Incorporation of lipoxygenase products into cholesteryl esters by acyl-CoA:cholesterol acyltransferase in cholesterol-rich macrophages

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Macrophages which were incubated with acetylated low-density lipoproteins, resulting in cholesteryl ester accumulation, incorporated the monohydroxyicosatetraenoic acids (5-, 15-, and 12-HETEs) into cholesteryl esters. The esterification of these hydroxy fatty acids to cholesterol by total membrane preparations of cholesterol-rich macrophages was dependent on the synthesis of the fatty acyl-CoA derivative, and was catalysed by acyl-CoA:cholesterol acyltransferase (ACAT). Stimulation of membrane ACAT activity by 25-hydroxycholesterol increased the synthesis of cholesteryl 12-HETE by 40%. In contrast, inhibiting ACAT activity by progesterone and compound 58–035 decreased cholesteryl 12-HETE production by 60% and 90% respectively. Although 5-, 15- and 12-HETE were esterified to cholesterol by ACAT, these monohydroxy fatty acids were less optimal as substrates compared with oleic acid or arachidonic acid. The hydrolysis and release of 12-HETE and the other monohydroxyicosatetraenoic acids from intracellular cholesteryl esters and phospholipids occurred at a faster rate than for the more conventional fatty acids, oleate and arachidonate. Cholesteryl esters which contain hydroxy fatty acids therefore provide only a transient storage for lipoxygenase products, as these fatty acids are released into the medium as readily as hydroxy fatty acids found in phospholipids and triacylglycerols. The data provide evidence, for the first time, of an ACAT-dependent esterification of the lipoxygenase products 5-, 15- and 12-HETEs to cholesterol in the macrophage-derived foam cell. The channelling of these monohydroxy fatty acids to cholesteryl esters provides a mechanism which can alter the amount of lipoxygenase products incorporated into cellular phospholipids, thus averting deleterious changes to cell membranes. ACAT, by catalysing the esterification of monohydroxyicosatetraenoic acids to cholesterol, could play a key role in regulating the amount of lipoxygenase products in the pericellular space of the cholesterol-enriched macrophage.

INTRODUCTION

Mouse peritoneal macrophages metabolize exogenous arachidonic acid to 5-, 12- and 15-hydroxyicosatetraenoic acids (HETEs) via the lipoxygenase pathway (Mathur *et al.*, 1985; Rabinovitch *et al.*, 1981). 12-HETE is the major monohydroxy fatty acid released. Cultured macrophages which are incubated with acetylated low-density lipoproteins (acetyl-LDL) to promote the formation of 'foam cells' release significantly more 12-HETE into the medium from the metabolism of arachidonic acid than the amount released by unmodified macrophages (Mathur *et al.*, 1985). Because 12-HETE has been shown to stimulate the migration of smooth-muscle cells and is a potent chemotactic factor for polymorphonuclear leucocytes (Nakao *et al.*, 1982; Stenson & Parker, 1979), it has been suggested that this monohydroxy fatty acid could play a role in the initiation and progression of an atherosclerotic plaque (Mathur *et al.*, 1985; Mathur & Field, 1987). If so, regulating the amount of 12-HETE in the pericellular space of the macrophage within the arterial wall could alter the formation of an atheroma.

Mouse macrophages also have the capacity to metabolize monohydroxyicosatetraenoic acids. 12-HETE is taken up and incorporated predominantly into mem-

brane phospholipids in unmodified macrophages (Mathur & Field, 1987; Pawlowski *et al.*, 1982). In contrast, in macrophage-derived foam cells, most of the 12-HETE which is taken up by the cell is incorporated into neutral lipids (Mathur & Field, 1987). One of the intracellular neutral lipids is cholesteryl 12-HETE, a product which could be generated by the action of the microsomal enzyme acyl-CoA:cholesterol acyltransferase (ACAT). This key intracellular enzyme regulates the amount of free and esterified cholesterol within the cells (Brown & Goldstein, 1983; Spector *et al.*, 1979). In macrophages incubated with acetyl-LDL, the activity of ACAT is increased, suggesting a need by the cell to divert the incoming excess cholesterol to a storage form (Brown & Goldstein, 1983). It has been suggested that the formation of cholesteryl 12-HETE by the cholesterol-enriched macrophage is a way to protect the cell's membrane from perhaps an injurious lipid modification and/or to regulate the amount of 12-HETE in the pericellular space by channelling this fatty acid into a more non-accessible form (Mathur & Field, 1987).

