresh medium for the indicated times, and the amount of [$^3$H]AA in PI (▼), PC (●), and PE (○) was measured. Data are given as means ± SEM of at least three independent determinations.

| | U937 | Mon. |
|---|---|---|
| PI(16:0/[$^2$H]AA) | blue | blue |
| PI(18:1/[$^2$H]AA) | blue | blue |
| PI(18:0/[$^2$H]AA) | blue | blue |
| PI([$^2$H]AA/[$^2$H]AA) | blue | blue |
| | | |
| PE(P-16:0/[$^2$H]AA) | blue | yellow |
| PE(P-18:1/[$^2$H]AA) | blue | yellow |
| PE(P-18:0/[$^2$H]AA) | blue | yellow |
| PE(18:1/[$^2$H]AA) | blue | yellow |
| | | |
| PC(16:0/[$^2$H]AA) | yellow | blue |
| PC(18:2/[$^2$H]AA) | yellow | blue |
| PC(18:1/[$^2$H]AA) | blue | blue |
| PC(18:0/[$^2$H]AA) | blue | yellow |
| PC([$^2$H]AA/[$^2$H]AA) | yellow | blue |

**Fig. 3.** Analysis of phospholipid species containing [$^2$H]AA in U937 and human monocytes. Cells were incubated with 1 μM [$^2$H]AA for 30 min. Afterward total lipids were extracted and [$^2$H]AA-containing glycerophospholipids were detected (blue boxes) or not (yellow boxes) by HPLC/MS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(18:0/[$^2$H]AA) was detected in U937 cells but still not in monocytes at 30 min, despite this species constituting the major reservoir of endogenous AA among PC species under equilibrium conditions [11]. The detection of diarachidonoylated PC molecular species in monocytes but not in U937 cells has been discussed elsewhere [29].

Phorbol esters such as PMA are very effective in inducing the differentiation of U937 cells towards mature monocyte-like cells [27]. To assess the effect of differentiation on the processes of AA incorporation and remodeling, we incubated the PMA-differentiated U937 cells with [$^3$H]AA, and the movement of the fatty acid between phospholipids was followed at different times. The data, as shown in Fig. 4, indicated that, although phospholipid AA incorporation was somewhat lower in the PMA-differentiated cells compared with the undifferentiated cells, the remodeling of AA in the differentiated U937 cells was almost identical to that previously documented for the fresh undifferentiated U937 cells (cf. Figs. 1 and 4). These data suggest that AA remodeling in U937 cells is not influenced by the maturation degree of the cells. Thus U937 cells remodel AA among phospholipids very rapidly regardless of whether or not the cells are committed to differentiate.

A major difference between U937 cells and monocytes is that the former, as cells in culture, are typically deficient in AA [43,44]. Thus, we studied whether the very fast remodeling of U937 cells was somehow related to a diminished AA content. To this end, we incubated the U937 cells with exogenous AA (100 μM, complexed with BSA 5:1) for 20 h, which raised the cellular AA content to levels similar to those found in mature blood monocytes (Fig. 5). After the 20-h incubation, the cells were pulse-chased with 1 μM [$^3$H]AA and the movement of the fatty acid among phospholipids was followed at different times. The amount of AA incorporated into phospholipids was 200 ± 20 pmol per $10^6$ cells at 120 min, which was similar to that found in U937 cells not loaded with AA or monocytes (Fig. 6). Interestingly, incorporation of AA into TAG, while still low, now

