Novel Effects of the Acyl-Coenzyme A:Cholesterol Acyltransferase Inhibitor 58-035 on Foam Cell Development in Primary Human Monocyte–Derived Macrophages

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Abstract—We examined the effect of acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitors on intracellular cholesterol stores in primary human monocyte–derived macrophages (HMMs) during foam cell formation. HMMs were exposed to acetylated low density lipoprotein (acLDL, 500 μg protein per mL) with or without 58-035 (1 to 10 μg/mL) or CI-976 (2 μg/mL) for 2 to 48 hours. Total cholesterol (TC) and esterified cholesterol (EC) mass was significantly lower while unesterified cholesterol (UC) increased slightly in cells incubated with acLDL plus ACAT inhibitors. Sterol mass was also measured in cells coincubated with acLDL (500 μg protein per mL) with or without 58-035 (2 μg/mL), high density lipoprotein (HDL, 400 μg protein per mL), or HDL+58-035 for 48 hours. TC and EC were 23% and 55% lower, respectively (P<0.0004), while UC was 11% higher (P<0.04) in cells incubated with acLDL plus 58-035. In contrast, coincubation with HDL alone did not significantly affect TC, EC, or UC mass compared with acLDL alone. The effect of 58-035 could not be explained by cytotoxicity, because adenine release, secreted lactate dehydrogenase, glucose utilization, and cell protein were similar in cells exposed to acLDL regardless of the presence of 58-035. We investigated several potential mechanisms for the decreased TC mass, including increased UC efflux and decreased acLDL binding and uptake. Efflux was measured in cells exposed to [1,2-3H]cholesteryl oleate–labeled acLDL, unlabeled control acLDL, and native untreated acLDL (500 μg protein per mL) with or without 58-035 (5 μg/mL) for 24 or 48 hours. UC efflux increased in a time-dependent manner from cells exposed to acLDL plus 58-035 compared with cells exposed to acLDL alone (P<0.04). High-affinity binding was measured in cells exposed to 125I-acLDL (5 μg protein per mL) with or without excess unlabeled acLDL (100 or 500 μg protein per mL) for 4 hours at 4°C. Specific acLDL binding, uptake, and total degradation were significantly lower when 58-035 was present during cholesterol enrichment compared with cells exposed to acLDL alone (P<0.001). Unlike the effects of ACAT inhibitors on foam cell formation in rodent macrophages, these compounds lowered TC accumulation in HMMs during foam cell formation by limiting the uptake of acLDL and enhancing UC efflux. They may offer promise as drug therapies for atherosclerosis. (Arterioscler Thromb Vasc Biol. 1999;19:2199-2206.)

Key Words: atherosclerosis ■ cholesterol efflux ■ modified lipoproteins ■ human macrophages ■ foam cells

The predominant cells in atherosclerotic lesions include cholesteryl ester (CE)–enriched monocyte-derived macrophages and smooth muscle cells (foam cells).1 Brown et al2 showed that modified LDLs induced foam cell formation in mouse peritoneal macrophages. Modified LDL, once internalized, is hydrolyzed in lysosomes and generates unesterified cholesterol (UC), which is then reesterified to CEs in the cytoplasm by acyl-coenzyme A:cholesterol acyltransferase (ACAT).2 CE is hydrolyzed by neutral CE hydrolase, and the cycle of reesterification and hydrolysis continues unless ACAT is chemically inhibited or a UC acceptor (ie, HDL) is present.2 Others have since shown that in mouse peritoneal macrophages, excess UC initially accumulates within the plasma membrane and after exceeding a critical threshold, is esterified by ACAT.3 Thus, ACAT and neutral CE hydrolase regulate the formation of CE within these cells.

The role of ACAT in the pathogenesis of atherosclerosis is an important one. Knowledge of ACAT’s critical role in foam cell formation has led to the development of numerous inhibitors of this enzyme.4 These compounds have been investigated in a variety of in vivo and in vitro models of atherosclerosis and have generally been shown to reduce this process. In a study of New Zealand White rabbits fed a high-cholesterol, high-fat diet, Bocan et al5 investigated whether the ACAT inhibitor CI-976 would directly reduce atherosclerosis without affecting plasma cholesterol levels. They showed that CI-976 (5 mg/kg) did not significantly reduce plasma cholesterol levels but did...
decrease macrophage foam cell involvement in the il-eofemoral region by 27% to 29%. These authors were unable to assess whether the reduction in macrophage foam cell area was due to a reduction in the number of cells or the extent of CE enrichment. The reduction in atherosclerosis was, however, clearly independent of lowering plasma cholesterol levels.

