Effect of cholesterol enrichment on 12-hydroxyeicosatetraenoic acid metabolism by mouse peritoneal macrophages

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Abstract The metabolism of 12-hydroxyeicosatetraenoic acid (12-HETE) was investigated in mouse peritoneal macrophages enriched in cholesterol by incubation with acetylated low density lipoproteins. After incubating with labeled arachidonic acid, cholesterol-rich cells released more 12-HETE into the medium than unmodified macrophages. With time, however, 12-HETE decreased in the medium of both cell preparations suggesting reuptake of this monohydroxyfatty acid and perhaps further metabolism. When control macrophages were incubated with radiolabeled 12-HETE for 2 hr, almost 70% of the cell-associated 12-HETE label was incorporated into phospholipids. In contrast, in cholesterol-rich cells, only 31% of the 12-HETE label was incorporated into phospholipids. Bee venom phospholipase completely hydrolyzed the label, suggesting that the monohydroxyfatty acid was esterified at the *sn*-2 position of the phospholipid. In cholesterol-rich cells, 69% of the 12-HETE was diverted into neutral lipids. Two major neutral lipids were identified in cholesterol-rich macrophages. One neutral lipid band which migrated with an *Rf* value of 0.34 contained the hydroxylated fatty acid esterified to a glyceride. The other neutral lipid band having an *Rf* value of 0.49 contained cholesterol and by further analysis was found to contain predominantly cholesteryl-12-HETE. The labeled fatty acids in these two neutral lipids were mostly oxidized products of 12-HETE in contrast to the native 12-HETE observed in the phospholipids. Cholesterol-rich macrophages released 25% more products of 12-HETE metabolism than control macrophages. Two major products were observed in the medium which eluted in the area of a standard di-HETE, LTB_4, on high performance liquid chromatography (HPLC) analysis. We propose that the reincorporation of 12-HETE into these neutral lipids and the increased capacity for further metabolism of this biologically potent hydroxyfatty acid could be a mechanism by which the cholesterol-rich macrophage maintains its membrane function, and regulates the amount of 12-HETE in the pericellular space.—**Mathur, S. N., and F. J. Field.** Effect of cholesterol enrichment on 12-hydroxyeicosatetraenoic acid metabolism by mouse peritoneal macrophages. *J. Lipid Res.* 1987. **28:** 1166–1176.

Supplementary key words peritoneal macrophages • cholesterol • arachidonic acid • 12-hydroxyeicosatetraenoic acid • prostaglandins • foam cells • cholesteryl 12-hydroxyeicosatetraenoic acid ester

Macrophages are an integral part of an atherosclerotic lesion. It is the blood-borne monocyte, the immediate precursor to the tissue macrophage, which is first observed to adhere to the endothelial surface of a lesion (1). Following a rapid migration to the subendothelial space, the macrophage is transformed into a foam cell by the accumulation of lipids. It is this early lesion that is recognized as a fatty streak. With time, smooth muscle cells migrate to the area and accumulate lipids. The numbers of lymphocytes and polymorphonuclear leukocytes increase and a fibrous matrix is produced. Frequently, platelets will also aggregate at the surface of the lesion. It has been suggested that this progression to a mature atheroma resembles an ongoing inflammatory response (2). Since various eicosanoids are potent mediators of inflammation, these substances have been implicated in atherogenesis (3).

Macrophages, specifically mouse peritoneal macrophages, are known to be rich sources of eicosanoids derived from the metabolism of arachidonic acid (4–6). Exogenous arachidonic acid is converted to products of both the cyclooxygenase and lipoxygenase pathways in cultured macrophages; however, lipoxygenase products predominate (5). Although several monohydroxyeicosatetraenoic acids (HETEs) are generated, 12-HETE is the major lipoxygenase product synthesized (5, 6). Pawlowski et al. (7) and Stenson, Nickells, and Atkinson (8) observed that macrophages will also take up these monohydroxyfatty acids and incorporate them into cellular lipids.

We have previously demonstrated that the cholesterol-rich mouse peritoneal macrophage, a cell type thought to play an important role in the pathogenesis of atherosclerosis, produces more 12-HETE from exogenously added arachidonic acid than does the unmodified macrophage (9). Since this observation could have important implica-

Abbreviations: HPLC, high performance liquid chromatography; HETE, 12-hydroxyeicosatetraenoic acid; ACAT, acylcoenzyme A:cholesterol acyltransferase; HEPES, N-2-hydroxylethylpiperazine-N′-2 ethanesulfonic acid; TLC, thin-layer chromatography; LDL, low density lipoprotein.

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tions regarding the initiation and propagation of an atherosclerotic lesion, the metabolism of 12-HETE by cholesterol-enriched mouse peritoneal macrophages was examined. Our results indicate that the incorporation of 12-HETE into cellular lipids and its conversion to more polar products differs considerably in control and in cholesterol-enriched macrophages.

MATERIALS AND METHODS

Materials

[1-^{14}C]Arachidonic acid was purchased from Radiochemical Center, Amersham; [5,6,8,9,11,12,14,15-^{3}H]5-, 12-, and 15-hydroxyeicosatetraenoic acids, [14,15-^{3}H(N)]-leukotriene B_4, [4-^{14}C]cholesterol, and [^{14}C(U)] glycerol were obtained from New England Nuclear, Boston, MA. Unlabeled prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$, prostaglandin D_2, prostaglandin B_2, and 12-HETE were from Cayman Chemical Company, Ann Arbor, MI. Compound 58-035 was a gift from Sandoz Pharmaceuticals, E. Hanover, NJ. Progesterone was purchased from Sigma. All other reagents were reagent grade.