The present paper demonstrates for the first time an ACAT-dependent catalysed esterification of cholesterol to lipoxygenase products derived from the metabolism of arachidonic acid by macrophages. It is speculated that

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; HETEs, hydroxyicosatetraenoic acids; acetyl-LDL, acetylated low-density lipoproteins; VLDL very-low-density lipoproteins.

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macrophage ACAT could play a role in regulating the amount of extracellular monohydroxyeicosatetraenoic acids in the sub-endothelial space of the arterial wall.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-³H]-5-, -12-, and -15-HETE, [1-¹⁴C]palmitic acid, [5,6,8,9,11,12,14,15-³H]arachidonic acid, [9,10-³H]oleic acid and [4-¹⁴C]cholesterol were obtained from New England Nuclear, Boston, MA, U.S.A. [1-¹⁴C]Linoleic acid was purchased from Amersham, Arlington Heights, IL, U.S.A. Unlabelled [5(S),6E,-8Z,11Z,14Z]5-HETE, [5Z,8Z,10E,12(S),14Z]12-HETE and [5Z,8Z,11Z,13E,15(S)]15-HETE and arachidonic acid were obtained from Cayman Chemical Co., Ann Arbor, MI, U.S.A. Linoleic acid was from Nu Check Prep, Elysian, MN, U.S.A. Compound 58-035 was given by Sandoz Pharmaceuticals, E. Hanover, NJ, U.S.A. Progesterone was purchased from Sigma. 25-Hydroxycholesterol was from Steraloids Inc., Wilton, NH, U.S.A. All other reagents were reagent grade.

Cell culture

Swiss Webster mice weighing 25–30 g (Harlan, Madison, WI, U.S.A.) were injected with 2 ml of 5% (v/v) Brewer Thioglycollate medium (Difco Laboratories, Detroit, MI, U.S.A.). After 4 days, peritoneal cells were collected by washing the peritoneal cavity with 10 ml of sterile saline containing 10 units of heparin/ml. Primary cultures were established as described by Edelson & Cohn (1976). The cells were cultured and enriched with cholesterol by incubating them with acetyl-LDL equivalent to 25 µg of cholesterol/ml for 18 h as previously described (Mathur & Field, 1987). Each dish contained 104 ± 2 µg of protein.

Preparation of cholesterol-rich macrophage total membrane fraction

Monolayers of cholesterol-rich macrophages were washed with cold phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/0.5 mM-MgCl₂/8 mM-Na₂HPO₄/2 mM-KH₂PO₄, pH 7.4). Cells were harvested by scraping them from the dishes with a plastic scraper. Cells were suspended in 10 ml of 0.1 M-phosphate buffer, pH 7.4, and sonicated with a micro-tip probe for 2–3 min with pulses of 25 W-s by using a Vibra-cell 250 sonicator (Sonics and Materials Inc., Danbury, CT, U.S.A.). The homogenates were centrifuged at 106 000 g in a Beckman L7–55 ultracentrifuge. The pellet containing the total membrane fraction was suspended in 0.1 M-phosphate buffer, pH 7.4, at a concentration of 3–4 mg of protein/ml.

Preparation of lipoproteins

Acetyl-LDL was prepared as described previously (Mathur & Field, 1987; Basu *et al.*, 1976).

ACAT assay

[³H]12-HETE-CoA was synthesized by incubating 2 mM-ATP, 2 mM-CoA, 2 mM-MgCl₂, 31.2 µM-bovine serum albumin, [³H]12-HETE (31.2 µM; 89 d.p.m./pmol) and 0.02 unit of acyl-CoA synthetase (from *Pseudomonas* sp.; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) in 100 µl of 0.1 M-Tris/HCl buffer, pH 7.4, for 30 min at 37 °C. The ACAT reaction was started by

mixing 100 µl of this 'cocktail' with 100 µl of cholesterol-rich macrophage membranes in 0.1 M-phosphate buffer containing 150–180 µg of protein. When required, 25-hydroxycholesterol, progesterone or 58-035 was added to 100 µl of the membrane fraction in 1 µl of ethanol and equilibrated with the membrane for 30 min at 37 °C before adding the coenzyme 'cocktail'. The ACAT reaction was carried out for 60 min at 37 °C. The reaction was terminated by addition of 1 ml of acetone. To this mixture 0.3 ml of 0.1 M-phosphate buffer and 4 ml of hexane were added, and the mixture was vortex-mixed for 30 s. The hexane layer was removed and the aqueous phase re-extracted with 4 ml of hexane. The two hexane extracts were combined, dried under N₂, and dissolved in 30 µl of chloroform/methanol (1:1, v/v).