The effect of ACAT inhibitors during foam cell formation in different tissue-culture models has yielded variable results. Murakami et al.6 reported that rat smooth muscle cells simultaneously incubated with 10% hyperlipidemic serum and the ACAT inhibitor HL-004 had less cholesterol accumulation than did cells exposed to serum alone. In their study, it was not clear whether the reduced cholesterol accumulation occurred as a primary effect of the ACAT inhibitor or secondarily from the presence of HDL in the serum. However, these investigators subsequently reported that rat macrophages accumulated the same amount of cholesterol when incubated with lipoprotein-deficient serum and acLDL in the presence or absence of HL-004.7 Thus, in rat macrophages, the net reduction of cholesterol accumulation did not occur during foam cell formation and ACAT inhibition. Lastly, in studies of mouse peritoneal macrophages, investigators have shown that the ACAT inhibitor octimibate, when present during foam cell formation, actually increased the uptake and degradation of acLDL and the accumulation of cholesterol in these cells.8

ACAT inhibitors have also been studied in cultured primary human monocyte-derived macrophages (HMMs). HMMs can become lipid enriched when incubated with modified LDL9 and have also been shown to release UC in the absence of added cholesterol acceptors in the medium.10 This net cholesterol efflux to serum-free medium may be unique to human macrophages, because it has not been shown to occur in cultured rodent macrophages. In their study of cholesterol efflux to serum-free medium, Zhang et al.11 examined the effect of the ACAT inhibitor 58-035 on HMM foam cells. They found that 58-035, when added after foam cell formation, produced a shift in the cholesterol pools, but cell mass and UC efflux were not significantly different compared with control cells.

In the aforementioned studies, the effect of ACAT inhibitors on cholesterol accumulation in HMMs was measured after the cells had been cholesterol enriched. To our knowledge, the effect of ACAT inhibitors during foam cell formation in HMMs has not been reported. A review of the literature revealed a report by Kogushi et al.12 who examined the effects of the ACAT inhibitor E5324 during foam cell formation in the transformed human THP-1 macrophage. THP-1 macrophages were exposed to acLDL and varying doses of E5324 for up to 6 days. TC and EC masses were significantly reduced while UC was significantly increased when these cells were reincubated in 10% human AB serum for an additional 6 days before use in experiments. We used western analysis for SR type A expression to show that the predominant cell types were macrophages maximally expressing this surface marker by day 10.

Materials

Tissue-culture plates (Falcon Primaria) were purchased from Becton Dickinson. Heat-inactivated, pooled, human type AB serum was purchased from PelleFreeze. RPMI-1640 medium was purchased from Life Technologies, Ficoll-Paque was purchased from Pharmacia Biotech, Inc. NuPAGE (polyacrylamide gel electrophoresis) gradient gels (4% to 12%) were purchased from Novex. Western blots were visualized using a Gibco-BRL Photoblot chemiluminescent kit. [1,2-3H]cholesteryl oleate (55.0 mCi/mmol) was purchased from Amersham Life Sciences, Inc. All other chemicals were reagent grade or higher. Class A scavenger receptor (SR) antiserum was generously provided by Dr T. Kodama (University of Tokyo, Tokyo, Japan). CD36 antiserum was generously provided by Dr John McGregor (INSERM, Lyon, France), and SR BI antiserum was provided by Dr Monty Krieger (Massachusetts Institute of Technology, Cambridge, Mass.). Anti-apoE was purchased from the University of Ottawa Heart Institute (Ontario, Canada). ACAT inhibitors were generously provided by Sandoz Inc, East Hanover, NJ (now Novartis; 58-035) and Parke-Davis, Inc, Ann Arbor, Mich (CI-976).

Cell Culture

HMMs were isolated from lymphocyte preparations obtained from the Johns Hopkins Hemapheresis Center, Baltimore, Md. Lymphocyte preparations were diluted with an equal volume of PBS. This mixture (21 mL) was underlayered with 19 mL of Ficoll-Paque and centrifuged at 500g for 10 minutes, and then the lymphocytes were harvested from the interface. The cells were then plated in 20% heat-inactivated human AB serum, and HMMs were allowed to adhere to the plastic dishes. After 2 hours, nonadherent cells were aspirated and the monolayer washed 4 times with 10% human AB serum. The cells were then maintained in 20% human AB serum for ~4 days, rinsed 4 times with 10% human AB serum, and then reincubated in 10% human AB serum for an additional 6 days before use in experiments. We used western analysis for SR type A expression to show that the predominant cell types were macrophages maximally expressing this surface marker by day 10.