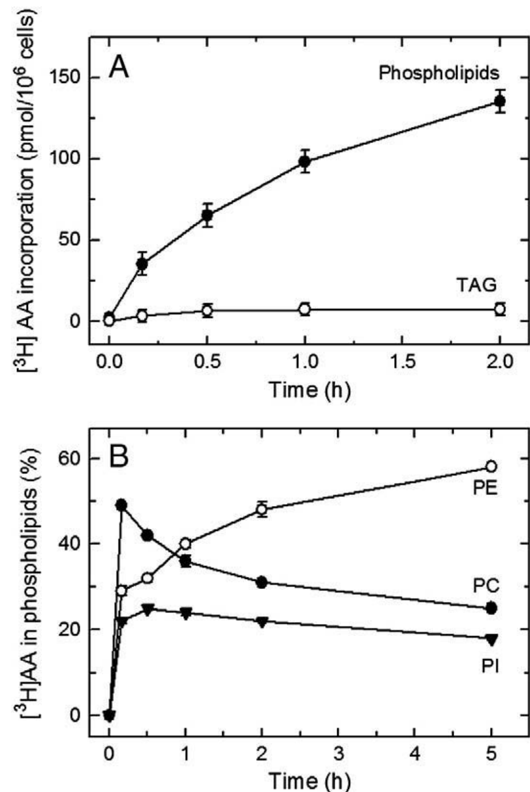


**Fig. 4.** [$^3$H]AA incorporation and remodeling into monocyte-like U937 cells. Cells, previously incubated with 35 ng/ml PMA for 24 h, were exposed to 1 μM [$^3$H]AA (0.25 μCi/ml) for the indicated times. Afterward total lipids were extracted and [$^3$H]AA incorporation in phospholipids (●) and TAG (○) (Fig. 4A) or remodeling in PI (▼), PC (●), and PE (○) (Fig. 4B) was measured. Data are given as means ± SEM of at least three independent determinations.

became significant and was comparable to that of monocytes (cf. Figs. 2 and 6). These data suggest that, under these conditions, saturation of AA entry into phospholipids was reached and the fatty acid is diverted to TAG [45,46]. Importantly however, the remodeling of AA among phospholipids was much slower than that of U937 cells not loaded with AA, becoming quite similar to that of monocytes. The remodeling time under these conditions was 15 h (Fig. 6). In experiments utilizing differentiated U937 cells enriched with AA, the remodeling of this fatty acid was essentially the same as that found in the undifferentiated U937 cells enriched with AA (data not shown), thus providing further evidence that this process is not influenced by the degree of maturation of the phagocyte.

Collectively, the earlier mentioned data indicate that U937 cells remodel AA between phospholipids at a much faster rate than monocytes and that this phenomenon appears to be related with the AA content of the cells. Studies in many laboratories have defined that the enzyme responsible for AA remodeling from PC to PE in mammalian cells is CoA-IT [20–25]. Unfortunately, the nucleotide sequence of the gene coding for CoA-IT still remains unknown, which prevents us from using genetic approaches to characterize the regulation of CoA-IT by cellular AA levels. Thus in the current experiments, CoA-IT was studied by following its enzymatic activity by in vitro assay. Homogenates from untreated U937 cells and from cells that had been incubated with exogenous AA for 20 h were prepared, and the CoA-IT substrate [$^3$H]lysoPAF was added at different concentrations. The results are shown in Fig. 7 and indicate that the measurable CoA-IT activity of homogenates from AA-enriched cells was significantly lower than that of homogenates of untreated U937 cells. Double reciprocal plots of the saturation data presented in Fig. 7 revealed an apparent $K_M$ of 6.14 ± 0.05 μM and $V_{max}$ of 263 ± 24 pmol min$^{-1}$ mg$^{-1}$ for the enzymatic activity of untreated U937 cell homogenates. In cells treated with AA, these parameters were 6.25 ± 0.07 μM for the $K_M$, and 182 ± 18 pmol min$^{-1}$ mg$^{-1}$ for the $V_{max}$. Thus, incubating the U937 cells with exogenous AA so as to enrich the intracellular phospholipid pools with this fatty acid (see Fig. 5) results in decreased activity but preserved substrate affinity of the CoA-IT of the corresponding homogenate. To determine the selectivity of the decrease in CoA-IT activity in AA-loaded cells, the activities of all enzymes putatively involved in phospholipid AA remodeling in U937 cells were assessed. These were $Ca^{2+}$-dependent and-independent $PLA_2$, arachidonoyl-CoA synthetase, and lysoPC:acyl-CoA acyltransferase. Assay conditions for all these activities have been validated previously for U937 cell homogenates [14,32–39,41]. However, none of these activities was found to appreciably change in homogenates after loading the cells with AA (data not shown).