Cell culture

Peritoneal cells from male Swiss Webster mice weighing 25–30 g (Harlan, Madison, WI) were collected by washing the peritoneal cavity with 10 ml of sterile saline containing 10 units of heparin/ml. The primary cultures were established as described by Edelson and Cohn (10). The cells were cultured in Medium 199 containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, and 2.5% fetal bovine serum. The control cells were incubated with 2 ml of this medium for 24 hr at 37°C. To obtain cholesterol-rich cells the medium was supplemented with human acetyl-low density lipoprotein at the concentrations described (11–13). At the end of 24 hr incubation, the monolayers were washed four times with 2 ml of fresh Medium 199 containing 10 mM HEPES buffer, pH 7.4 (medium). This procedure yielded viable macrophage monolayers of 99% purity (14). Each 35-mm dish had 98 ± 3.4 μg of cellular protein. All procedures were done under sterile conditions and the cells were incubated in an atmosphere of 5% CO_2 and 95% air at 37°C.

Metabolism of exogenously supplied [^{14}C]arachidonic acid or [^{3}H]12-HETE

The washed monolayers of control and cholesterol-rich macrophages were incubated with 1 ml of fresh medium containing appropriate amounts of the sodium salt of the fatty acid as indicated for each experiment. The sodium salt of the fatty acid was prepared and added to the medium in 5 μl of ethanol/ml of medium (7). The incubations were terminated by aspirating the medium and transferring it into tubes containing 2 ml of acetone. The cells were washed twice with 1 ml of ice-cold phosphate-buffered saline (NaCl, 136.9 mM; KCl, 2.7 mM; $MgCl_2 \cdot 6H_2O$, 0.5 mM; KH_2PO_4, 1.5 mM; Na_2HPO_4, 8 mM) pH 7.4. The cells were harvested as described by Brown and Goldstein (12).

Lipid extraction

Cellular total lipids were extracted with 20 volumes of chloroform–methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (15). Following separation and isolation of the chloroform phase, the solvent was evaporated under nitrogen and the lipid residue was dissolved in 50 μl of chloroform–methanol 2:1 (v/v).

The metabolites of arachidonic acid and 12-HETE contained in the medium were extracted by the method described by Salmon and Flower (16). Briefly, 1 ml of the medium was added to a tube containing 2 ml of acetone. The aqueous layer then was acidified to pH 3 with citric acid and extracted twice with 2 ml of chloroform. The recovery of the radioactive products was monitored for each experiment by counting a portion of lipid extract and aqueous phase from each dish, as well as by parallel extraction of authentic tritium-labeled compounds. The recoveries for samples from control and cholesterol-rich cells were identical. Based on recoveries of radioactive standard compounds, 89% of 6-keto $PGF_{1\alpha}$, 97% of PGE_2, 83% of 12-HETE, 88% of 15-HETE, and 94% of 5-HETE were extracted into the chloroform phase.

Separation of the lipids by thin-layer chromatography

The lipids were separated on 20 × 20 cm Adsorbosil plus prekotes without binder (Alltech Associates, Deerfield, IL) in the appropriate solvent systems described below. TLC solvent A: organic phase of ethyl acetate–isooctane–acetic acid–water 110:50:20:100 (v/v). TLC solvent B: hexane–diethyl ether–acetic acid 60:40:1 (v/v). TLC solvent C: chloroform–methanol–acetic acid–water 60:50:1:4 (v/v).

The radioactive metabolites were visualized by autoradiography or fluorography with EN^{3}HANCE (New England Nuclear, Boston, MA) using KODAK X-Omat AR film (Eastman-Kodak Co., Rochester, NY). The radioactive zones were scraped and counted in a liquid scintillation spectrometer.

Separation of the oxygenated products by high performance liquid chromatography (HPLC)

Reverse phase HPLC was performed on an Ultrasphere ODS, 5 μ, 4.6 × 25 cm column. The cyclooxygenase and lipoxygenase products were separated by gradient chromatography (17, 18). Developing solvents were HPLC-grade acetonitrile (EM Science, Cherry Hill, NJ) and water (Fisher Scientific Co., Fairlawn, NJ); the

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pH was adjusted to 3.5 with H_3PO_4. For the first 25 min following injection of the sample in 20 μl methanol, the acetonitrile concentration was 35% (v/v). A linear increase in acetonitrile was initiated at 25 min to 75% over 5 min and at 50 min to 95% over 4 min. The 95% acetonitrile mobile phase was kept for 6 min and then reduced to 35% over 5 min. The column was reequilibrated for 30 min prior to reuse. Usually 0.5-ml or 1-ml fractions were collected at a flow rate of 1 ml/min and were counted in 5 ml of Budget Solve (Research Produce International Corp., Mount Prospect, IL) using a Packard scintillation spectrometer (Downers Grove, IL). HPLC solvent system B: lipoxygenase products were also separated by gradient elution with a mobile phase of acetic acid-ammonium hydroxide buffer, pH 5.8 and methanol (19). The percent of methanol was 52% for the first 4.5 min. This was increased to 80% over 1.5 min and to 85% over the next 15 min. Between 21 and 27 min, it was increased to 100%. This was followed by a reduction to 52% over 2 min. The flow rate was 0.8 ml/min.

Preparation of lipoproteins

Human low density lipoproteins (d 1.019-1.063 g/ml) were isolated by differential ultracentrifugation and acetylated as described by Basu et al. (20). In experiments in which the lipoprotein was to be labeled, [4-^{14}C]cholesterol (57 mCi/nmol) was added in ethanol (5 μl/ml medium) to the lipoprotein solution and incubated for 24 hr at room temperature with gentle mixing under sterile conditions (21).

Identification of fatty acids incorporated into cellular lipids

The total phospholipids and neutral lipids were separated by thin-layer chromatography using TLC solvent B. After staining and outlining the areas of the gel, the corresponding bands were scraped. The silical gel was transferred to disposable pipettes plugged with glass wool, and the lipids were eluted 4 times with 2 ml of a mixture of chloroform-methanol-acetic acid-water 60:50:1:4 (v/v). By this procedure 95-98% of the lipids were recovered in the eluant. To 8 ml of the eluant 2.8 ml of chloroform and 2.2 ml of 0.04 N HCl was added, and the contents of the tube were mixed with a vortex mixer. The lipids in the chloroform phase were collected for further analysis.