Lipoprotein Isolation

LDL (1.019 to 1.063 g/mL) and HDL (1.063 to 1.210 g/mL) were isolated from healthy male and female donors by using sequential density ultracentrifugation.13 Before ultracentrifugation, 5,5'-dithiobis(2-nitrobenzoic acid) was added to the plasma samples to inhibit lecithin:cholesterol acyltransferase activity.14 Preparations were subjected to a second ultracentrifugation at their respective densities (1.063 g/mL for LDL and 1.210 g/mL for HDL) and then dialyzed extensively in 0.15 mol/L NaCl–0.3 mmol/L EDTA at 4°C before use. HDL preparations were subjected to heparin-Sepharose affinity chromatography to remove apoB- and apoE-containing lipoproteins.15 LDL was acetylated (acLDL) as described by Frankel-Conrat,16 and the completeness of acetylation and purity of the acLDL were assessed by agarose electrophoresis. Complete acetylation of LDL was confirmed by increased migration of the treated preparation compared with native LDL. acLDL was stored at 4°C and used for experiments within 2 months of preparation.

Lipid Enrichment of Cells

Macrophages were CE enriched by exposure to RPMI medium containing acLDL (500 μg protein per mL) with or without the ACAT inhibitor 58-035 (1 to 10 μg/mL) or CI-976 (2 μg/mL) or HDL (400 μg protein/mL) for varying periods of time (2 to 48 hours). In some experiments, acLDL was incubated with UC-phospholipid liposomes (225 μg UC per mL) and BSA (10 mg/mL) overnight at 37°C before use in experiments.17 During the course of these experiments, we found that the addition of liposomes and BSA to the medium containing acLDL did not further increase esterified cholesterol (EC) accumulation in HMMs, and these agents were subsequently omitted from the incubation medium. The concentration of acLDL used in these studies was based on our previous work in human THP-1 macrophages and on the work of other investigators studying foam cell formation in HMMs.18,19 There were differences in

Methods
the absolute values of cholesterol mass accumulation in the different experiments, and we attributed these differences to variability of the acLDL preparations. Dimethyl sulfoxide was the vehicle used to dissolve the ACAT inhibitors, and equal quantities were added to other experimental media (final concentration, 0.1%). Cellular lipids were extracted with hexane/isopropanol (3:2, vol/vol) for 1 hour, and stigmasterol (1 mg/mL) was used as an internal standard. UC and total cholesterol (TC) masses were quantified by gas-liquid chromatography, and EC was calculated as the difference between the 2 measurements. Lipid values were normalized to cell protein as measured by the Markwell modification of the Lowry method.

**Radiolabeling of acLDL:**

[1,2-3H]Cholesterol Labeling

acLDL was radiolabeled with [1,2-3H]cholesteryl oleate by the method described by Faust et al. In brief, 1 mCi of [1,2-3H]cholesteryl oleate was dried under N₂ and then redissolved in 100 μL of dimethyl sulfoxide. acLDL (20 mg protein) was then added to the solution and allowed to incubate for 2 hours at 40°C. Control acLDL (20 mg protein) was similarly treated but without addition of the radiolabeled CE. At the end of the incubation period, the lipoprotein preparations were dialyzed in 0.15 mol/L NaCl–0.3 mmol/L EDTA for 4 exchanges (4 L each) at 4°C. Lipids were extracted from the lipoprotein preparations with chloroform/methanol (1:1, vol/vol), and radiolabeled CE in the acLDL preparation was verified by subjecting an aliquot of the extract to thin-layer chromatography (TLC) in a solvent system of hexane/glacial acetic acid (80:20, vol/vol). Identified UC and EC bands were counted by liquid scintillation spectrometry, and EC was calculated as the difference between the 2 measurements. Lipid values were normalized to cell protein as measured by the Markwell modification of the Lowry method.

**Binding, Uptake, and Degradation Experiments:**

125I-acLDL Binding

Cells were preincubated with RPMI medium containing acLDL (500 μg protein per mL) in the presence or absence of 58-035 for 48 hours. At the end of this period, the cells were rinsed with RPMI medium and then incubated in lipoprotein-deficient serum (5 mg/mL) for 30 minutes at 37°C. The cells were then changed to the same medium containing 125I-acLDL (5 μg/mL) with or without unlabeled acLDL (100 or 500 μg protein per mL) and incubated for 4 hours at 4°C. The medium was discarded and the cells were washed 5 times with PBS containing 0.5% BSA and 3 times with PBS alone. The cells were dissolved in 1.0 mL of 1.0 mol/L NaOH, dried overnight, and resuspended with 1.0 mL of distilled water. Aliquots were taken for the measurement of bound 125I-acLDL and cell protein. Specific binding was calculated as the difference between 125I-acLDL bound in the presence and the absence of unlabeled acLDL.

