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Biochimica et Biophysica Acta 1259 (1995) 211–219

BBA Biochimica et Biophysica Acta

Acetylated low density lipoprotein inhibits the incorporation of arachidonic acid in phospholipids with a concomitant increase of cholesterol arachidonate in rat peritoneal macrophages

Cécile Pollaud [a], Siegfried Krause [a,1], Jean-Claude Lepert [a], Claudine Orfila [a], Marie-Hélène Séguélas [a], Didier Festal [b], Jacques Decerprit [b], Bernard Pipy [a,*]

[a] *Laboratoire de la Signalisation et de la Différenciation des Macrophages, INSERM CJF91-07, Toulouse, France*
[b] *Centre de Recherche et de Développement, LIPHA S.A., Lyon, France*

Received 13 April 1995; accepted 17 July 1995

Abstract

The aim of our work was to evaluate the influence of native low density lipoproteins (LDL) and LDL chemically modified by acetylation (acLDL) on incorporation and release of arachidonic acid (AA) in rat peritoneal macrophages. Compared to a control group without treatment, 100 μg/ml of acLDL for 15 h considerably increased the incorporation of [^{3}H]AA in cholesterol-ester (CE) of rat peritoneal macrophages and induced a decrease of ^{3}H-labeled membrane phospholipids (PL). No effect was shown with LDL treatment. In the presence of acLDL, LS3251 (100 nM), an acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor, inhibited the [^{3}H]AA incorporation into CE in macrophages. [^{3}H]AA-prelabeled macrophages cultured for 15 h with acLDL (compared to macrophages untreated or treated with LDL) showed an increase of labeled CE and a decrease of labeled PL and of cyclooxygenase and lipoxygenase eicosanoid production. After zymosan stimulation of macrophages prelabeled with [^{3}H]AA and treated with or without LDL or acLDL, AA release and eicosanoid production increased in all groups of macrophages. The inhibition of eicosanoid production in foam cells does not seem to be linked to an inhibition of phospholipase but rather paralleled to an increase of the cholesterol [^{3}H]arachidonate. A significant portion of cellular arachidonate released from phospholipids, in particular from phosphatidylcholine, could serve as a substrate to ACAT in this foam cell.

Keywords: Native low density lipoprotein; Acetylated low density lipoprotein; Arachidonic acid; Macrophage; Icosanoid; Cholesteryl ester; Phospholipid

1. Introduction

Macrophages have been shown to play a key role in atherogenesis. Monocytes entering the intima of the artery are activated into macrophages under participation of modified low density lipoproteins (LDL) [1]. Uptake of modified LDL by the macrophages will lead then to foam cell formation and may alter expression of many substances in the macrophages [2].

Macrophages are also essential components of the inflammatory process; they are the source of different inflammatory mediators (eicosanoids, RLO, cytokines) and growth-regulatory molecules in the atheromatous plaque environment [3].

Eicosanoids derived from arachidonic acid (AA) have potent biological actions in a number of areas, e.g., hemostasis and inflammation [4,5]. Production of eicosanoids and their alteration during atherosclerosis have been demonstrated by numerous investigators; nevertheless, little is known about the exact role of the eicosanoids in the genesis of atherosclerosis [6].

Particularly the investigations of the interaction of macrophages with LDL or modified LDL have given quite controversial results concerning AA metabolism and the synthesis of eicosanoids.

It has been reported that oxidized LDL stimulate the synthesis of the prostaglandins PGE_2, 6-keto-$PGF_{1\alpha}$ and the leukotriene LTC_4 in murine peritoneal macrophages and that native LDL have no effect on eicosanoid synthesis

* Corresponding author. Fax: +33 61 322293.

[1] Present adress is Research Centre for Vascular Biology and Medicine Erfurt, University of Jena, Nordhäuser Str.78, D-99089 Erfurt (Germany).

SSDI 0005-2760(95)00164-6

[7]. On the other hand, it is described that native LDL stimulate the production of LTB_4 in monocyte-derived macrophages [8] and thromboxane TXB_2, PGI_2, $PGF_{2\alpha}$, PGE_2 are synthesised in a mouse macrophage cell line [9]. Others found a decreased arachidonate metabolism with a decreased synthesis of PGE_2, LTC_4, 6-keto-$PGF_{1\alpha}$ in murine macrophages after cell incubation with oxidized LDL and suggest an inhibition of the phospholipase A_2 in the lipid-laden macrophages [10]. Another aspect that has remained unclear until now is the involvement of different phospholipids and neutral membrane lipids in the AA metabolism of macrophages during interactions with LDL and chemically modified LDL.

In the present study, we therefore investigated the interaction of rat peritoneal macrophages with native LDL and LDL modified by acetylation (acLDL) and their influence on incorporation and release of AA in/from cellular phospholipids and neutral membrane lipids as well as on the synthesis of eicosanoids in macrophages.

2. Materials and methods

2.1. Materials

The following were used: labeled [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA), spec. act. 208 Ci/mmol (Amersham-France); [1-^{14}C]arachidonic acid ([1-^{14}C]AA), spec. act. 55 mCi/mmol (Amersham-France); 199 medium with Hanks' salts (Gibco, Cergy-Pontoise, France); Dulbecco's modified Eagle medium (DMEM) (Gibco, Cergy-Pontoise, France); zymosan (Sigma Chimie, La Verpillière, France). The acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor LS3251 was synthesized by LIPHA (L'Industrie Pharmaceutique Lyonnaise), Lyon, France. All reagents and solvents used were of analytical grade.

