methods

Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness

Aaron T. Lada,* Matthew Davis,* Carol Kent,* James Chapman,† Hiroshi Tomoda,§ Satoshi Ōmura,§ and Lawrence L. Rudel[1,*]

Department of Pathology,* Arteriosclerosis Research Program, Wake Forest University School of Medicine, Winston-Salem, NC; College of Pharmacy,† University of South Carolina, Columbia, SC; and The Kitasato Institute,§ Tokyo, Japan

Abstract **Acyl CoA:cholesterol acyltransferase 1 (ACAT1) and ACAT2 are enzymes responsible for the formation of cholesteryl esters in tissues. While both ACAT1 and ACAT2 are present in the liver and intestine, the cells containing either enzyme within these tissues are distinct, suggesting that ACAT1 and ACAT2 have separate functions. In this study, NBD-cholesterol was used to screen for specific inhibitors of ACAT1 and ACAT2. Incubation of AC29 cells, which do not contain ACAT activity, with NBD-cholesterol showed weak fluorescence when the compound was localized in the membrane. When AC29 cells stably transfected with either ACAT1 or ACAT2 were incubated with NBD-cholesterol, the fluorescent signal localized to the nonpolar core of cytoplasmic lipid droplets was strongly fluorescent and was correlated with two independent measures of ACAT activity. Several compounds were found to have greater inhibitory activity toward ACAT1 than ACAT2, and one compound was identified that specifically inhibits ACAT2. The demonstration of selective inhibition of ACAT1 and ACAT2 provides evidence for uniqueness in structure and function of these two enzymes.** **To the extent that ACAT2 is confined to hepatocytes and enterocytes, the only two cell types that secrete lipoproteins, selective inhibition of ACAT2 may prove to be most beneficial in the reduction of plasma lipoprotein cholesterol concentrations.**—Lada, A. T., M. Davis, C. Kent, J. Chapman, H. Tomoda, S. Ōmura, and L. L. Rudel. **Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness.** ***J. Lipid Res.*** **2004.** 45: **378–386.**

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In tissues, cholesterol is esterified by the enzyme acyl CoA:cholesterol acyltransferase (ACAT). The two identified forms, termed ACAT1 and ACAT2, along with acyl-CoA:diacylglycerol acyltransferase 1, (DGAT1) make up the ACAT gene family (1–5). ACAT1 and ACAT2 are more than 50% similar in primary amino acid sequence; however, significant differences in membrane topology have been identified (6). The two enzymes also differ in their tissue distribution, with ACAT2 protein present only in the liver and intestine, whereas ACAT1 is more ubiquitously expressed (2, 4, 5). Furthermore, in the liver and intestine, where both enzymes are present, the cellular location of the two enzymes is distinct. In nonhuman primates, ACAT2 was found in hepatocytes and ACAT1 was localized to Kupffer cells (7). In the primate intestine, ACAT1 was found in goblet cells, macrophages, and Paneth cells, whereas ACAT2 was localized to the apical third of the mucosal cells (7).

These differences in the cellular localization of ACAT1 and ACAT2 suggest different functions of the two enzymes. ACAT2 is thought to function in intestinal cholesterol absorption and transport in chylomicrons and in providing cholesteryl esters (CEs) for VLDL assembly in the liver. ACAT1 appears to function to maintain free cholesterol (FC) balance in cells that accumulate and store extra cholesterol as CEs. Studies in mice support these roles for ACAT1 and ACAT2. ACAT1-deficient mice lack CEs in macrophages and in the adrenal cortex but have normal cholesterol absorption and hepatic cholesterol esterification (8). ACAT2-deficient mice have reduced cholesterol absorption and were resistant to diet-induced hypercholesterolemia by a high-fat, high-cholesterol diet (9). In mice fed a fat- and cholesterol-enriched diet, ACAT1 deficiency did not protect against atherosclerosis (10, 11), and in one study, ACAT1 deficiency apparently led to increased atherosclerosis, possibly as a result of FC

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Abbreviations: CE, cholesteryl ester; FC, free cholesterol; PPPA, pyripyropene A.

[1] To whom correspondence should be addressed.
e-mail: lrudel@wfubmc.edu

toxicity in macrophages (12). In contrast, in apolipoprotein E-deficient mice, ACAT2 deficiency protected against atherosclerosis (13).