**Uptake and Degradation**

After the initial 48-hour preincubation period described above, the cells were incubated with 125I-acLDL (5 μg/mL) in the presence or absence of unlabeled acLDL (100 or 500 μg/mL, as indicated) for 6 hours at 37°C. At the end of the incubation period, the cells were cooled to 2°C, and the medium was transferred to 0.1 volume of ice-cold TCA (1 g/mL, wt/vol) and allowed to stand in an ice bath for 18 hours. The precipitated proteins were sedimented by centrifugation at 10,000 g for 10 minutes, and the appearance of TCA-soluble radioactivity was measured in an aliquot of the supernatant. A separate aliquot of the supernatant was diluted to 1.2 mL with distilled water and treated with 30 μL of 40% (wt/vol) KI and 40 μL of 30% H₂O₂ to convert unbound 125I to 125I₂, which was then extracted with chloroform. 125I products were measured in an aliquot of the aqueous phase. The cells were washed 5 times with 0.5% BSA–PBS and 3 times with PBS and dissolved in 1 mL of 1 mol/L NaOH, and an aliquot of the mixture was used to measure cell-associated radioactivity and cell protein as described above. Specific uptake and degradation were calculated as the difference between that which occurred in the presence and the absence of excess unlabeled acLDL.

**Cytotoxicity Assays**

We used 3 measures of cytotoxicity to assess the potential toxicity of 58-035 during the experiments. [U-14C]Adenine Release

The cellular release of radiolabeled adenine was measured and calculated as described by Warner et al. In brief, HMMs were incubated with [1,2-3H]cholesteryl oleate–labeled acLDL, control acLDL, or native, untreated acLDL (500 μg protein per mL) with or without 58-035 (5 μg/mL) for 24 or 48 hours. The purpose of labeling cells with [1,2-3H]cholesteryl oleate was to measure the appearance of UC, generated from the hydrolysis of cholesteryl oleate, into the medium at the various time points. At each time point the medium was collected, centrifuged at 500g to remove floating cells, and then extracted with chloroform/methanol. The extracted lipid pools were dried under N₂, and then redissolved in 1 mL of hexane. An aliquot of 100 μL was measured as total counts by liquid scintillation spectrometry. The remainder of the extract was again dried under N₂ and redissolved in 50 μL of hexane. Aliquots (15 μL) were subjected to TLC with the use of plastic-backed silica G plates in a solvent system of hexane/glacial acetic acid (80:20, vol/vol). Identified UC and EC bands were counted by liquid scintillation. Recovery of labeled cholesterol by TLC was ~94% of the total extracted medium counts. Values of radiolabeled UC were corrected for dilution and normalized to cell protein. Intracellular lipids were extracted as described above.

**Western Blotting**

After incubation with acLDL (500 μg protein per mL) with or without 58-035 (2 μg/mL) for 48 hours, HMMs were rinsed 3 times with cold PBS; solubilized in a buffer containing 50 mmol/L Tris-HCl (pH 6.8), 5% SDS, aprotinin (10 μg/mL), leupeptin (20 μg/mL), pepstatin (10 μg/mL), and PMSF (1 mg/mL); and then boiled for 10 minutes. Samples (20 μg protein per lane) were subjected to NuPAGE (4% to 12%) and then transferred onto polyvinylidene fluoride membranes overnight at 4°C in 10 mmol/L CAPS, pH 10.6. Blots were reacted with either polyclonal anti-SR-A, anti-CD36, or anti-SR-BI at 37°C for 1 hour. Blots were then rinsed 4 times with Tris-buffered saline–Tween 20 (0.05%, vol/vol) and reacted with a biotinylated secondary antibody for an additional hour. Bands were visualized by using a chemiluminescence kit and quantified by densitometric measurement. Transfer efficiency was determined by staining membranes with Ponceau S and gels with Coomassie blue.
Lactate Dehydrogenase (LDH)

We measured LDH released into the medium from cells incubated with acLDL (500 μg protein per mL) with or without 58-035 (5 μg/mL) for 48 hours. Experiments were terminated by removing and centrifuging the medium at 500g to remove floating cells. The medium was stored at −20°C until analyzed for LDH with the use of a clinical chemistry assay. In brief, in the presence of LDH, the reaction measures the equimolar conversion of NAD+ and lactate to pyruvate and NADH. The rate of NADH formation is measured by an increase in absorbance and is directly proportional to enzyme activity.