2.2. Cell culture

Resident peritoneal macrophages were harvested from male Sprague-Dawley rats (purchased form IFFA-CREDO, l'Arbresle, France) aged 5–6 weeks with a weight 200–250 g as described by Cohn and Benson [11]. Briefly, the peritoneal cavity was washed with 100 ml of sterile 199 medium with Hanks' salts containing 20 IU heparin per ml. The cells collected were centrifuged at $800 \times g$ for 10 min and resuspended in DMEM with 44 mM $NaHCO_3$ buffer (pH 7.2) containing 1% of fetal calf serum (FCS). The cells were plated out in 24-well tissue culture plates at 10^6 cells in a total volume of 0.3 ml per well. About 60% of the cells in the peritoneal perfusate were nonspecific esterase-positive. After 5 h of culture, more than 98% of the adherent cells were nonspecific esterase-positive and had the morphological appearance of macrophages when examined by May-Grünwald and Giemsa staining. Cell viability was assessed by Trypan blue exclusion assay.

2.3. Preparation of low density lipoprotein (LDL)

LDL was isolated from the serum of normal volunteers in the density range 1.006–1.063 g/ml by ultracentrifugation as previously described by Paumay et al. [12]. Isolated LDL were dialyzed against 150 mM NaCl containing 5 mM Tris and 0.3 mM EDTA (pH 7.4) at 4°C for 48 h. The lipoproteins were filtered through a 0.45-μm filter (Sartorius) and the apolipoprotein concentrations were determined by kinetic turbidimetry (Behring Turbitimer). The LDL was used within 3 weeks of isolation.

2.4. Modification of LDL

Fresh LDL was acetylated using excess acetic anhydride [13]. The acetylated LDL (acLDL) were passed through a G25 PD-10 Sephadex column (Pharmacia) and were filtered using a 0.45-μm filter (Sartorius). The acLDL were used within 3 weeks of modification.

2.5. Treatment of macrophages with LDL or acLDL

The macrophages were allowed to adhere by incubating for 5 h at 37°C in an atmosphere of 5% CO_2 and 95% air. Non-adherent cells were removed by washing the dishes with DMEM. The adherent cells were then incubated without or with 1–100 μg/ml of LDL or acLDL in DMEM containing 1% FCS in the CO_2 incubator for 15 h.

In some experiments, adherent macrophages were preincubated with the inhibitor of the acyl-coenzyme A:cholesterol acyltransferase (ACAT) LS3251 (final concentration 100 nM) for 15 min and then the cells were incubated with 100 μg/ml acLDL in the presence of 100 nM of LS3251 for 15 h.

2.6. Incorporation of labeled AA in lipoprotein-treated macrophages

In some experiments, after incubation of macrophages with or without LDL or acLDL, the supernatant was removed and 300 μl of DMEM containing 1% FCS and 1 μCi [^{3}H]AA were added. The cells were then incubated with labeled AA for 5 h.

2.7. AA release during lipoprotein treatment

To investigate the effect of lipoprotein treatment on AA release, macrophages were prelabeled in a second set of experiments with [^{3}H]AA prior to treatment with LDL or acLDL. Briefly, adherent macrophages (10^6 per well) were incubated with DMEM (300 μl) containing 1% FCS and 1 μCi [^{3}H]AA for 5 h in the CO_2 incubator. Then the cells were washed three times with 300 μl DMEM containing 1% FCS; afterwards the cells were treated or not with 100 μg/ml of LDL or acLDL in DMEM containing 1% FCS and incubated in the CO_2 chamber for a further 15 h.

For determination of [^{3}H]AA incorporation, part of the cells was removed from the wells immediately after [^{3}H]AA treatment and subsequent washing and the phospholipids and neutral membrane lipids were extracted as described below.

2.8. AA release and eicosanoid synthesis during cell stimulation by opsonized zymosan

Macrophages labeled with [^{3}H]AA and then treated or not with 100 μg/ml of LDL or acLDL for 15 h, were stimulated with opsonized zymosan for 2 h and the AA release and eicosanoid production were determined. For each assay, opsonized zymosan (100 μg/ml) in DMEM without FCS was added to the cell layer of 10^6 macrophages per well and the cells were incubated at 37°C and 5% CO_2 for 2 h.

2.9. Double labeling assay

To compare the metabolism of cell-incorporated AA with that of extracellular AA, a double labeling assay using [^{3}H]AA as well as [1-^{14}C]AA were performed. Adherent macrophages (10^6 cells per well) were labeled with 0.5 μCi [1-^{14}C]AA in 300 μl DMEM containing 1% FCS for 5 h at 37°C and at 5% CO_2. The macrophages were then washed three times with DMEM with 1% FCS and incubated with or without 100 μg/ ml of LDL or acLDL in DMEM containing 1% FCS in the CO_2 incubator for 15 h. After washing of the cell layer, 1 μCi [^{3}H]AA in 300 μl DMEM containing 1% FCS were added and the cells were finally incubated for 5 h at 37°C and at 5% CO_2.

2.10. Extraction and assay of phospholipids and neutral membrane lipids

The extraction of the phospholipids and neutral membrane lipids to determine the incorporation and the release of labeled arachidonic acid used a technique described by Bligh and Dyer [14].