Lipid extraction

Total cellular lipids were extracted *in situ* by using hexane/propan-2-ol/water (30:20:1, by vol.) (Via *et al.*, 1985). By this procedure over 99% of the radioactive lipids were extracted from the cells. The lipid extract was made up to a known volume. A portion was taken for measuring total radioactivity. The remaining was evaporated and dissolved in 30 µl of chloroform/methanol (1:1, v/v) for separation by t.l.c.

The medium was adjusted to pH 3 with 10 µl of 1 M-citric acid. The lipids were extracted from the medium by adding an equal volume of acetone and 2 vol. of chloroform. The chloroform extraction was repeated and the two chloroform extracts were combined. The solvent was removed under N₂, and the lipids were dissolved in 30 µl of chloroform/methanol (1:1, v/v).

Separation of lipids by t.l.c.

The lipids were separated on 20 cm × 20 cm silica-gel G plates (Fisher Scientific, Pittsburgh, PA, U.S.A.) in the solvent system hexane/diethyl ether/acetic acid (60:40:1, by vol.). The radioactive metabolites were identified by a Radiomatic TLC Scanner (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.). The lipids containing radioactivity were scraped off and counted for radioactivity in a liquid-scintillation counter.

Separation of the 12-HETE metabolites by h.p.l.c.

The native fatty acid and its metabolites released into the medium from the cellular lipids were analysed by reverse-phase h.p.l.c. using a Vydac 201TP54 C18 5 µm column (250 mm × 4.6 mm). The solvents were (A) 0.003 M-H₃PO₄ in water and (B) acetonitrile. The gradient was formed by eluting the column at a flow rate of 1 ml/min with 50% acetonitrile for 0–19 min, increasing to 98% acetonitrile at 21 min holding it until 25 min. The acetonitrile concentration was lowered to 48% at 27 min. The elution times were: 15-HETE, 14.8 min; 12-HETE, 17 min; 5-HETE, 18.8 min; arachidonic acid, 27.4 min. The radioactivity in the effluent was monitored by a Flow-one model HP radioactivity detector (Radiomatic).

Protein

Protein was measured by the method of Lowry *et al.* (1951). The protein residue in the dishes after extraction with hexane/propan-2-ol/water was dissolved in 0.2 ml of 0.2 M-NaOH and used for protein determination.

RESULTS

5-, 15- and 12-HETE esterification to cholesterol in control and cholesterol-rich macrophages

Sodium salts of labelled monohydroxyeicosatetraenoic acids were incubated individually with control macrophages and macrophages enriched in cholesterol after incubating them with acetyl-LDL. After 4 h at 37 °C, lipids were extracted from the cells and separated by t.l.c. Fig. 1 shows the rate of incorporation of 5-, 15- and 12-HETE into cholesteryl esters. For each monohydroxy fatty acid, the rate of synthesis of cholesteryl 5-, 15- and 12-HETEs was significantly higher in macrophages enriched in cholesterol than in unmodified cells.

ACAT activities as measured by the incorporation of [14 C]oleoyl-CoA into cholesteryl oleate in membranes prepared from control and cholesterol-rich macrophages were 35 ± 2 and 269 ± 20 pmol/min per mg of protein respectively.