Glucose Utilization

We examined the effects of a 48-hour preincubation with acLDL and 58-035 on the ability of cells to metabolize glucose during a subsequent 6-hour incubation at 37°C. Cells were preincubated with RPMI containing glucose (2 mg/mL) and acLDL (500 μg protein per mL) with or without 58-035 (2 or 5 μg/mL) for 48 hours. After the preincubation period, the medium was removed and the monolayer rinsed 3 times with RPMI. The cells were then incubated in RPMI medium containing 5, 100, or 500 μg protein/mL acLDL but no 58-035 for an additional 6 hours at 37°C. At the end of the incubation period, an aliquot of the medium was removed and diluted with an equal volume of distilled water, and glucose was analyzed on a Hitachi 740 clinical chemistry analyzer (Boehringer-Mannheim Diagnostics) using an enzymatic method supplied by the same manufacturer (catalog No. 704035). In this method, glucose is converted to glucose-6-phosphate in an ATP-requiring reaction catalyzed by hexokinase. Glucose-6-phosphate is then oxidized in the presence of glucose-6-phosphate dehydrogenase and NADP to produce gluconate-6-phosphate and NADPH+H+. Glucose utilization was calculated as the difference between that present in the medium at the beginning minus that at end of the 6-hour incubation period.

Statistical Analysis

Unpaired Student’s t tests were used to compare group means. A value of P<0.05 was considered statistically significant.

Results

The purpose of the present studies was to examine the effect of ACAT inhibition on TC mass accumulation in HMMs. In initial experiments, HMMs were incubated in acLDL (500 μg protein per mL) complexed with liposomes (225 μg free cholesterol per mL) and BSA (10 mg/mL) or the same medium plus 58-035 (2 μg/mL) for 48 hours. The results from 3 independent experiments are shown in Figure 1A. TC and EC masses were 42% (P<0.0003) and 91% (P<0.0001) lower, respectively, in cells exposed to acLDL plus 58-035 compared with acLDL alone. UC mass did not change significantly in cells exposed to acLDL plus 58-035 compared with acLDL alone. Similar results were obtained when HMMs were exposed to acLDL plus CI-976 (2 μg/mL) for 48 hours (Figure 1B). TC mass was 52% lower (P<0.0001), EC was 87% lower (P<0.0001), and UC was not significantly different in cells exposed to acLDL plus CI-976 compared with acLDL alone. Thus, in HMMs exposed to acLDL plus ACAT inhibitors, TC and EC mass was lower in the presence of ACAT inhibition. It should also be noted that the cellular accumulation of cholesterol in the control condition in Figure 1B was similar to that in Figure 1A despite the omission of liposomes and BSA from the medium. For this reason, liposomes and BSA were omitted from subsequent experiments.

We next measured the dose response of 58-035 in lowering TC and EC mass. HMMs were incubated with RPMI medium containing acLDL (500 μg protein per mL) and 58-035 (0 to 10 μg/mL) for 48 hours. As shown in Figure 2, the effect of 58-035 in lowering TC and EC mass was dose dependent and was maximal at 2 μg/mL. We then measured the time dependence of 58-035 on TC and EC mass accumulation. HMMs were exposed to control RPMI medium, acLDL (500 μg protein per mL), 58-035 (2 μg/mL), or acLDL plus 58-035 for varying periods of time (2 to 48 hours). The data shown in Figure 3 are representative of 2 independent experiments. In Figure 3A, the accumulation of cholesterol induced by acLDL increased at about the same rate for the
Sterol accumulation in HMMs exposed to acLDL (500 μg protein per mL)±58-035 (5 μg/mL) for various periods of time (0 to 48 hours). Results are the mean±SE of triplicate wells and are representative of 2 independent experiments. A, TC (μg/mg cell protein); B, UC; and C, EC. *P<0.01, **P<0.001, ***P<0.009 compared with acLDL alone. Error bars not shown are within the symbols.

Figure 3. Sterol accumulation in HMMs exposed to acLDL (500 μg protein per mL)±58-035 (5 μg/mL) for various periods of time (0 to 48 hours). Results are the mean±SE of triplicate wells and are representative of 2 independent experiments. A, TC (g protein per mL); B, UC; and C, EC. *P<0.01, **P<0.001, ***P<0.009 compared with acLDL alone. Error bars not shown are within the symbols.