Phospholipid analysis was carried out as previously described [15,16]. Briefly, the cell layer was removed by scratching using a rubber policeman in acidified methanol and the cellular lipids were extracted with chloroform-methanol-water (1:1:1, v/v). The chloroform phase, which contains the cellular lipids, was evaporated to dryness under nitrogen. A sample of 80 μl of methanol was added to the residues and applied to preactivated (1 h at 100°C) thin-layer silica-gel plates (LK-6-DF Whatman). The cellular phospholipids were separated in a first run over 9 cm using chloroform-methanol-water-triethylamine (30:34:8:35, v/v). The plates were dried and a second run over 15 cm was performed for separation of the cholesteryl-ester (CE) and neutral lipids (monoacylglycerol (MG), diacylglycerol (DG)) using the solvent system petroleum ether/diethyl ether/acetic acid (50:50:1, v/v). The labeled metabolites were identified using the Berthold LB2821 analyzer.

In experiments where cells were labeled with [1-^{14}C]AA and [^{3}H]AA, the separated metabolites were scratched from the TLC plates, transferred to scintillation vials and counted with a liquid scintillation counter.

2.11. Extraction and assay of eicosanoids

The culture medium and the supernatants used to wash the cells were collected and centrifuged at $3000 \times g$ for 10 min at 4°C. The various AA oxidation products (lipoxygenase and cyclooxygenase metabolites) released by the cells into culture medium were measured by thin-layer chromatography as previously described [15–17]. Briefly, supernatants were acidified to pH 5.4 with 1 M HCl and chromatographically extracted using Varian Bond Elut C18 columns (Analytichem). AA metabolites were eluted with methanol. The methanol samples were evaporated to dryness under nitrogen. The residues were dissolved in 80 μl of methanol and applied to thin-layer silica-gel plates (LK-6-DF Whatman), which had been previously activated (1 h at 100°C). The solvent system used for the separation of eicosanoids was the organic phase of ethyl acetate-water-isooctane-acetic acid (110:100:50:20, v/v) as previously described [18]. Samples were monitored by rapid scanning with the Berthold LB2821 TLC-linear analyzer.

2.12. Microscopic examination

Macrophages were made to adhere to a 16-well Lab-Tek chamber slide (Nunc) and then incubated for 15 h with or without 100 μg/ml of acLDL, in the same way as in 24-well tissue culture plates. For electron microscopy, the cells were fixed in 2% glutaraldehyde for 30 min, then post-fixed in 2% osmium tetroxide and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope Corinth 275 (A.E.I., UK).

2.13. Statistics

Data are expressed as mean $\pm$ standard error. For each experiment, the data were analyzed by one-way analysis of variance and the multiple comparison method of Tukey [19].

3. Results

3.1. Effect of pretreatment with native LDL and acetylated LDL on incorporation of [^{3}H]AA in phospholipids and other cellular lipids of macrophages

Macrophages were incubated with different concentrations of LDL or acLDL (1, 10, 100 μg/ml) for 15 h and

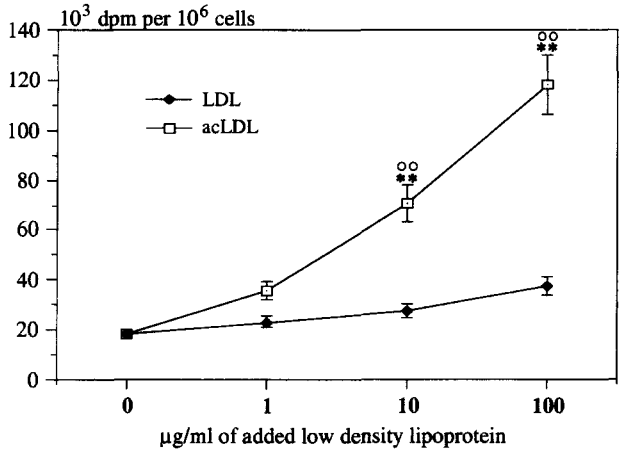

Fig. 1. Effect of different doses of native LDL or acLDL on incorporation of [^{3}H]AA in cholesteryl-ester of macrophages. Rat peritoneal macrophages (10^6 cells per well) were incubated with or without 1, 10 or 100 μg/ml of LDL or acLDL for 15 h. [^{3}H]AA was added to the cell medium (1 μCi to 300 μl medium per well) and the macrophages were incubated for an additional 5 h. The extraction and assay of cellular lipids were performed as described under Section 2. Each result is the mean ± standard error of three experiments. ** $P < 0.01$ indicates a significant difference compared to control group, °° $P < 0.01$ indicates a significant difference compared to LDL-treated macrophages.

then labeled with [^{3}H]AA for 5 h. The incorporation of [^{3}H]AA in phospholipids and neutral lipids was evaluated. We found that pretreatment with acLDL augmented the incorporation of [^{3}H]AA into the cholesteryl-ester (CE) of macrophages dose-dependently (Fig. 1). In macrophages pretreated with 1, 10 or 100 μg/ml of acetylated LDL, the incorporation of [^{3}H]AA in CE was increased significantly with respect to the control without LDL by about 96%, 277% and 530% respectively. No significant changes in the labeling of CE were observed when the macrophages were pretreated with native LDL. To achieve a maximum incorporation of [^{3}H]AA in CE, we used, in the next experiments, pretreatment with 100 μg/ml acLDL.