Assay for measuring the rate of cholesteryl 12-HETE formation

ACAT activity was measured by an acyl-CoA generating system by using whole membrane pre-

Fig. 1. Incorporation of 5-, 15- and 12-HETE into cholesteryl esters by control and cholesterol-rich macrophages

Macrophages were enriched in cholesterol by incubating them with 25 μ g of acetyl-LDL for 18 h in M199 medium containing 2.5% fetal-calf serum. Acetyl-LDL was not added to control cells. The monolayers were washed and the macrophages were incubated with 1 μ M-[3 H]-5-HETE (930 d.p.m./pmol), -15-HETE (1080 d.p.m./pmol) or -12-HETE (850 d.p.m./pmol) for 4 h in a protein-free medium at 37 °C. After thorough washing of the cells, the lipids were extracted from the medium and cells as described in the Materials and methods section, and resolved by t.l.c. The R_f values were: 5-HETE phospholipids, 0.00; 5-HETE acylglycerols, 0.24; cholesteryl 5-HETE, 0.29; 15- and 12-HETE phospholipids, 0.00; 15- and 12-HETE acylglycerols, 0.34; cholesteryl 15- and 12-HETE, 0.49. The data represent means $\pm$ S.E.M. for five dishes from two separate experiments. ▨, control macrophages; ▩, cholesterol-rich macrophages; * P < 0.001 versus control macrophages.

Fig. 2. Effect of 12-HETE concentration on the synthesis of cholesteryl [3 H]12-HETE by macrophage membranes

ACAT activity was assayed in total membranes prepared from cholesterol-rich macrophages. The complete assay system consisted of 2 mM-ATP, 2 mM-CoA, 2 mM-MgCl₂, 31.2 μ M-bovine serum albumin, 0.02 unit of acyl-CoA synthetase and 31.2 μ M-[3 H]12-HETE (sp. radioactivity 90 d.p.m./pmol) in a total volume of 0.2 ml. The mixture was incubated for 1 h at 37 °C. Cholesteryl [3 H]12-HETE was isolated by t.l.c. as described in the Materials and methods section. The amount of 12-HETE added to the assays was varied as indicated. Each value is an average of duplicate assays.

parations from cholesterol-rich macrophages. The complete assay system contained 2 mM-ATP, 2 mM-CoA, 2 mM-MgCl₂, 31.2 μ M-bovine serum albumin, 0.02 unit of acyl-CoA synthetase, 31.2 μ M-[3 H]12-HETE (90 d.p.m./pmol) and 0.18 mg of membrane protein in a total volume of 0.2 ml. The rate of formation of cholesteryl [3 H]12-HETE in the complete system was 235 pmol/h per mg. To determine cofactor requirements for the assay, enzyme activities without ATP or CoA were 4.1 ± 0.4 and 5.1 ± 0.2 pmol/h per mg (P < 0.001) respectively. In the absence of exogenously added acyl-CoA synthetase, 105 ± 14 pmol of cholesteryl [3 H]12-HETE/h per mg was formed, suggesting the presence of this enzyme in the macrophage membranes. In the complete assay system, the synthesis of cholesteryl [3 H]12-HETE was linear with time for 60 min and up to 0.6 mg of membrane protein per assay.

Fig. 2 shows a substrate-concentration experiment for 12-HETE in which the fatty acid/albumin ratio remained constant. Maximal rates of cholesteryl 12-HETE formation were observed at a fatty acid concentration of 35 μ M.

Additional experiments were performed with macrophage membranes which were first preincubated with [14 C]cholesterol (Goodman *et al.*, 1964; Lichtenstein & Brecher, 1980). The labelled cholesterol was added to macrophage membranes in a small amount of acetone and allowed to incubate for 30 min at 37 °C before addition of 30 μ M unlabelled 12-HETE and the necessary cofactors to initiate the reaction. The characteristics of the incorporation of [14 C]cholesterol into [14 C]cholesteryl 12-HETE were similar to the characteristics of the

Fig. 3. Effect of different fatty acids on ACAT activity

The conditions of the assay are the same as described in Fig. 2 except that the following fatty acids were added at a concentration of $31.25 \mu\text{M}$: C_{18:1} (60 d.p.m./pmol), C_{18:2} (47 d.p.m./pmol), C_{20:4} (75 d.p.m./pmol), 12-HETE (55 d.p.m./pmol), 5-HETE (60 d.p.m./pmol), 15-HETE (67 d.p.m./pmol). The data represent averages of duplicate assays.