Alkaline hydrolysis

The total phospholipids and the neutral lipid compounds were hydrolyzed overnight with 1 ml 0.5 M NaOH in 90% methanol at room temperature under a nitrogen atmosphere (22). To this mixture 0.75 ml of water was added and the pH was adjusted to 3.5 with 5 N HCl, followed by 2 ml of chloroform. The chloroform phase was collected and the aqueous phase was extracted again with 2 ml of chloroform. The two chloroform extracts were combined and analyzed by TLC using solvent system B.

Hydrolysis with triacylglycerol acyl hydrolase

The neutral lipid fractions isolated by TLC were suspended in 25 μl of ethanol and added to 1 ml of phosphate buffered saline, pH 7.4, containing 400 units of triacylglycerol acyl hydrolase (Sigma, EC 3.1.1.3, from *Rhizopus arrhizus*, sp act: 431,000 units/mg protein) and incubated at 37°C for 1 hr. The pH was adjusted to 3.5 with 6 N HCl and lipids were extracted with chloroform-methanol. The lipids were then resolved by TLC using solvent system B.

Hydrolysis with sterol-ester acyl hydrolase

The neutral lipids isolated by TLC were suspended in 25 μl of ethanol and treated with 1 ml of phosphate-buffered saline, pH 7.4, containing 0.5 units of sterol-ester acyl hydrolase (Sigma, EC 3.1.1.13, from *Pseudomonas fluorescens*, sp act: 400,000 units/g protein), 0.05% Triton X-100, and 5 mM sodium taurocholate for 1 hr at 37°C. The lipids were resolved as described above.

Hydrolysis with phospholipase

The total phospholipid fraction was treated with bee venom phospholipase A_2 (EC 3.1.1.4) (Sigma, St. Louis, MO) to determine the positional specificity and identity of the esterified ^{3}H-labeled 12-HETE and its metabolites. The phospholipids were dissolved in 0.2 ml of diethyl ether and incubated overnight at room temperature with 0.01 ml of bee venom stock solution (1 mg, 800 units, lyophilized bee venom in 1 ml of 5 mM $CaCl_2$) (8). After evaporation of the solvent, the lipid residue was taken up in 10 μl of chloroform-methanol-water 1:1:1 (v/v) and analyzed by thin-layer chromatography using solvent system B.

Synthesis of cholesteryl-12-HETE ester

The cholesteryl-12-HETE ester was synthesized by the method of Murthy and Ganguly (23) using the reverse reaction of cholesterol esterase. The reaction mixture contained 1 ml of 0.1 M phosphate buffer, pH 6.1, 5 μM cholesterol, 5 μM 12-HETE, 5 mM sodium taurocholate, and 0.25 units of bovine pancreatic cholesterol esterase (Sigma, St. Louis, MO). The formation of [^{14}C]cholesteryl-12-HETE or cholesteryl [^{3}H] 12-HETE was followed by including 0.4 μCi of [^{14}C]cholesterol or 0.6 μCi of [^{3}H]12-HETE in the incubation mixture for 1 hr at 37°C. The lipids were extracted and analyzed as described above using TLC solvent B. As a control, the formation of [^{14}C]cholesteryl oleate was measured in a separate reaction mixture containing oleic acid in lieu of 12-HETE.

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Chemical analysis

Cholesterol was determined by gas-liquid chromatography with cholestane as an internal standard. Gas-liquid chromatography was done on a Hewlett Packard 5840A gas chromatograph at 280°C with a 3-foot glass column packed with 3% SP-2100 on 100/120 Supelcoport (24). Total cellular phospholipids were measured according to the method of Chalvardjian and Rudnicki (25). Triglycerides were measured with an AutoAnalyzer II as described by the Lipid Research Clinics (26). Protein was estimated by the method of Lowry et al. (27) with bovine serum albumin as the standard.

RESULTS

Arachidonic acid metabolism in cholesterol-rich macrophages

Macrophages were enriched in cholesterol by incubating the cells with 25 μg of acetyl-LDL for 24 hr (9). **Table 1** shows the lipid composition of these cells. Phospholipids and acyl glycerols were significantly increased in macrophages incubated with acetyl-LDL. The major lipid which accumulated in these cells, however, was cholesterol. Unesterified cholesterol was increased twofold, whereas the amount of cholesteryl ester was 25-fold higher in cells incubated with acetyl-LDL compared to controls.

Control and cholesterol-rich macrophages were then exposed to [1-^{14}C]arachidonic acid (40 μM) complexed to bovine serum albumin (15 μM) for 2 hr at 37°C. The amount of cell-associated radioactivity and the amount of radioactivity that remained in the medium over the 2-hr time span were identical in the two cell populations. At 2 hr, the distribution of labeled arachidonic acid in the cellular lipids was analyzed in control and cholesterol-rich macrophages **(Table 2)**. Except for a significant increase in the incorporation of arachidonic acid into cholesteryl esters in the cholesterol-rich macrophages, the distribution of the remaining radioactivity was very similar. The percent distribution of the arachidonate label in individual phospholipid classes was also determined by thin-layer chromatography (solvent system C) and found to be similar in the two cell populations. Approximately 78% of the radioactivity was incorporated into phosphatidylcholine followed by phosphatidylinositol 10%, phosphatidylethanolamine 9%, and phosphatidylserine 2.5%.

The oxygenated products released into the medium from arachidonic acid metabolism by control and cholesterol-rich macrophages were separated by thin-layer chromatography using solvent system A. As observed previously (9), more oxygenated products were released from the cholesterol-rich cells compared to unmodified macrophages (1585 pmol/dish per 10 min vs. 835, $P < 0.01$). The amount of products derived from the cyclooxygenase pathway were similar in the two macrophage populations. The increase in arachidonate products observed in cholesterol-rich macrophages was secondary to an increase in the synthesis of lipoxygenase products (1293 pmol/dish per 10 min vs. 563, $P < 0.01$). Since 12-HETE was the major product released by both macrophage populations (9), the quantity of 12-HETE in the medium, as measured by HPLC, was determined over a 2-hr incubation with [1-^{14}C]arachidonic acid. **Fig. 1** shows data from a representative experiment. Both control and cholesterol-rich cells released maximal amounts of 12-HETE in the first 10 to 20 min. At each time point, cholesterol-rich macrophages produced considerably more of this monohydroxyfatty acid than did control macrophages. The amount of 12-HETE which remained in the medium subsequently decreased as the incubation continued over the 2-hr period.