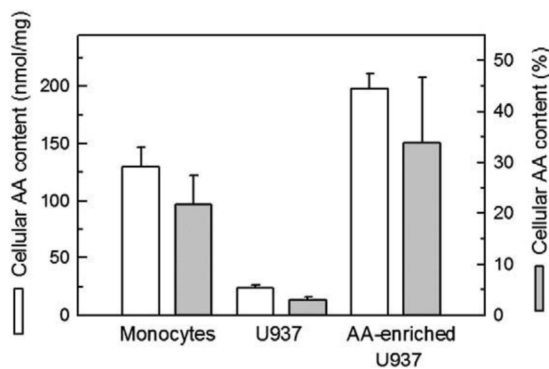


**Fig. 5.** Total AA content in U937, human monocytes and AA-enriched U937 cells. Total lipids were extracted and transmethylated with 0.5 M KOH in MeOH. The resulting fatty acid methyl esters were analyzed by GC/MS. Total mass of AA per mg protein (white bars) or % of AA respect to total amount of fatty acid (grey bars) are shown. Data are given as means ± SEM of at least three independent determinations.

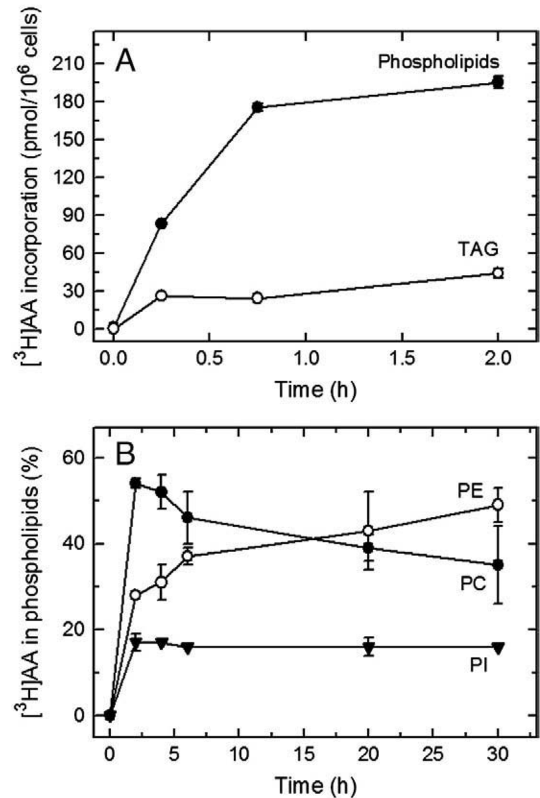


**Fig. 6.** [$^3$H]AA incorporation and remodeling into AA-enriched U937 cells. U937 incubated with medium containing AA 100 μM complexed with BSA (ratio 5:1) for 20 h were washed and labeled with 1 μM [$^3$H]AA (0.25 μCi/ml) for the indicated times. Afterward total lipids were extracted and [$^3$H]AA incorporation in phospholipids (●) and TAG (○) (A). For the remodeling assay (B), the cells were washed with medium containing BSA after 30 min incubation with 1 μM [$^3$H]AA (0.25 μCi/ml) and [$^3$H]AA label was measured in PI (▼), PC (●), and PE (○). Data are given as means ± SEM of at least three independent determinations.

## 4. Discussion

In this study we have compared the incorporation and remodeling of AA within membrane phospholipids of human U937 promonocyte-like cells and human peripheral blood monocytes. The importance of understanding cellular AA dynamics in human phagocytic cells stems from the fact that AA and its oxygenated derivatives, the eicosanoids, are key regulators of immunoinflammatory reactions. Remodeling of AA between cellular phospholipids is important to achieve the appropriate distribution of the fatty acid into the specific pools which will be acted upon by phospholipases $A_2$ to initiate eicosanoid

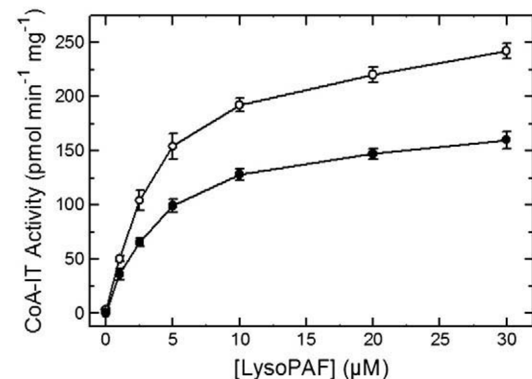


**Fig. 7.** CoA-IT activity is decreased in homogenates from AA-enriched U937 cells. Homogenates were prepared from untreated cells (○) or AA-enriched cells (●). Data are given as means ± SEM of at least three independent determinations.

biosynthesis [3,4]. There is now strong evidence that CoA-dependent acyl transferase and CoA-independent transacylases all participate sequentially in the process of phospholipid AA remodeling [3,4]. CoA-dependent acyl transferases mediate the initial incorporation of AA into phospholipids, which in turn is followed by the action of CoA-IT which redistributes the AA among phospholipid classes. Importantly, inhibition of these pathways has been demonstrated to profoundly alter cellular homeostasis and induce cell death [17–19].