Fig. 4. Effect of 25-hydroxycholesterol, progesterone and 58-035 on the synthesis of cholesteryl [^3H]12-HETE by macrophage membranes

The assay conditions are the same as those described in Fig. 2, except that the macrophage membranes were preincubated for 30 min with $250 \mu\text{M}$ -25-hydroxycholesterol, $100 \mu\text{M}$ -progesterone or $10 \mu\text{g}$ of 58-035 before adding the complete assay system. The data represent means $\pm$ S.E.M. for five assays from two different membrane preparations: * $P < 0.05$ versus control; ** $P < 0.001$ versus control.

Fig. 5. Net loss of [^3H]12-HETE from cholesteryl esters and loss of cholesteryl ester mass in cholesterol-rich macrophages

Macrophages were incubated with $1 \mu\text{M}$ -[^3H]12-HETE (11 d.p.m./pmol) for 4 h in protein-free medium. The medium was then removed and the cells were washed six times. A batch of dishes were harvested immediately to determine the amount of label incorporated into cellular lipids and to measure cholesteryl ester mass, i.e. zero time point. The other dishes were incubated for up to 36 h in 1 ml of medium with (■) or without (●) 10% fetal-calf serum. At the time indicated, the medium was collected, and the lipids were extracted and analysed by h.p.l.c. as described in the Materials and methods section. The cell lipids were extracted and separated by t.l.c. as described in the Materials and methods section. The zero-time value is an average of four dishes. The other time points represent averages of duplicate dishes.

incorporation of labelled 12-HETE into cholesteryl 12-HETE (results not shown).

Fatty acid substrate specificity

Fig. 3 shows the rates of formation of the respective cholesteryl esters when different fatty acids at a concentration of $31 \mu\text{M}$ were added as substrates. Of the fatty acids tested, oleic acid was the preferred substrate for ACAT. The rates of formation of cholesteryl esters after adding C_{20:4} and C_{18:2} acids were very similar. Of the three monohydroxyicosatetraenoic acids, 12-HETE was the best substrate, followed by 15-HETE and then 5-HETE.

Regulating cholesteryl 12-HETE formation

Macrophage membranes from cholesterol-rich cells were preincubated for 30 min with 250 μ M-25-hydroxycholesterol, 100 μ M-progesterone or 10 μ g of 58-035. The reaction was initiated by adding the complete assay system and [3 H]12-HETE. Fig. 4 shows these results. Compared with control membranes, 25-hydroxycholesterol caused a 40% increase in the rate of synthesis of cholesteryl 12-HETE. In contrast, the ACAT inhibitors progesterone and 58-035 resulted respectively in a 60% and 90% decrease in the rates of formation of cholesteryl 12-HETE.

[3 H]12-HETE incorporation into cellular lipids and its release into the medium by cholesterol-rich macrophages

The esterification of lipoxygenase products to cholesterol through the ACAT pathway could be a mechanism by which the cholesterol-enriched macrophage diverts these more polar fatty acids away from the membrane and allows the cell to store these potentially harmful fatty acids. To investigate this, cholesterol-rich macrophages were prelabelled with [3 H]12-HETE and the turnover of this label in intracellular cholesteryl esters, triacylglycerol and phospholipids was determined. Fig. 5 shows the loss of the 12-HETE label from cellular

Fig. 6. Net loss of [3 H]12-HETE from triacylglycerols and phospholipids in cholesterol-rich macrophages

The experimental conditions are the same as those described in Fig. 5: ■, with fetal-calf serum; ●, without fetal-calf serum.

cholesteryl esters (a) as well as the loss of cholesteryl ester mass (b) in the presence and absence of a cholesterol acceptor in the medium, 10% (v/v) fetal-calf serum. The presence of the cholesterol acceptor resulted in an increase in the rate of disappearance of cholesteryl ester mass and labelled cholesteryl 12-HETE from the cell. The rate of loss of [^3H]12-HETE from cholesteryl esters was significantly more than the rate of loss of the cholesteryl ester mass. By 12 h, more than 90% of the 12-HETE label was lost from cholesteryl esters, whereas cholesteryl ester mass only decreased by 33% in the presence of a cholesterol acceptor. Fig. 6 shows the rate of turnover of the 12-HETE label from triacylglycerols and phospholipids and its appearance in the medium as native 12-HETE or its more polar products (Mathur & Field, 1987; Pawlowski *et al.*, 1982). The disappearance of the label from phospholipids was very rapid, similar to what was observed in labelled cholesteryl esters. There was also significant release of the 12-HETE label from triacylglycerol, although the rate appeared to be somewhat slower than that observed for cholesteryl esters and phospholipids. The amount of label appearing in the medium mirrored the loss of the label from the cellular lipids. In the presence of 10% fetal-calf serum, signifi-

cantly more of the fatty acid that was released was native 12-HETE.