12-HETE metabolism in cholesterol-rich macrophages

The decrease in the amount of 12-HETE observed over time in the medium of control and cholesterol-rich macrophages suggested that this monohydroxyfatty acid was being reincorporated into the cells. **Table 3** shows

TABLE 1. Lipid composition of macrophages

Lipid Fraction	Control Cells	Cholesterol-rich Cells	Fold Increase
	nmol/mg protein		*cholesterol-rich/control*
Phospholipids	237 ± 21	313 ± 9[a]	1.32
Acyl glycerols	78 ± 10	121 ± 12[a]	1.55
Unesterified cholesterol	91 ± 8	196 ± 8[b]	2.15
Esterified cholesterol	11 ± 3	277 ± 5[b]	25.2

Macrophages were incubated with or without 25 μg of acetyl-LDL for 18 hr at 37°C. After washing the cells four times with cold phosphate-buffered saline, the lipids were extracted and analyzed as described in Methods. The values represent the average ± SEM of 12 dishes.

[a]Significantly different from control, $P < 0.05$.

[b]Significantly different from control, $P < 0.001$.

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TABLE 2. Incorporation of arachidonic acid into cellular lipids of control and cholesterol-rich macrophages

Lipid Fraction	Arachidonic Acid Incorporation		
	Control	Cholesterol-Rich	Fold Increase
	pmol/2 hr per dish		*cholesterol-rich/control*
Total lipids	12380 ± 490[a]	14660 ± 1380	1.18
Phospholipids	3910 ± 490	3250 ± 330	.83
Acyl glycerols	8470 ± 770	8490 ± 2980	1.00
Cholesteryl esters	6 ± 3	2470 ± 370[b]	412

Control and cholesterol-rich macrophages were incubated with [1-^{14}C]arachidonic acid (40 μM), specific activity 25 dpm/pmol, complexed to bovine serum albumin (15 μM) for 2 hr at 37°C. The macrophages were washed thoroughly with phosphate-buffered saline and the lipids were extracted and separated by thin-layer chromatography (system B) as described in Methods.
[a]Values represent the average ± SEM of six dishes.
[b]Significantly different from control, $P < 0.001$.

data from the incorporation of 4 nM [^{3}H]12-HETE (0.16 μCi) into cellular lipids and its metabolism by control and cholesterol-rich macrophages after 2 hr of incubation. In the medium from cholesterol-rich cells, there was a 21% increase in the synthesis and release of products that were more polar than 12-HETE. These products were characterized further by HPLC **(Fig. 2)**. Both macrophage populations synthesized two major metabolites which eluted earlier than 15-HETE and just prior to the elution of the diHETE, LTB_4. Smaller amounts of three other products were also observed. The chromatograms suggested that similar metabolites of 12-HETE were produced by both control and cholesterol-rich macrophages. Since none of these products formed in the absence of cells, the metabolites shown in Fig. 2 were generated from the metabolism of 12-HETE by the macrophages.

The distribution of the 12-HETE label within the cellular lipids also showed marked differences between the two cell populations (Table 3). Phospholipids isolated from control macrophages contained 1066 fmol of 12-HETE per dish compared to 412 fmol/dish in cholesterol-rich cells. Since cholesterol-rich cells contained more phospholipids per mg cell protein, the amount of 12-HETE per nmol of phospholipid was significantly decreased in cholesterol-rich cells compared to unmodified macrophages. In contrast, significantly more 12-HETE was incorporated into two neutral lipid bands migrating with R_f values of 0.34 and 0.49 in cholesterol-rich cells. Very little of the 12-HETE label comigrated with standards triolein or cholesteryl oleate in either cell preparation. Because the pools of triglycerides and cholesterol were greatly expanded in cholesterol-rich cells (Table 1), we investigated the possibility that the bands which migrated with R_f values of 0.39 and 0.49 contained lipids in which 12-HETE was esterified to a glycerol or a cholesterol molecule.

Characterization of the neutral lipids accumulated by cholesterol-rich cells

Since a characteristic feature of the cholesterol-rich macrophage is the accumulation of massive amounts of esterified cholesterol due to a significant increase in the rate of cholesterol esterification, the possibility that the monohydroxyfatty acid may be esterified to cholesterol was evaluated. Routine thin-layer chromatography separation may not detect cholesteryl-12-HETE as the hydroxylated fatty acid could cause the ester to migrate with a lower R_f value than the standard cholesteryl oleate. To investigate this possibility, cells were incubated with 25

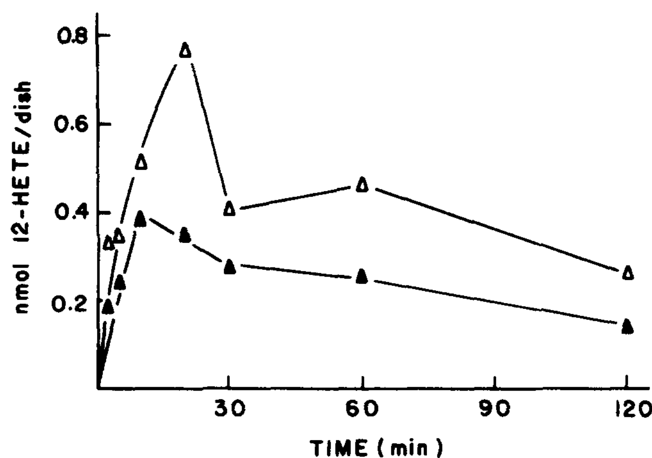

Fig. 1. Time-dependent release of 12-HETE into the medium by macrophages treated with exogenous arachidonic acid. Control (closed triangles) and cholesterol-rich (open triangles) macrophages were incubated with 40 μM, 0.5 μCi [^{14}C]arachidonic acid complexed with 15 μM bovine serum albumin for the indicated times at 37°C. The medium was removed and the lipids extracted with chloroform-methanol 2:1 (v/v) and the amount of 12-HETE was determined by HPLC as described in Methods.