Table 1 shows the results of incorporation of [^{3}H]AA in the other membrane lipid fractions (phospholipids and neutral lipids) of macrophages pretreated with or without LDL or acLDL. Pretreatment of macrophages with 100 μg/ml of native LDL has no effect on incorporation in most membrane lipids. We only observed a slight, but significant, decrease of incorporation in diacylglycerol (DAG) and increase in triacylglycerides (TG) (Table 1). On the other hand, the incorporation of [^{3}H]AA after pretreatment with acLDL into phosphatidylcholine (PC), phosphatidylserine (PS) and diacylglycerol (DAG) was significantly decreased by about 26%, 35% and 57%, respectively.

Electron microscopy revealed that macrophages treated under these conditions with 100 μg/ml acLDL showed an accumulation of oil droplets in contrast to the untreated macrophages (Fig. 2).

3.2. *Effect of an inhibitor of the acyl-coenzyme A:cholesterol acyltransferase (ACAT) on the AA-incorporation*

To investigate the possible involvement of ACAT activity in the synthesis of cholesterol arachidonate, we incubated the macrophages with LS3251, an ACAT inhibitor synthesized by LIPHA, and looked for the incorporation of [^{3}H]AA in CE. Macrophages were preincubated with 100 nM LS3251 for 15 min and then treated, in the presence of 100 nM LS3251, for 15 h with acLDL, as indicated. As shown in Table 2, the inhibition of ACAT abolishes the enhancing effect of acLDL on AA incorporation into the CE (Table 2).

3.3. *Release of [^{3}H]AA from phospholipids and other cellular lipids and AA reincorporation during LDL treatment of macrophages*

After incubation of non-treated macrophages with [^{3}H]AA for 5 h, the AA was incorporated mainly into PC [(291.0 ± 22.5) · 10^3 dpm per 10^6 cells], PS [(47.4 ± 7.1) · 10^3 dpm per 10^6 cells] and CE [(21.5 ± 1.3) · 10^3 dpm per 10^6 cells]. During further incubation of macrophages without LDL for 15 h most of the incorporated AA was

Table 1
Effect of pretreatment with native LDL or acLDL on incorporation of [^{3}H]AA in phospholipids and other cellular lipids of macrophages

(10^3 dpm per10^6 cells)	Control	LDL	P [1]	acLDL	P [1]	P [2]
PC	274.6 ± 19.3	275.8 ± 7.6	n.s.	203.0 ± 14.8	< 0.05	< 0.05
PS	59.6 ± 2.1	60.1 ± 2.2	n.s.	38.5 ± 3.2	< 0.01	< 0.01
PI/PE	9.1 ± 1.5	11.1 ± 2.0	n.s.	9.0 ± 1.6	n.s.	n.s.
PGL	9.9 ± 1.5	9.0 ± 2.4	n.s.	9.1 ± 1.8	n.s.	n.s.
DAG	8.9 ± 0.3	4.8 ± 0.8	< 0.01	3.8 ± 0.2	< 0.01	n.s.
TG	0.8 ± 0.2	1.8 ± 0.2	< 0.05	1.5 ± 0.1	n.s.	n.s.
AA	1.7 ± 0.5	1.8 ± 0.1	n.s.	1.5 ± 0.1	n.s.	n.s.

Adherent rat peritoneal macrophages (10^6 cells per well) were incubated with 100 μg/ml of LDL or acLDL for 15 h and then incubated with cell medium containing [^{3}H]AA (1 μCi to 300 μl medium per well) for 5 h. The extraction and assay of phospholipids and other cellular lipids were performed as described under Section 2. Each result is the mean ± standard error of three experiments. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PGL, phosphatidylglycerol; DAG, diacylglycerol; AA, arachidonic acid; TG, triacylglycerol.
[1] P: significance with respect to the untreated control; [2] P: significance between LDL and acLDL-treated macrophages; n.s., non-significant.

released from these lipids, in particular 57% from PC ($P < 0.01$) and 74% from CE ($P < 0.01$) (Table 3). When macrophages were incubated with native LDL for 15 h, the release of [^{3}H]AA from these cellular lipids was not different from the control (Table 3). In contrast to native LDL, during treatment with acLDL, the amount of [^{3}H]AA in the fraction of PC was significantly decreased (29%; $P < 0.05$) in respect to control macrophages (cultivated 15