Figure 4. Sterol accumulation in HMMs exposed to RPMI medium containing acLDL (500 μg protein per mL)±HDL (400 μg protein per mL)±58-035 (2 μg/mL)±58-035 for 48 hours. Results are the mean±SE of 4 independent experiments, each done in triplicate. *P<0.001, **P<0.03, ***P<0.0004 compared with acLDL alone.

It has been proposed that the cardioprotective effect of HDL resides in its ability to retard cholesterol accumulation in macrophages. In our next series of experiments, we compared the effect of HDL versus 58-035 on TC mass accumulation during the CE enrichment period. HMMs were exposed to acLDL (500 μg protein per mL), with or without 58-035 (2 μg/mL), HDL (400 μg protein per mL), or HDL plus 58-035 for 48 hours. Cellular cholesterol mass results are shown in Figure 4, and are the mean±SE of 4 independent experiments. TC and EC masses were 23% lower (P<0.0004) and 55% lower (P<0.00002), respectively, and UC mass 11% higher (P<0.04) in macrophages incubated with acLDL with 58-035 compared with control. By comparison, HDL coincubated with acLDL did not significantly affect TC, EC, or UC mass compared with control. The results suggested that 58-035 was more effective than HDL in limiting cholesterol accumulation in HMMs.

Under certain experimental conditions, 58-035 may induce cytotoxity as a consequence of an excessive accumulation of UC, as suggested by findings in mouse peritoneal macrophage foam cells. For this reason, we next assessed whether the reduced TC mass during foam cell formation by 58-035 was due to cytotoxity by the inhibitor. HMMs were preincubated with RPMI medium containing [U-14C]adenine for 2 hours and then exposed to RPMI medium or the same medium containing acLDL (500 μg protein per mL), 58-035 (5 μg/mL), or acLDL plus 58-035 for 48 hours. The release of radiolabeled adenine to the medium was measured and the results are shown in Figure 5. There was an increase in adenine release from cells that had been exposed to either acLDL (5-fold, P<0.0003) or acLDL plus 58-035 (6-fold) compared with control (P<0.0004). However, there was no significant increase in adenine release from cells exposed to 58-035 alone. Additionally, there was no significant increase in adenine release from cells exposed to 58-035 compared with 58-035 compared with acLDL alone. We also measured release of LDH as another means of assessing cytotoxity by 58-035. HMMs were exposed to RPMI medium or the same medium containing acLDL (500 μg protein per mL), 58-035 (5 μg/mL), or acLDL plus 58-035 for 48 hours. LDH levels were measured in the medium pooled from replicate dishes (n=6), and the results are shown in Figure 6. LDH levels increased by ~2-fold when cells were exposed to acLDL or 58-035 compared with control. The addition of 58-035 to the medium containing acLDL did not further increase LDH levels. Finally, glucose utilization was measured in cells
preincubated with acLDL with or without 58-035 for 48 hours. This was assessed by measuring the ability of cells to metabolize glucose during the 6-hour incubation period after being preincubated with acLDL (500 μg protein per mL) with or without 58-035 (2 μg/mL) for 48 hours. There were no significant differences in glucose utilization in cells preincubated with acLDL and 58-035 compared with acLDL alone (63.7 versus 63.9 μg/mg cell protein per 6 hours, respectively, data not shown). In a separate experiment in which the cells had been preincubated for 48 hours with a higher concentration of 58-035 (5 μg/mL) during the CE enrichment period, the results were the same. Prior exposure to the ACAT inhibitor for 48 hours did not reduce glucose utilization during the subsequent 6-hour incubation from which 58-035 had been omitted. These findings suggested that the metabolic competence of the cells had not been compromised by the 48-hour preincubation with the ACAT inhibitor, and the reduction of TC mass in cells exposed to 58-035 was not explained by cytotoxicity of the inhibitor.

Figure 5. [U-14C]Adenine release from HMMs exposed to RPMI, 58-035 (5 μg/mL), acLDL (500 μg protein per mL), and acLDL+58-035 for 48 hours. Percent adenine release was calculated as (medium dpm of the treatment group/total cellular dpm at time zero)×100. Results are the mean±SE of 2 independent experiments, each performed in triplicate. *P<0.0004 compared with RPMI. Results of the adenine release from cells exposed to acLDL were not significantly different from cells exposed to acLDL+58-035.

Figure 6. LDH levels from the medium of HMMs exposed to acLDL (500 μg protein per mL)+58-035 (5 μg/mL) for 48 hours (data from 48-hour time point in Figure 3). Results are from pooled media from 6 wells. Cellular lipid mass measurements are from the 48-hour time point shown in Figure 3.