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TABLE 3. Metabolism of 12-HETE by control and cholesterol-rich macrophages

Lipid Fraction	Control		Cholesterol-Rich		Cholesterol-Rich/ Control Ratio
	fmol of 12-HETE/2 hr per dish				
Cell-associated total lipids	1521 ± 89	(100)	1348 ± 102	(100)	0.88
Cell-associated individual lipids					
R_f 0.00 Phospholipids[a]	1066 ± 50	(70.2)[b]	412 ± 39[c]	(30.7)	0.39
R_f 0.13	85 ± 8	(5.6)	71 ± 5	(5.3)	0.84
R_f 0.21 Cholesterol[a]	158 ± 27	(10.4)	109 ± 16	(8.1)	0.69
R_f 0.28 1,2 Diolein[a]	46 ± 9	(3)	79 ± 11	(5.9)	1.74
R_f 0.34	131 ± 32	(8.6)	213 ± 19[c]	(15.9)	1.63
R_f 0.49	30 ± 9	(2)	443 ± 35[c]	(33)	14.8
R_f 0.72 Triolein[a]	5 ± 2	(0.3)	19 ± 4	(1.4)	3.8
R_f 0.82 Cholesteryl oleate[a]	1 ± 0.3	(0.001)	1.8 ± 0.2	(0.05)	1.8
Medium					
Total lipids	2646 ± 245[d]		3399 ± 202[e]		
12-HETE	354 ± 45		624 ± 77[f]		
More polar products	2291 ± 212		2774 ± 191[e]		

Control and cholesterol-rich macrophages were incubated with 4 nM [^{3}H]12-HETE (0.16 μCi) for 2 hr. The lipids from the cells and medium were extracted and analyzed using TLC solvent system B as described in Methods. The R_f values for the lipid standards were: phospholipids, 0.00; LTB_4, 0.04; monoolein, 0.06; triricinolein, 0.10; cholesterol, 0.21; 12,15-HETE, 0.21; 1,3 diolein, 0.21; 12-HETE methyl ester, 0.27; 1,2 diolein, 0.28; arachidonic acid, 0.46; estolides, 0.72; triolein, 0.72; and cholesteryl oleate, 0.82.

[a]Standard compounds used to identify individual lipids or lipid classes.
[b]Percent of cell-associated 12-HETE radioactivity.
[c]Significantly different from control, $P < 0.001$.
[d]Values represent the average ± SEM of nine dishes.
[e]Significantly different from control, $P < 0.05$.
[f]Significantly different from control, $P < 0.01$.

μg/ml of acetyl-LDL for 16 hr. The cells were then washed and incubated for 6 hr with 0.1 μM of unlabeled 12-HETE and 1 μCi of [^{14}C]cholesterol complexed with 2.5 μg of acetyl-LDL (sp act, 340 dpm/pmol cholesterol). Lipids were extracted from the cells and analyzed as described for Table 3. Compared to the background radioactivity observed at an R_f value of 0.49 from medium not containing cells, there was a sevenfold enrichment of the [^{14}C]cholesterol label observed in the cellular lipid fraction which migrated with this R_f value.

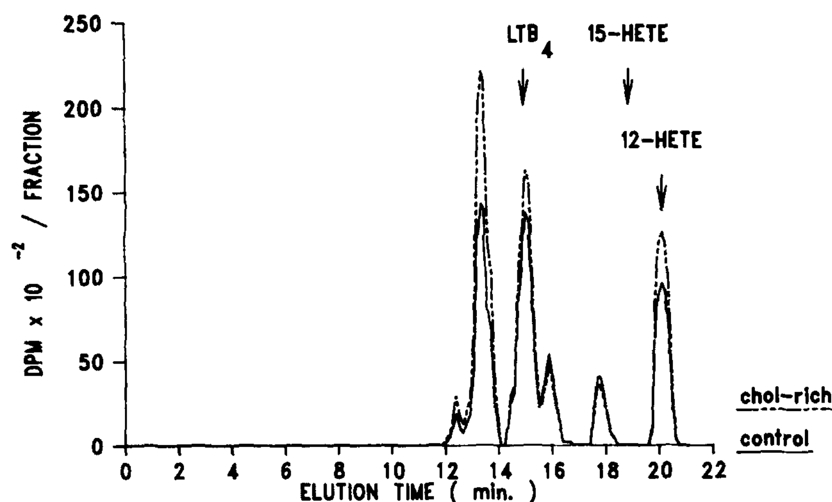

Fig. 2. HPLC elution profile of 12-HETE metabolites in the medium. Control (solid line) and cholesterol-rich (dotted line) macrophages were incubated with 4 nM [^{3}H]12-HETE (40 Ci/mmol) in protein-free medium for 2 hr at 37°C. The lipids in the medium were extracted with chloroform–methanol 2:1 (v/v) and resolved by HPLC as described in Methods. Elution times for authentic LTB_4 (15 min), 15-HETE (19 min), and 12-HETE (20 min) are indicated by the arrows.

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This suggested that the hydroxylated fatty acid, 12-HETE, was most likely being esterified to cholesterol in cholesterol-enriched macrophages and the resulting cholesteryl-12-HETE was migrating well below cholesteryl oleate after separation by thin-layer chromatography.

To prove that this was a 12-HETE cholesteryl ester, the following experiment was done. [^{14}C]Cholesterol, 5 μM, 0.4 μCi or [^{3}H]12-HETE, 5 μM, 0.6 μCi was incubated in a total volume of 1 ml of 0.1 M phosphate-buffered saline, pH 6.1, containing 5 mM sodium taurocholate, the unlabeled 12-HETE or cholesterol, and 0.25 units of bovine pancreatic cholesterol esterase. After 1 hr at 37°C, the lipids were extracted from the reaction mixture and separated by TLC as described for Table 3. The lipid which migrated at an R_f value of 0.49 was found to contain radioactivity irrespective of whether the label was on the sterol or the fatty acid. In the absence of cholesterol esterase or 12-HETE in the reaction mixture, no radioactivity was observed and no band was visualized in this region. After elution and saponification of the lipid at 0.49, the label then migrated with cholesterol or 12-HETE depending upon which label was used.