Fatty acids esterified to cholesterol undergo continued hydrolysis and re-esterification. To investigate if cholesteryl monohydroxy fatty acid esters, particularly cholesteryl 12-HETE, were handled differently from the more commonly observed cholesteryl esters, i.e. cholesteryl oleate and arachidonate, by cholesterol-rich macrophages, the release of these fatty acids from cholesteryl esters was determined in the presence of 10% fetal-calf serum as a cholesterol acceptor. Fig. 7 shows these data. The lipoxygenase products 5-, 15- and 12-HETE which were esterified to cholesterol disappeared significantly faster from this lipid fraction compared with labelled cholesteryl arachidonate. Cholesteryl oleate disappeared rapidly also, but, like cholesteryl arachidonate, it was conserved within the cell. By 6 h, 74% of 5-HETE, 85% of 15-HETE and 91% of 12-HETE were lost from cholesteryl esters, compared with 56% and 27% for oleate and arachidonate respectively.

The loss of each of these fatty acids from cellular triacylglycerols did not differ substantially. In contrast, 12- and 15-monohydroxyeicosatetraenoic acids were lost very rapidly from phospholipids compared with the

Fig. 7. Net loss of radiolabelled fatty acids from cholesteryl esters, triacylglycerols and phospholipids in cholesterol-rich macrophages

Macrophages were incubated with $1\ \mu\text{M}$ of the ^3H -labelled fatty acids for 4 h at 37°C in protein-free medium. At the end of 4 h 206 pmol of 12-HETE, 140 pmol of 15-HETE, 332 pmol of 5-HETE, 213 pmol of $\text{C}_{18:1}$ acid and 264 pmol of $\text{C}_{20:4}$ acid were incorporated into total cellular lipids. The cells were washed extensively, and a batch of cells was harvested immediately to determine the amount of radiolabel in the cellular lipids (represents zero time). The rest of the dishes were incubated for up to 24 h with 1 ml of medium containing 10% fetal-calf serum. At the times indicated, the medium was collected and the lipids were extracted and analysed by h.p.l.c. as described in the Materials and methods section. The cell lipids were extracted and separated by t.l.c. Each value represents the mean of three dishes at each time point. Fatty acid: *, 12-HETE; $\circ$, 15-HETE; $\square$, 5-HETE; $\triangle$, $\text{C}_{18:1}$; $\blacksquare$, $\text{C}_{20:4}$.

other fatty acids. 5-HETE, oleic acid and arachidonic acid were conserved in this lipid class. In data not shown, the appearance of the fatty acid labels in the medium mirrored the disappearance of the label from the cell.

DISCUSSION

This study clearly demonstrates an ACAT-dependent esterification of the lipoxygenase products 5-, 15- and 12-HETE to cholesterol. The assay conditions, which utilized an acyl-CoA-generation system, were adapted from the assay described for ACAT by Goodman *et al.* (1964) in rat liver. Similarly to their observations with other fatty acids as substrates, essentially no cholesteryl 12-HETE was generated in the absence of CoA or ATP. This verified the requirement of the fatty acid to be first 'activated' to its CoA derivative before esterification, a requirement necessary for a fatty acid to be utilized as substrate by ACAT. Unfortunately, the rate of cholesterol esterification as determined by this assay does not necessarily reflect the activity of ACAT alone. It is also dependent on the activity of fatty acyl-CoA synthetase. In order to compare the rates of cholesterol esterification among the fatty acids used in this study, it has to be assumed that each fatty acid is an equally good substrate for this synthetase. Despite this shortcoming of the assay, it is a valid method for measuring CoA-dependent cholesterol esterification and agrees favourably with the more popular method which uses a commercially available labelled fatty acyl-CoA substrate (Goodman *et al.*, 1964; Brown *et al.*, 1979; Lichtenstein & Brecher, 1980). The assay was also validated in the present study by utilizing endogenous membrane cholesterol as the labelled substrate (Goodman *et al.*, 1964; Lichtenstein & Brecher, 1980).