Figure 7. Efflux of radiolabeled UC into the medium from cells exposed to [1,2-3H]cholesteryl oleate–acLDL (500 μg protein per mL)±58-035 (5 μg/mL) for 24 or 48 hours. Results are the mean±SE of media from triplicate wells for the 24-hour time point and the mean±SE of media from 6 wells for the 48-hour time point. *P<0.04 compared with acLDL alone. At the 24-hour time point, TC mass for cells exposed to 1,2-[3H]cholesteryl oleate–acLDL, control acLDL, and native acLDL was 73.1±5, 70.3±2, and 73.3±2 μg/mg cell protein, respectively. TC mass for cells exposed to the 3 acLDL preparations+58-035 was 54.5±1, 59.1±3, and 60.1±3 μg/mg cell protein, respectively. At the 48-hour time point, TC mass for cells exposed to [1,2-3H]cholesteryl oleate–acLDL, control acLDL, and native acLDL was 109.2±5, 112.7±4, and 106.6±4 μg/mg cell protein, respectively. TC mass in the presence of 58-035 was 72.9±9, 81.8±4, and 69.3±4 μg/mg cell protein, respectively.

We considered that the lower TC mass accumulation in cells exposed to acLDL plus 58-035 might have occurred secondarily to the effects of ACAT inhibition and the availability of increased UC for efflux. Therefore, cholesterol efflux was measured in cells incubated with [1,2-3H]cholesteryl oleate–labeled acLDL (500 μg protein per mL) in the presence or absence of 58-035 (5 μg/mL) for various periods of time (24 or 48 hours). After each time point the medium was collected and centrifuged at 500g to pellet the floating cells. The appearance of [1,2-3H]UC in aliquots of the medium was measured and is shown in Figure 7. By 48 hours, UC efflux from cells exposed to acLDL and 58-035 increased significantly compared with acLDL alone (P<0.04). As indicated in the legend to Figure 7, TC mass was =35% lower in cells exposed to acLDL plus 58-035. The radiolabeled acLDL had similar effects on foam cell formation as did unlabeled control and native untreated acLDL.

Another possible explanation for the effects of 58-035 on TC mass accumulation in cells exposed to acLDL could be that the compound may have operated, at least in part, to regulate acLDL uptake and limit foam cell formation. For this reason, we next examined the effect of 58-035 on high-affinity binding, uptake, and degradation of acLDL in HMMs preincubated with acLDL (500 μg protein per mL) with or without 58-035 (5 μg/mL) for 48 hours. High-affinity binding was measured by incubating cells with 125I-acLDL (5 μg protein per mL) with or without excess unlabeled acLDL (500 μg protein per mL) for 4 hours at 4°C. Uptake and degradation were measured in cells exposed to 125I-acLDL (5 μg protein per mL) with or without excess unlabeled acLDL (500 μg protein per mL) for 6 hours at 37°C. The results of observations in 3 different HMM
preparations are shown in the Table. The data in this table are given in terms of specific binding, uptake, and degradation. In a preliminary experiment, cells were preincubated with acLDL alone for 48 hours, and then 58-035 was added to the medium during the binding, uptake, and degradation assays. Binding at 4°C and uptake and degradation at 37°C were unchanged in the presence of 58-035 (data not shown). In subsequent experiments, the ACAT inhibitor was omitted from the binding, uptake, and degradation assays. In contrast, when cells were preincubated with acLDL plus 58-035 (2 or 5 μg/mL) for 48 hours, there was a decrease of 30% in the specific binding at 4°C (the Table, experiments 1 and 3; P<0.001). Similarly, the cellular uptake of 125I-acLDL at 37°C, which reflects binding plus internalization of the labeled lipoprotein, was reduced by ~25% (Table, experiments 2 and 3; P<0.001). 125I-acLDL degradation was also 1/3 lower than when 58-035 was present during foam cell formation (the Table, experiments 2 and 3).

Overall, the findings indicated that when 58-035 was added during the time course of the binding assay, it had no direct inhibitory effect on acLDL binding, but when present during foam cell formation, this ACAT inhibitor produced a significant decrease in the ability of cells to bind acLDL, probably accounting for the observed reduction in acLDL uptake and degradation. Thus, the presence of 58-035 during foam cell formation appeared to limit acLDL uptake.