Since intracellular cholesterol esterification is catalyzed by acylcoenzyme A:cholesterol acyltransferase (ACAT), the effect of regulating ACAT activity on 12-HETE incorporation into cellular lipids was studied **(Table 4)**. Only the lipid classes that migrated with R_f values of 0.00, 0.34, and 0.49 are shown since ACAT regulation had no significant effect on the incorporation of 12-HETE into lipids with R_f values of 0.13, 0.21, 0.28, 0.72, or 0.82 (Table 3). As previously shown in Table 3, acetyl-LDL resulted in both an increase in the incorporation of 12-HETE into lipids that migrated with R_f values of 0.34 and 0.49 and a decrease in the incorporation of 12-HETE into phospholipids. However, when the ACAT inhibitors, progesterone (2.5 μg/ml) and 58-035 (10 μg/ml), were added to the incubation, there was a marked decrease in the incorporation of 12-HETE into the lipid band migrating with an R_f value of 0.49. Under these same conditions, the incorporation of 12-HETE into the lipid band of R_f value 0.34 was, if anything, slightly increased. More of the label now appeared in the phospholipid band (R_f 0.00). When entry of acetyl-LDL was blocked by dextran sulfate (25 μg/ml), the incorporation of 12-HETE into cellular lipids resembled that which was observed in control cells. 25-Hydroxycholesterol (50 μg/ml), a known stimulator of ACAT activity, increased by threefold the incorporation of 12-HETE into the lipids with an R_f value of 0.49 without affecting the incorporation of this fatty acid into the lipids with an R_f value of 0.34. Autoradiograms of this experiment substantiate and, perhaps, better illustrate graphically these results. As shown in **Fig. 3,** the compounds migrating at the R_f value of 0.49 were not detected when the incubation mixture contained dextran sulfate or the ACAT inhibitors, 58-035 or progesterone.

To monitor the activity of ACAT in this experiment, the incorporation of oleic acid into cholesteryl oleate was concomitantly measured. Progesterone, 58-035, and dextran sulfate inhibited ACAT activity by 92%, 92%, and 94%, respectively. 25-Hydroxycholesterol resulted in a 40-fold increase in ACAT activity. Therefore, under experimental conditions whereby ACAT activity was being significantly altered, the incorporation of 12-HETE into the lipids migrating with an R_f of 0.49 was being similarly regulated. This strongly suggests that ACAT regulates the formation of a product or products that migrate at this R_f value.

To examine whether the other neutral lipid band with an R_f value of 0.34 contained a glyceride attached to the hydroxyfatty acid, control and cholesterol-rich cells were incubated for 2 hr with 0.3 μCi of [^{3}H]12-HETE, 8 nM, or with 1 μCi of [^{14}C]glycerol, 115 μM. After the incuba-

TABLE 4. Effect of ACAT regulation on 12-HETE incorporation into macrophage lipids

	12-HETE Incorporated per Dish		
	R_f 0.00	R_f 0.34	R_f 0.49
	% of total cell-associated radioactivity		
Control	67 ± 2	14 ± 1	4 ± 1
Control + 25-hydroxycholesterol	62 ± 3	13 ± 1	12 ± 3
Acetyl-LDL	25 ± 3	27 ± 4	37 ± 1
Acetyl-LDL + 58-035	41 ± 4	37 ± 4	10 ± 1
Acetyl-LDL + progesterone	31 ± 2	48 ± 2	8 ± 2
Acetyl-LDL + dextran sulfate	58 ± 2	19 ± 2	10 ± 1

Macrophages were incubated with 25 μg/ml of acetyl-LDL in 2.5% fetal bovine serum for 16 hr. One set of dishes also contained 25 μg/ml of dextran sulfate. The cells were then washed twice and 12-HETE, 0.1 μM, 0.2 μCi, was added with either 2.5 μg/ml of acetyl-LDL alone or in combination with 10 μg/ml of 58-035, 2.5 μg/ml progesterone, or 25 μg/ml dextran sulfate. 25-Hydroxycholesterol, 50 μg/ml, was added to control dishes. The incubations were continued for another 6 hr before cell lipids were extracted and analyzed as described in Methods using solvent system B. For simplicity, only lipids migrating with R_f values 0.00, 0.34, or 0.49 are shown. The values are averages ± SEM of three dishes.

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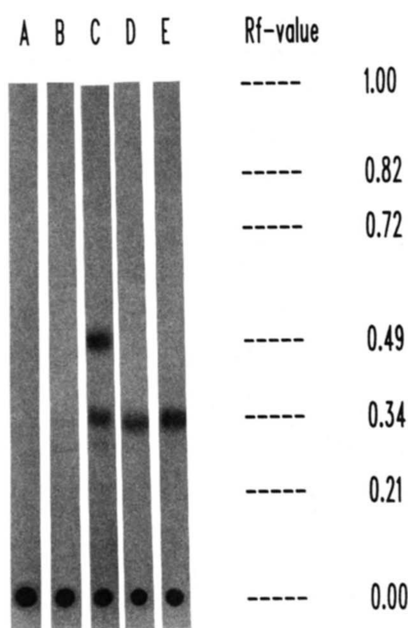

Fig. 3. Autoradiogram of 12-HETE-labeled cellular lipids. Control and cholesterol-rich macrophages were incubated with 0.1 μM, 0.2 μCi [^{3}H]12-HETE for 6 hr at 37°C. The cellular lipids were resolved by thin-layer chromatography using hexane–diethylether–acetic acid 60:40:1 (v/v). The thin-layer plates were sprayed with EN^{3}HANCE and exposed to X-Omat AR film for 3 days at − 70°C. A, Control; B, acetyl LDL + dextran sulfate; C, acetyl LDL; D, acetyl LDL + 58-035; E, acetyl LDL + progesterone.