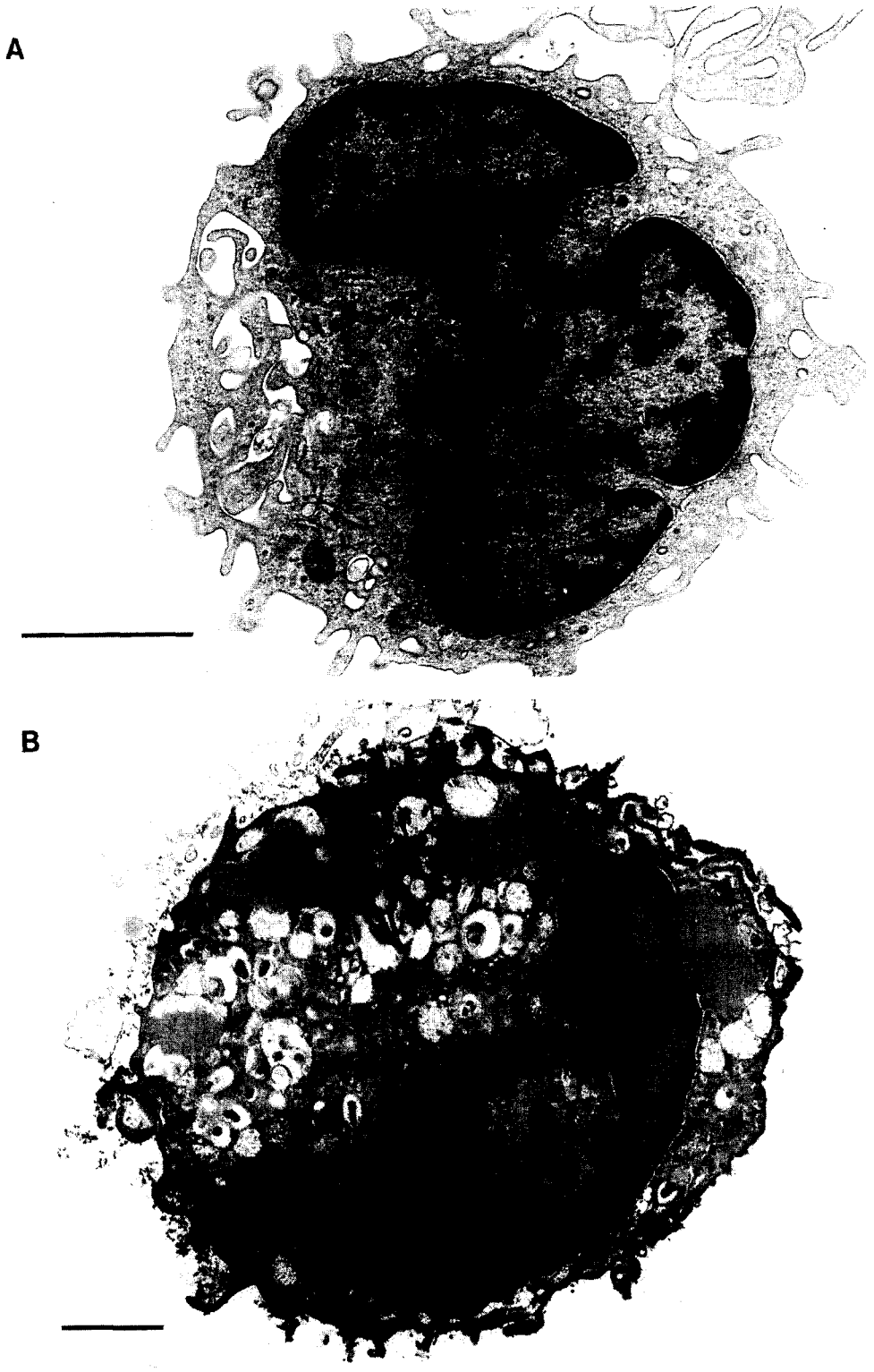

Fig. 2. Photomicrographs of adherent rat macrophages. The cells were incubated for 15 h with medium alone (panel A) or with 100 μg/ml acetylated LDL (panel B). The bar indicates 2 μm and ★ indicates oil droplets. Panel A, ×16000; panel B, ×9600.

Table 2
Effect of the inhibitor of the acyl-coenzyme A:cholesterol acyltransferase (ACAT) LS3251 on incorporation of [^{3}H]AA in CE in macrophages treated with acLDL

Incorporation of [^{3}H]AA in cholesteryl-ester			
(10^3 dpm per 10^6 cells)		P [1]	P [2]
Control	9.9 ± 1.4		
LS3251 only	10.6 ± 0.9	n.s.	
acLDL	41.7 ± 9.0	< 0.01	
acLDL + LS3251	10.3 ± 0.7	n.s.	< 0.01

Adherent macrophages were preincubated with the ACAT-inhibitor LS3251 (final concentration 100 nM) for 15 min and then the cells were incubated with or without 100 μg/ ml acLDL in the presence of 100 nM of LS3251 for 15 h. [^{3}H]AA (1 μCi to 300 μl medium per well) was incorporated into the cells for 5 h and then cellular lipids were extracted. Each result is the mean ± standard error of three experiments.
[1] P: significance with respect to the control; [2] P: significance with respect to the acLDL-treated macrophages; n.s.: non-significant.

h after treatment without acLDL or LDL) (Table 3). In parallel, this treatment with acLDL increases cholesterol arachidonate (656%; $P < 0.01$) with respect to control macrophages after treatment (Table 3). Radiolabeled arachidonate incorporated into PC and PS is redistributed into CE but not into other phospholipids.

We have also looked at the amount of free [^{3}H]AA and its metabolites in the supernatant of macrophages after treatment with native LDL and acLDL. We found that macrophages treated with acLDL but not with native LDL exhibit a decrease of free AA release of about 38% ($P < 0.01$) with respect to control macrophages (Fig. 3). On the other hand, the pattern of the eicosanoids produced most is slightly changed; we found a significant decrease of the production of 6-keto-PGF1α (35%; $P < 0.05$), $PGF_{2\alpha}$ (35%; $P < 0.05$), PGE2 (65%; $P < 0.05$), LTB_4 (41%; $P < 0.05$) and HETEs (42%; $P < 0.05$) (Fig. 3).