In macrophages incubated with acetyl-LDL, ACAT activities were significantly higher than those in unmodified cells. It has been shown that there is a massive accumulation of cholesteryl esters within these cells as more and more of the incoming cholesterol is re-esterified (Brown & Goldstein, 1983). A large expansion of the cholesterol substrate pool for the ACAT enzyme is thought to be the primary mechanism for the up-regulation of ACAT activity (Field & Mathur, 1983b; Hashimoto *et al.*, 1983). Although membrane cholesterol is not the only regulator of ACAT activity, it is certainly an important and potent one. The significant increases which were observed in the rates of esterification of cholesterol to 5-, 15- and 12-HETEs in membranes prepared from cholesterol-enriched macrophages were likely to be secondary to an increase in ACAT activity from the expansion of the membrane cholesterol pool.

Not all fatty acids are equal substrates for ACAT. Within cells, oleic acid is the fatty acid which is utilized most for esterification to cholesterol (Brown & Goldstein, 1983; Yla-Herttuala, 1986). This fatty acid also gives the highest ACAT activity in assays performed *in vitro* with microsomal preparations from liver or intestine (Goodman *et al.*, 1964; Haugen & Norum, 1976). In the present study, a change in chain length or a change in the number of double bonds within a fatty acid only modestly altered ACAT activity. The addition of an hydroxyl group, as in 12-HETE, however, significantly decreased ACAT activity as compared with ACAT activity with a fatty acid such as C_{20:4} with comparable chain length

and saturation. The data therefore suggest that the monohydroxyeicosatetraenoic acids are poorer substrates than the non-hydroxylated fatty acids. Unlike small modifications of the sterol nucleus, which can radically alter its specificity for ACAT, hydroxylation of a fatty acid, which could be considered a major modification, does not result in the same degree of change in ACAT activity as that observed with sterol modifications (Field & Mathur, 1983a; Tavani *et al.*, 1982).

Agents which are commonly used to regulate ACAT activity are 25-hydroxycholesterol, progesterone and the Sandoz compound 58-035 (Lichtenstein & Brecher, 1980; Ross *et al.*, 1984). All three compounds are thought to regulate ACAT activity by a different mechanism. 25-Hydroxycholesterol is a potent stimulator of ACAT activity, whereas progesterone and 58-035 inhibit it. The observation that these compounds regulated the esterification of 12-HETE to cholesterol in a predictable manner is strong supportive evidence that ACAT is responsible for catalysing this reaction.

Diverting lipoxygenase products through the ACAT pathway can be viewed as a mechanism by the macrophage-derived foam cell to store the fatty acids and to divert them away from membrane phospholipids which could potentially cause harmful lipid modifications of the membrane. The data suggest, however, that channelling the monohydroxy fatty acids into cholesteryl esters or even triacylglycerols, which are stored in cytoplasmic lipid droplets, do not result in long-term storage of the fatty acids within the cell. The monohydroxy fatty acids that were esterified to cholesterol were actually hydrolysed faster than the more conventional fatty acids, oleate and arachidonate. The presence of the hydroxyl group did not preclude them from undergoing a continuous cycle of esterification and rehydrolysis. From this standpoint, these fatty acids were not treated differently by the cholesterol-rich macrophage. Interestingly, arachidonic acid, the parent compound for the lipoxygenase products, appeared to be conserved in the cellular lipids, more so than the monohydroxy fatty acids (Fig. 7).

This is the first description of an ACAT-catalysed esterification of products derived from the lipoxygenase pathway of arachidonic acid metabolism to cholesterol. Cholesteryl 5-, 15- and 12-HETEs are products that are synthesized by macrophages enriched in cholesterol after incubation with acetyl-LDL. These lipoxygenase products are diverted not only into cholesteryl esters but also into triacylglycerol and phospholipids, which provide transient storage for the monohydroxy fatty acids and serve as sustained sources of the fatty acids for release into the pericellular space. The macrophage-derived foam cell in an atherosclerotic plaque therefore has a means of regulating the amount of lipoxygenase products in the sub-endothelial space. ACAT, by catalysing the esterification of the lipoxygenase products to cholesterol, may play a significant role in this regulation.

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