In the aggregate, the results from the above experiments suggested that 58-035 may have regulatory effects on SR expression. To further explore this possibility, western blotting analysis was performed in HMMs exposed to acLDL (500 μg protein per mL) with or without 58-035 (2 μg/mL) for 48 hours. Cell lysates from pooled triplicate dishes were subjected to SDS-PAGE, and membranes were reacted with polyclonal antibodies to several known SRs (SR-A, SR-BI, and CD36). Within the limits of this semiquantitative assay, the expression of SR-A, SR-BI, and CD36 was the same in cells exposed to acLDL plus 58-035 compared with acLDL alone (data not shown).

Discussion

Our results have indicated that ACAT inhibitors exert multiple effects on HMMs during foam cell formation. In our studies, HMMs were exposed to acLDL in the presence and absence of the ACAT inhibitor 58-035 or CI-976 under various conditions. No known cholesterol acceptors (ie, serum, HDL, apoA1/phospholipid particles) were present during the incubation periods. TC mass showed a time- and dose-dependent lowering when cells were simultaneously incubated with acLDL plus 58-035. The reduction in TC mass by 58-035 was due to 2 effects: enhanced UC efflux and a reduction of high-affinity binding, uptake, and degradation of acLDL. Our finding of a reduction in TC mass and binding, uptake, and degradation of acLDL in HMMs was not correlated with changes in the protein expression of known SRs and was not due to increased cytotoxicity from the ACAT inhibitor.

Warner et al26 reported that cell toxicity was induced in mouse peritoneal macrophage foam cells that had been incubated with medium containing the ACAT inhibitor CP-113,818 (2 μg/mL) for varying time periods (6 to 36 hours). The increased release of [3H]adenine was correlated with the accumulation of UC.26 These authors concluded that excessive accumulation of UC was cytotoxic and was overcome by the presence of acceptors in the medium.26 In general, the effects of ACAT inhibitors on intracellular cholesterol metabolism and metabolic competence have generally been studied after macrophages were induced to foam cell formation. In our experimental model, ACAT inhibitors were added during foam cell formation with acLDL and did not show increased cytotoxicity compared with acLDL alone. UC mass did not significantly accumulate in response to ACAT inhibition during foam cell formation and thus, may explain the lack of increased cytotoxicity.

58-035 exerted multiple effects on intracellular cholesterol accumulation, including increased UC efflux. One plausible mechanism for the general lack of increased UC mass in HMM cells exposed to acLDL plus 58-035 compared with published reports for rodent macrophages is that secreted apoE may have acted as an acceptor of UC. ApoE secreted in the medium was measured by ELISA and was not found to be significantly different in cells exposed to acLDL plus 58-035 compared with acLDL alone (data not shown). We believe that the most likely explanation for this negative result was the fact that acLDL contained apoE (data not shown) and interfered with our ability to detect secreted apoE in the medium.

The presence of 58-035 during foam cell development in HMMs decreased the binding, uptake, and degradation of acLDL by ~30%. Our observations of reduced acLDL binding, uptake, and degradation by 58-035 were somewhat similar to those in J774 murine macrophages. White et al28 examined 125I-LDL metabolism in J774 murine macrophages preincubated for varying periods of time (0 to 24 hours) with the ACAT inhibitor cyclandelate. They showed that the effects of ACAT inhibition were time dependent, with 125I-LDL binding and degradation inhibited in the cells after 8 hours of incubation with the drug. These authors suggested that the drug acted indirectly by reducing LDL receptor number.
Given that high-affinity 125I-acLDL binding decreased in HMMs exposed to the combination of acLDL and 58-035, we also examined the possible decreased expression of the known SR proteins SR-A, SR-BI, and CD36. Western blot analysis failed to show significant differences in cells exposed to acLDL plus 58-035 compared with acLDL alone, suggesting that 58-035 did not affect expression of these proteins. It may be that western blotting was not sufficiently sensitive to detect a 30% reduction in the expression of a single receptor or of smaller reductions of several receptors. Alternatively, as suggested by Scheithle et al., there may exist another uncharacterized receptor that specifically binds acLDL in human macrophages.

In summary, ACAT inhibitors exerted multiple effects on TC accumulation in HMMs. UC efflux increased over time and may have been due to the presence of apoE in the medium. A novel finding was the decreased specific binding, and may have been due to the presence of apoE in the medium. 58-035 and CI-976 lowered uptake, and degradation of 125I-acLDL in cells preincubated with acLDL plus 58-035. That 58-035 and CI-976 lowered accumulation in HMMs may suggest a potential benefit of these compounds in the drug therapy of atherosclerosis.

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Novel Effects of the Acyl-Coenzyme A:Cholesterol Acyltransferase Inhibitor 58-035 on Foam Cell Development in Primary Human Monocyte–Derived Macrophages
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