3.4. Double labeling assay

This assay was performed to observe the mobilization of labeled arachidonic acid ([1-^{14}C]AA incorporated before LDL treatment) during the interaction of lipoproteins

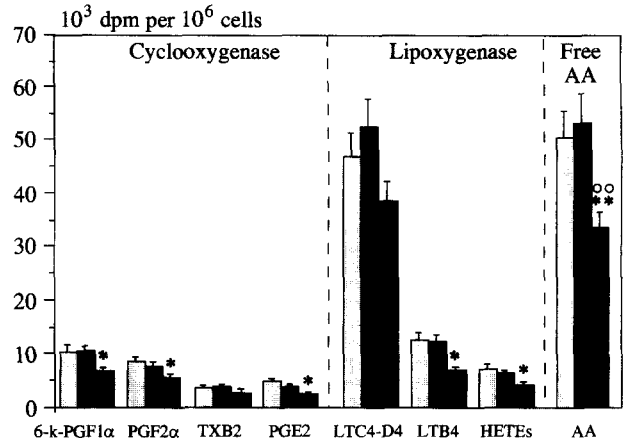

Fig. 3. Production of metabolites of cyclooxygenase and lipoxygenase and release of arachidonic acid (AA) by macrophages untreated or treated with native LDL (LDL) or acetylated LDL (acLDL). 1 μCi of [^{3}H]AA per 10^6 cells was incorporated in rat peritoneal macrophages for 5 h at 37°C. Then the cells were washed and incubated with or without LDL or acLDL (100 μg/ml) for 15 h. Eicosanoids released in the supernatant were determined. Each result is the mean ± standard error of three experiments. Control treatment (□), treatment with LDL (▤), treatment with acLDL (■). * (**) $P < 0.05$ ($P < 0.01$) indicates a significant difference compared to control treatment, °° $P < 0.01$ indicates a significant difference compared to LDL-treated macrophages.

with the cells and the following incorporation of extracellular [^{3}H]AA.

Like the findings with [^{3}H]AA, macrophages prelabeled with [1-^{14}C]AA and then incubated for 15 h with acLDL showed a significant change in AA distribution between phospholipids and CE. The incubation with acLDL produced a decrease in phospholipid labeling and a higher incorporation into CE compared with untreated or LDL-treated macrophages (results not shown). When the acLDL-treated macrophages prelabeled with [1-^{14}C]AA were cultivated with [^{3}H]AA, the content of [1-^{14}C]AA in the fraction of CE was significantly decreased from (5.6 ± 0.4) · 10^3 dpm/10^6 cells to (3.3 ± 0.8) · 10^3 dpm/10^6 cells ($P < 0.05$) and the incorporation of [^{3}H]AA in the CE fraction was strongly elevated to about (32.0 ± 2.1) ·

Table 3
Amount of [^{3}H]AA in PL and CE of macrophages before and after treatment with LDL or acLDL

(10^3 dpm per 10^6 cells)	Control before treatment	Control after treatment	P [1]	Treatment with LDL	P [1]	P [2]	Treatment with acLDL	P [1]	P [2]
PC	291.0 ± 22.5	125.3 ± 7.5	< 0.01	109.5 ± 7.1	< 0.01	n.s.	89.3 ± 3.9	< 0.01	< 0.05
PS	47.5 ± 7.1	8.6 ± 1.4	< 0.01	9.5 ± 0.5	< 0.01	n.s.	9.1 ± 0.6	< 0.01	n.s.
PI	4.9 ± 0.5	2.1 ± 0.4	< 0.01	1.9 ± 0.2	< 0.01	n.s.	2.0 ± 0.3	< 0.01	n.s.
PE	10.3 ± 0.9	4.1 ± 0.03	< 0.01	5.6 ± 1.3	< 0.05	n.s.	4.0 ± 0.7	< 0.01	n.s.
PGL	23.2 ± 2.2	8.6 ± 1.4	< 0.01	6.3 ± 0.3	< 0.01	n.s.	6.7 ± 0.1	< 0.01	n.s.
CE	21.5 ± 1.3	5.5 ± 0.5	< 0.01	6.2 ± 0.7	< 0.01	n.s.	36.6 ± 1.3	< 0.01	< 0.01

[^{3}H]AA (1 μCi/10^6 cells) was incorporated in rat peritoneal macrophages for 5 h at 37°C. Then the cells were washed and incubated with or without LDL or acLDL (100 μg/ml) for 15 h. Cells were extracted immediately after [^{3}H]AA treatment and after subsequent incubation over 15 h. The extraction and assay of phospholipids and other cellular lipids were performed as described under Section 2. Each result is the mean ± standard error of three experiments.
[1] P: significance with respect to the control group before treatment; [2] P: significance with respect to the control group after treatment; n.s., non-significant.

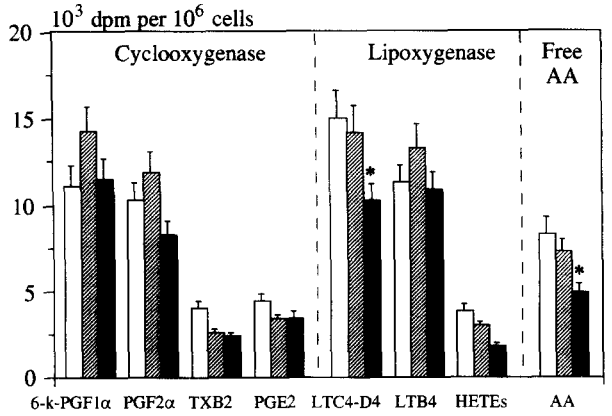

Fig. 4. Production of metabolites of cyclooxygenase, lipoxygenase and release of arachidonic acid (AA) by macrophages incubated for 2 h with zymosan after treatment with or without LDL or acLDL. 1 μCi of [^{3}H]AA per 10^6 cells was incorporated in rat peritoneal macrophages for 5 h at 37°C. Then, the cells were washed and incubated with or without LDL or acLDL (100 μg/ml) for 15 h. The cells were washed and incubated with zymosan for 2 h and eicosanoids released in the supernatant were determined. Each result is the mean ± standard error of three experiments. Control treatment (□), treatment with LDL (▨), treatment with acLDL (■). * $P < 0.05$ indicates a significant difference compared to control treatment.