tion, cellular lipids were extracted and separated by thin-layer chromatography as indicated in Table 3. The results are shown in **Table 5**. In cholesterol-rich cells, there was a four- to fivefold enrichment of the glycerol label into both neutral lipid bands having R_f values of 0.34 and 0.49. Since the previous experiment suggested that one of the lipid compounds migrating with R_f value of 0.49 was predominantly cholesteryl-12-HETE, the lipids migrating at R_f values of 0.34 and 0.49, and which contained the glycerol label, were analyzed further. **Table 6** shows the results obtained when these lipids were subjected to saponification and hydrolysis by sterol-ester acyl hydrolase and triacylglycerol acyl hydrolase. It is apparent from these data that the sterol-ester acyl hydrolase is not specific and hydrolyzes triolein as well as cholesteryl oleate. This has been recognized previously (28). However, the triacylglycerol acyl hydrolase is specific for triacylglycerols and hydrolyzes only the lipids that migrate with an R_f value of 0.34. There was no hydrolysis of the lipids migrating with an R_f value of 0.49 suggesting that the glycerol label observed here was not incorporated into the molecule containing the 12-HETE. Alkaline hydrolysis, as expected, hydrolyzed the lipids in both bands equally well. These data, therefore, taken together with the data shown in Table 5, suggest that in cholesterol-rich cells, 12-HETE is preferentially diverted into neutral lipids. One of the neutral lipids having an R_f value of 0.34 contains a glyceride, while one of the lipids migrating with an R_f value of 0.49 contains cholesterol and is cholesteryl-12-HETE.

Distribution of native 12-HETE and its metabolites in cellular lipids

The data in Fig. 2 and Table 3 demonstrate that 12-HETE is taken up, incorporated into cellular lipids, and metabolized to more polar products by these macrophages. To examine whether unmodified 12-HETE or metabolites of 12-HETE were being incorporated into cellular lipids, the distribution of 12-HETE and the metabolites of 12-HETE in phospholipid and neutral

TABLE 5. Incorporation of [^{3}H]12-HETE or [^{14}C]glycerol into cellular lipids of control and cholesterol-rich macrophages

	12-HETE		Glycerol	
Lipid Fraction	Control	Cholesterol-Rich	Control	Cholesterol-Rich
	pmol/2 hr per dish			
Total lipids	3.87 ± 0.21[a]	2.23 ± 0.14	1541 ± 84	1690 ± 212
Individual lipids				
R_f 0.00	3.01 ± 0.03	0.35 ± 0.02[b]	1187 ± 14	912 ± 216
R_f 0.34	0.18 ± 0.02	0.68 ± 0.10[c]	31 ± 12	142 ± 25[c]
R_f 0.49	0.08 ± 0.04	0.86 ± 0.12[c]	39 ± 25	203 ± 14[c]
R_f 0.72	0.02 ± 0.00	0.14 ± 0.01[b]	60 ± 10	384 ± 72[b]
R_f 0.82	ND	ND	ND	ND

Control and cholesterol-rich cells were incubated for 2 hr with 8 nM [^{3}H]12-HETE (0.32 μCi) or 115 μM [^{14}C]glycerol (1 μCi). After incubation, cellular lipids were extracted and analyzed by thin-layer chromatography using solvent system B. See Table 3 for explanation of R_f values.
[a]Values represent the average ± SEM of four dishes.
[b]Different from control cells, $P < 0.001$.
[c]Different from control cells, $P < 0.01$.
[d]None detected.

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TABLE 6. Hydrolysis of the two neutral lipids in cholesterol-rich macrophages

Treatment	R_f Value 0.34	R_f Value 0.49	Controls: Triolein	Controls: Cholesterol Oleate
	% hydrolysis			
Sterol-ester acylhydrolase	76[a]	74	88	60
Triacylglycerol acylhydrolase	76	0.2	89	0
Alkaline hydrolysis	83	73	98	99

The macrophages were incubated with 25 µg/ml of acetyl-LDL for 16 hr. After washing the cells, they were incubated with 0.1 µM, 0.5 µCi, of [^{3}H]12-HETE in serum-free medium for 6 hr. The lipids were resolved by thin-layer chromatography using solvent system B. The lipid fractions corresponding to R_f 0.34 and 0.49 were scraped and eluted from the gel and subjected to hydrolysis as described in Methods. Tri[1-^{14}C]oleoylglycerol and [1,2,6,7-^{3}H]cholesteryl oleate were used as controls to monitor the hydrolytic activity of the enzymes. Percent hydrolysis was determined by subtracting the radioactivity observed in the esterified lipid in the presence of the hydrolase from the amount of label found in this lipid in the absence of the enzyme, divided by the radioactivity without enzyme × 100.
[a]Average of two separate experiments.

lipid was studied (**Table 7**). Incubating the total phospholipid fraction isolated from control and cholesterol-rich cells with bee venom phospholipase caused the release of all the 12-HETE label, suggesting that the incorporated fatty acids were esterified at the *sn*-2 position. In both control and cholesterol-rich macrophages, the proportions of 12-HETE and its metabolites in phospholipids were similar, with 64-68% of the radioactivity present as unmodified 12-HETE and 32-36% as more polar products. A similar distribution was confirmed when the phospholipid fractions were hydrolyzed with alkali.

Alkaline hydrolysis of the two neutral lipids indicated that these compounds also contained native 12-HETE as well as more polar products. However, compared to the distribution observed in phospholipids, the distribution in the neutral lipids of cholesterol-rich cells was reversed, with 34-45% consisting of unmodified 12-HETE and the remainder being more polar products, 60-66%. These data suggest that an increase in the synthesis of neutral lipids in cholesterol-rich cells diverts more of 12-HETE, and especially its more polar products, into neutral lipids instead of phospholipids as occurs in control cells.