We demonstrate that U937 cells manifest a relatively high capacity to incorporate AA into phospholipids that is quantitatively similar to that of blood monocytes, and in both types of cells, import of AA into neutral lipids (TAG) is minor. In both U937 cells and monocytes, AA is incorporated into PC and PI first, followed by a transfer to PE at the expense of PC. Although this AA remodeling process is qualitatively similar to that reported in other cell systems [3], what makes it noteworthy in the U937 cells is that it is extremely rapid compared to that in monocytes.

U937 cells can be induced to differentiate towards mature monocyte-like cells by exposing them to a variety of agents, including the phorbol ester PMA. When exposed to PMA, U937 cells cease to divide, become adherent, form clumps and express phenotypic properties of mature monocytes, including the expression of surface antigens [27]. Importantly however, essentially the same exceedingly high phospholipid AA remodeling of the undifferentiated U937 cells is also seen in PMA-differentiated cells. Hence, these results dissociate the differentiation-induced changes in lipid metabolism and the accompanying decreased need for synthesis of phospholipids from AA trafficking between phospholipids, suggesting that these events are regulated in an independent manner.

The amount of AA present in normal blood monocytes represents about 20% of total cellular fatty acid (this study). In U937 cells however, AA represents roughly only 3%. Thus, the possibility arises that the remarkably high capacity of U937 cells to remodel AA in phospholipids is related to their relative 'deficiency' in endogenous AA as compared to normal monocytes. To investigate this hypothesis, we have prepared U937 cells enriched in AA by treating them with fatty acid/albumin complex, so that the intracellular acylated amount of AA was similar to that of normal monocytes. When exposed for a second time to exogenous AA, the AA-enriched U937 cells incorporate the fatty acid in a similar manner, both qualitatively and quantitatively, to both U937 cells or monocytes. Importantly however, our results clearly show that in these AA-enriched cells, the phospholipid AA remodeling process is much slower than that of otherwise untreated U937 cells, becoming similar to that of monocytes (remodeling time for AA-enriched U937 cells and monocytes being approx. 15 h).

With the recent cloning of mammalian $Mg^{2+}$-dependent phosphatidate phosphatases (lipins) [47] and platelet-activating factor acetyl transferase [48], CoA-IT remains as the last lipid signaling and remodeling enzyme whose sequence remains unknown. Thus, currently the only manner to study the cell regulation of CoA-IT is by measuring its enzyme activity. By doing this, we have found that CoA-IT activity levels appear to be negatively regulated by the intracellular level of AA. Thus, in homogenates from cells enriched in AA, CoA-IT activity is significantly lower than in homogenates from AA-deficient cells. After replenishing the cells with AA, CoA-IT activity of homogenates from these cells displays a decreased $V_{max}$ with respect to homogenates from cells untreated with AA, but the $K_M$ was found to be the same in both cases. It is interesting to note here that extensive studies of the CoA-IT activity from broken neutrophils carried out by Winkler and colleagues have shown that such an activity is not directly inhibited by phospholipid substrates or phospholipid products [49]. Thus, the most straightforward explanation for our data is that enriching the cells with AA may reduce the cellular mass of CoA-IT and hence its measurable activity in homogenates. This leads to the intriguing question of whether an AA-containing phospholipid that is present in the AA-enriched cells but not in the otherwise untreated cells may affect the expression levels of CoA-IT. In this regard, recent studies have highlighted the idea that particular phospholipid molecular species may exert profound effects on gene expression by directly interacting with transcription factors [50], and our recent lipidomic studies in human monocytes and U937 have unveiled the existence of a very rapid turnover of particular AA-containing species depending on the activation state of the cell [10]. Collectively, the results reported here suggest that the role of CoA-IT in AA signaling may be more complex than merely regulating the transfer of fatty acid between phospholipid pools, and suggest interesting implications in terms of defining possible novel strategies to regulate lipid mediator formation by controlling the cellular levels of AA.

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