10^3 dpm per 10^6 cells ($P < 0.01$) with respect to the macrophages incubated without LDL (control group) or with native LDL [$(4.5 \pm 0.1) \cdot 10^3$ dpm and $(5.9 \pm 0.2) \cdot 10^3$ dpm per 10^6 cells, respectively].

3.5. Release of [^{3}H]AA from phospholipids and other cellular lipids and eicosanoid synthesis during stimulation of the macrophages with zymosan

When prelabeled macrophages were stimulated with zymosan for 2 h after the LDL-treatment, the release of AA as well as the production of eicosanoids was increased, independently of the treatment with lipoproteins (Fig. 4). After zymosan stimulation, we observed a significant decrease in the production of LTC_4-D_4 (31%; $P < 0.05$) and free AA (39%; $P < 0.05$) in the acLDL-treated macrophages with respect to the control (Fig. 4). The other eicosanoids produced did not differ significantly in the three groups.

The content of incorporated AA in phospholipids and neutral membrane lipids of untreated macrophages hardly changes after zymosan stimulation. Table 4 indicates that the incubation from macrophages for 2 h with zymosan produces a decrease in PC labeling (25% with respect to the control without zymosan (results not shown)). Incubation of macrophages for 2 h with zymosan after the acLDL-treatment produced a decrease in PC labeling (33%; $P < 0.05$) and a higher incorporation in CE (319%; $P < 0.01$) compared with untreated macrophages stimulated by zymosan (Table 4).

4. Discussion

Our transmission electron microscopic study demonstrated that peritoneal macrophages pretreated by acLDL for 15 h are transformed into foam cells containing large accumulation of lipid droplets. These cells showed a significant decrease in the amounts of [^{3}H]AA incorporated into PL and an increase in the amounts of [^{3}H]AA incorporated into CE. The esterification of cholesterol in mammal cells was catalyzed by the acyl-coenzyme A:cholesterol acyltransferase (ACAT) and there is evidence that ACAT is involved in foam cell formation [1,20,21]. LS3251, an ACAT inhibitor, clearly diminished [^{3}H]AA incorporation into the CE of acLDL-treated macrophages with no decrease of the amounts of [^{3}H]AA incorporated into PL (results not shown). It suggests that acLDL stimulates cholesterol [^{3}H]arachidonate synthesis and ACAT activity and in parallel, decreases AA labeling of PL. The increase of [^{3}H]AA incorporation induced by acLDL is consistent with reports which clearly demonstrate that acLDL stimulated cholesteryl [^{14}C]oleate synthesis [10]. The mechanism involved in labeled phospholipid decrease is unclear. One explanation could be that acLDL induces a decrease of the incorporation of [^{3}H]AA into membrane phospholipids. Arachidonic acid binds to PL through the action of two enzymes, an acyl-CoA synthase and an acyltransferase [17]. Another explanation could be that [^{3}H]AA incorpo-

Table 4
Amount of [^{3}H]AA in PL and CE of macrophages incubated for 2 h with zymosan after treatment with or without LDL or acLDL

(10^3 dpmper 10^6 cells	Control treatment	Treatment with LDL	P [1]	Treatment with acLDL	P [1]	P [2]
PC	55.6 ± 7.9	47.8 ± 1.6	n.s.	37.1 ± 2.3	< 0.05	n.s.
PS	7.6 ± 1.9	6.3 ± 1.1	n.s.	7.5 ± 0.8	n.s.	n.s.
PI	2.1 ± 0.4	1.8 ± 0.3	n.s.	0.9 ± 0.1	n.s.	n.s.
PE	4.7 ± 0.6	3.2 ± 0.2	n.s.	2.4 ± 0.4	n.s.	n.s.
PGL	7.2 ± 1.0	4.7 ± 0.6	n.s.	3.4 ± 0.3	< 0.05	n.s.
CE	7.2 ± 0.5	9.7 ± 0.5	n.s.	30.2 ± 2.1	< 0.01	< 0.01

1 μCi of [^{3}H]AA per 10^6 cells was incorporated into rat peritoneal macrophages for 5 h at 37°C. Then the cells were washed and incubated with or without LDL or acLDL (100 μg/ml) for 15 h. The cells were washed and incubated with zymosan for 2 h and extracted. The extraction and assay of phospholipids and other cellular lipids were performed as described under Section 2. Each result is the mean ± standard error of three experiments.
[1] P: significance with respect to the control treatment; [2] P: significance with respect to the LDL treatment; n.s., non-significant.

rated into PL is rapidly released by a phospholipase A2 and immediately esterified with cholesterol by ACAT.