DISCUSSION

In an earlier study (9), we demonstrated that the cholesterol-enriched mouse peritoneal macrophage synthesized more 12-HETE from exogenously supplied arachidonic acid than did the unmodified macrophage. In this study, we have investigated the metabolism of 12-HETE by both macrophage populations. Significant differences were observed. There were marked differences in the esterification of arachidonic acid and 12-HETE into cellular lipids in control and cholesterol-rich cells. In unmodified macrophages, there was substantial incorporation of labeled arachidonic acid and 12-HETE into phospholipids. This finding supports previous observations in resting mouse peritoneal macrophages (7) and the macrophage-like cell line J774 (8). In cholesterol-rich macrophages, there was also a substantial incorporation of arachidonic acid into phospholipids but, in addition, a large amount was incorporated into cholesteryl ester indicating an increase in ACAT activity in these cells, an observation we have made before in rabbit peritoneal macrophages (21). In contrast to control macrophages, there is a diversion of 12-HETE from polar lipids to neutral lipids in cholesterol-rich macrophages. In another cell type, the neutrophil, Stenson and Parker (29) also observed that 88% of cell-associated 12-HETE was incorporated into triglycerides rather than into phospholipids. Neutrophils, therefore, selectively incorporate 12-HETE into cellular neutral lipids, somewhat similar to the cholesterol-enriched macrophage.

TABLE 7. Distribution of 12-HETE and its metabolites in phospholipids and neutral lipids

	Control	Cholesterol-Rich
	pmol/dish per 2 hr[a]	
Phospholipids		
Native 12-HETE	1.93	0.23
Polar products of 12-HETE	1.08	0.11
Neutral lipids		
Native 12-HETE	0.16	0.58
Polar products of 12-HETE	0.11	0.77

[^{3}H]12-HETE was incubated with control and cholesterol-rich macrophages for 2 hrs. Intracellular lipids were extracted and separated as described in Methods. The phospholipid and two neutral lipid bands were eluted from the TLC plate. A portion of the lipids was hydrolyzed overnight in 1 ml of 0.5 M NaOH in 90% methanol at room temperature under nitrogen. The lipids were re-extracted with chloroform-methanol as described in Methods. The saponified lipids were separated by TLC. Another portion of phospholipids was dissolved in 0.2 ml diethyl ether and added to 0.01 ml of bee venom (1 mg of lyophilized bee venom in 1 ml 5 mM $CaCl_2$) and incubated overnight at room temperature. The solvent was evaporated and the lipids separated by TLC.
[a]Mean of two separate experiments.

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In the cholesterol-rich macrophage, a large proportion of the 12-HETE was incorporated into two neutral lipid bands having R_f values of 0.34 and 0.49. Based on the incorporation of [^{14}C]glycerol, susceptibility to hydrolysis by triacylglycerol acyl hydrolase, and lack of inhibition by ACAT inhibitors, one of the neutral lipids of R_f value 0.34 is a glyceride containing 12-HETE. The neutral lipid band at R_f value 0.49 contains cholesteryl-12-HETE ester. It is formed only in cells having high ACAT activity; its synthesis is inhibited by specific inhibitors of ACAT; it contains cholesterol; it is hydrolyzed by a sterol-ester acyl hydrolase; and it comigrates with synthesized cholesteryl-12-HETE. Moreover, the 15-fold increase of cholesteryl-12-HETE observed in cholesterol-rich cells parallels the 25-fold increase in the amount of cholesteryl esters accumulated by these cells (Tables 1 and 3).

The esterification of 12-HETE into the *sn*-2 position of phospholipids in both control and cholesterol-enriched macrophages is consistent with the usual pattern of esterification of other polyunsaturated fatty acids. This is in agreement with the observations of Stenson, Nickells, and Atkinson (8) in J774 cells. Similar to that report, we also observed that, after phospholipase treatment of the phospholipids, most of the recovered label was still present as native 12-HETE and not a metabolic product. In contrast, however, the neutral lipids of cholesterol-enriched macrophages contained substantially more of the metabolic products of the monohydroxyfatty acid suggesting some degree of specificity of the acyltransferase for neutral lipid and phospholipid synthesis.

The results also demonstrate the metabolism of 12-HETE to more polar products by both the unmodified and cholesterol-enriched macrophage. This has been shown previously in control macrophages by Pawlowski et al. (7) and in granulocytes by Stenson and Parker (29). In contrast, J774.2 cells, a mouse macrophage-like tumor cell line, do not metabolize 12-HETE further (8). Very similar to the observations made in the granulocyte (29), two major metabolic products of 12-HETE metabolism were observed. These products were more polar than 15-HETE and migrated in the vicinity of a di-HETE, LTB_4. Attempts are presently being made to identify these two products. It is speculated that they are further hydroxylation products of the hydroxylated fatty acid. The cholesterol-enriched macrophage released more products from the metabolism of 12-HETE than did the unmodified macrophage. Perhaps, this could be a potential mechanism for the cholesterol-enriched macrophage to metabolize excessive amounts of 12-HETE which are being secreted into the pericellular environment of an atheroma by other macrophages or platelets.

The pathological implications of an increase in the synthesis of mono-HETEs by the cholesterol-rich macrophage are uncertain. However, in an atherosclerotic lesion in which there is an arterial injury, it is possible that an increased production of lipoxygenase products by cholesterol-rich macrophages present in the subendothelial space could contribute to the ongoing inflammatory response. The differences observed in the incorporation of arachidonic acid and 12-HETE into cellular lipids in the cholesterol-enriched macrophage could also give rise to a functionally distinct population of cells with biological properties that differ from unmodified macrophages. Mahoney et al. (30) have demonstrated in mouse peritoneal macrophages, that an increase in the saturated to unsaturated ratio of phospholipid fatty acids within cellular membranes resulted in a decrease in fluid-phase pinocytosis and receptor-mediated phagocytosis. Likewise, Schroit and Gallily (31) observed an increase in phagocytic ingestion of *Shigella flexneri* by mouse peritoneal macrophages whose membranes had been enriched in polyunsaturated fatty acids. The incorporation of an hydroxylated polyunsaturated fatty acid into the membranes of a macrophage, therefore, could lead to profound changes in cellular function. The incorporation of the monohydroxyfatty acid into neutral lipids rather than into membrane phospholipids may be a mechanism whereby the cholesterol-rich macrophage prevents any further change in its membrane lipid composition and thereby protects its biological activity. It could also be a mechanism to regulate the amount of 12-HETE in the pericellular environment. This possibility that different patterns of intracellular distribution of monohydroxyfatty acids may lead to differences in biological activity has been suggested previously (8, 32).

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