Our results suggest that the decrease in the amounts of [^{3}H]AA labeled PL is correlated with an increase of the cholesteryl [^{3}H]arachidonate synthesis. Indeed, when macrophages were prelabeled with [^{3}H]AA, acLDL treatment during 15 h produced a decrease in [^{3}H]AA labeling of PL and an increase in the labeling of CE. During this culture time, with or without native or acLDL, eicosanoid synthesis was activated. The rate-limiting step in eicosanoid synthesis is thought to be the release of free AA from phospholipids [22]. Our results indicate that acLDL increases AA mobilization by phospholipase. Indeed, the release of [^{3}H]AA from membrane phospholipids was significantly increased in the macrophages treated with acLDL with respect to untreated macrophages. In contrast, a decrease of free [^{3}H]AA and of both lipoxygenase and cyclooxygenase products of AA appears with respect to untreated macrophages. The mechanism involved in both free [^{3}H]AA and [^{3}H]eicosanoid decrease after macrophage pretreatment by acLDL could be [^{3}H]AA release from PL by a phospholipase and rapid esterification of free [^{3}H]AA into CE by ACAT. In murine macrophages and J774 macrophages, treated with acLDL, the plasma membrane free cholesterol became accessible to ACAT as substrate, the result being an accumulation of CE [23]. In foam cells derived from rat peritoneal macrophages by preincubation with acLDL, a significant amount of cellular free cholesterol was released and could serve as an efficient substrate for ACAT [24]. Our results indicate that a significant portion of cellular arachidonate released from phospholipids also could serve as a substrate to ACAT in foam cells derived from rat peritoneal macrophages.

Just like Yokode et al. [7], our results show that native LDL and acLDL had no stimulatory effect on prostaglandin or leukotriene synthesis by [^{3}H]AA-prelabeled macrophages. The release of [^{3}H]AA from membrane phospholipids is catalyzed by phospholipase activity. In our study, increased arachidonate mobilization in the membrane phospholipid fraction was found in macrophages that incorporated acLDL when arachidonate metabolites were decreased. A different result was reported by Arai et al. in mouse peritoneal macrophages with oxidized LDL [10] and by Mathur et al. in mouse peritoneal macrophages with acLDL [25]. Pomerantz and Hajjar [26] reported that lipid rich smooth muscle cells with cationized LDL produced less PGI2 and PGE2 than control cells; for these authors potential mechanisms for this effect include competitive inhibition of eicosanoid production by linoleate derived from LDL, direct inhibition of PLA2 activity by cholesterol and decrease in cyclooxygenase activity. It is suggested by Arai et al. [10] that the decreased arachidonate metabolism in lipid-enriched macrophages stimulated by zymosan is caused by the inhibition of phospholipase A_2-activity. These authors [10] determined the phospholipase activity from peritoneal macrophages after foam cell transformation with oxidized or acetylated LDL and then labeling by [^{14}C]AA for 1 h. The phospholipase activity was calculated as a percent of the radioactivity in the medium after zymosan stimulation of macrophages per total cell radioactivity. In our study, the inhibition of eicosanoid production observed in lipid-enriched macrophages unstimulated by zymosan was not linked to decrease of the release of [^{3}H]AA from membrane phospholipids but was parallel to an increase of the cholesterol [^{3}H]arachidonate. We suggest that eicosanoid inhibition by lipid-enriched macrophages could be linked at a decrease of free AA available for cyclooxygenase and 5-lipoxygenase; cellular free AA released from PL by a PLase could serve as an efficient substrate for cholesterol esterification. This hypothesis was also suggested by the results obtained after zymosan stimulation of three groups of macrophages treated or not with LDL or acLDL and after double labeling assay. Indeed, the decrease of phospholipase activity in lipid-enriched macrophages was not observed after stimulation of macrophages by zymosan. This inflammatory agent activates both the mobilization of AA from the phospholipids and the production of eicosanoids [16]. In lipid-enriched macrophages, a weak inhibition of free AA and LTC4 production appears with a concomitant increase of cholesterol arachidonate.

After incorporating acLDL, unstimulated macrophages secrete less PGE2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, LTB4. These eicosanoids have different biological actions that may play a role in atherogenesis, e.g., LTB_4 has chemotactic properties [8,27], $PGF_{2\alpha}$ acts as a proinflammatory [9]. It is interesting to note some relations between ACAT activity and AA metabolism. PGE_2 inhibits the ACAT activity in smooth muscle cells and macrophages by raising intracellular cyclic AMP levels [10]. 6-keto-$PGF_{1\alpha}$ is the stable metabolite of prostacyclin (PGI_2), which has a well-known antiatherogenic potential [6]. Recently, it has been reported that two prostacyclin agonists reduce foam cell formation in vivo and suppress macrophage scavenger receptor-mediated uptake of acLDL and increase cyclic adenosine monophosphate levels [28]. So, it may be suggested that prostacyclin also has an ACAT-inhibitory action and this may be involved in the known anti-atherogenic potential of prostacyclin. However, it is difficult to say which consequences are due to the alterations of eicosanoid production of the acLDL-treated macrophages in our experiments.

In summary, our data clearly demonstrate that CE enrichment reduces eicosanoid metabolism by unstimulated macrophages. Based on the results presented here, we conclude that the diminished AA release and metabolism in macrophages treated with acLDL is caused by an enhanced incorporation of AA especially in the CE, but not by a decreased AA mobilization from the phospholipids. Further studies to examine PLA2, cyclooxygenase and 5-lipoxygenase activities will provide insight into the processes involved in arachidonate metabolism in lipid-enriched macrophages.

Acknowledgements

We would like to acknowledge Gilles Favre and Samadi-Baboli for preparing the low density lipoproteins and for helping by the acetylation of the LDL. We thank M.F. Frisach for technical assistance. This work was supported by INSERM and LIPHA.

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