Numerous ACAT inhibitors have been identified. However, the different functions and potential roles in atherosclerosis development suggest a need for specific inhibitors of ACAT2. Current methods for assaying ACAT activity are laborious and time-consuming, involving the use of radioactive substrates in live cells or in incubations of cell homogenates or microsomes with the isolation of the radioactive CE products by TLC and subsequent quantification. To facilitate the identification of potential ACAT-specific inhibitors, we have developed a more rapid and high-throughput cell-based assay. The assay uses 22-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol), a fluorescent sterol analog in which the NBD moiety replaces the terminal segment of the alkyl tail of cholesterol. NBD-cholesterol has been shown to mimic native cholesterol absorption in hamsters (14), intracellular lipid transport (15, 16), and esterification in vivo and by cultured cells (14).

The relative fluorescence of NBD-cholesterol is dependent on its environment. When in a polar environment, NBD-cholesterol is weakly fluorescent, whereas in a nonpolar environment, it is strongly fluorescent (17–19). This property of NBD-cholesterol was used to measure ACAT activity in cells stably expressing ACAT1 or ACAT2. These cells readily form lipid droplets rich in CE, providing the opportunity to compare the effects of potential inhibitors separately on the two enzymes. Identification of inhibitors specific for either enzyme should facilitate the study of ACAT activity in tissues that contain both enzymes and in the longer term will assist in the development of compounds that can be used to selectively inhibit ACAT enzymes in vivo, helping to define the roles of ACAT1 and ACAT2 in atherosclerosis.

METHODS

Cell culture

All cell lines were maintained at 37°C in 5% CO_2 in Ham's F-12 medium supplemented with 1% Eagle's vitamins, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FBS. AC29 cells, a Chinese hamster ovary cell line with no ACAT activity, was a generous gift from T-Y. Chang (Dartmouth College of Medicine, Hanover, NH). For transfections, AC29 cells were grown to 50–75% confluence on 100 mm dishes. Transfections were carried out with FuGENE (Boehringer Mannheim Biochemicals, Indianapolis, IN) and African green monkey ACAT1 or ACAT2 cDNA in pOPSRV1 plasmid (Stratagene, La Jolla, CA) at a 3 μl:1 μg FuGENE/DNA ratio. Cells were selected with geneticin (G418), and monoclonal populations expressing ACAT1 or ACAT2 were isolated. AC29 cells were also transfected with human ACAT1 or ACAT2 in a similar manner.

Fluorescence microscopy

NBD-cholesterol (Molecular Probes, Eugene, OR) was solubilized in ethanol for a stock solution of 1 mg/ml. AC29 cells and AC29 cells stably expressing African green monkey ACAT1 or ACAT2 were seeded onto 35 mm culture dishes. The next day, 2 ml of medium containing 1 μg/ml NBD-cholesterol in ethanol (final ethanol concentration, 0.1%) was added, and cells were incubated for 2 h at 37°C in a CO_2 incubator. To prepare cells for microscopy, dishes were washed three times with PBS and fixed with 1 ml of 3.7% formaldehyde in PBS for 20 min at room temperature. Dishes were washed and drained, and two drops of *p*-phenylenediamine (1 mg/ml) in glycerol was added as a mounting medium to each dish. Cells were examined using a Zeiss Axioplan microscope using a neofluor (numerical aperture, 1.3) oil immersion objective (63×) with green channel filters (488 nm excitation, 540 nm emission).

High-performance TLC

High-performance TLC was used to separate free and esterified NBD-cholesterol. Cellular isopropanol lipid extracts were dried under nitrogen and brought up in 20 μl of chloroform. Ten microliters was spotted onto a 10 × 20 TLC plate (Whatman, Inc., Clifton, NJ) using a Camag Linomat IV plate spotter, and lipids were separated using a 50:50:1 hexane-ether-acetic acid solvent system. Stock NBD-cholesterol was run as a standard to identify the free NBD-cholesterol peak. Plates were scanned in a Camag TLC Scanner II with a mercury lamp set at 469 nm excitation wavelength with a 560 nm filter for emission. Peaks were quantitated using the TLC Evaluation Software CATS version 3.19 from Camag.

Fluorescent ACAT assay

A total of 30,000 cells per well were plated on Falcon 96-well culture plates and allowed to recover overnight. Assays were done with cells at least 80% confluent. Cells were incubated in medium containing 1 μg/ml NBD-cholesterol for 2–6 h as indicated in the figure legends. NBD-cholesterol was added from a 1 mg/ml stock in ethanol, and ethanol concentrations did not exceed 0.1%. AC29 cells incubated with NBD-cholesterol or ACAT-expressing cells incubated with NBD-cholesterol and the ACAT inhibitor CP113 were used to determine background fluorescence attributable to free NBD-cholesterol, as indicated in the figure legends. After incubation, medium was removed, and the cells were washed two times with cold balanced salt solution (BSS). Plates were read

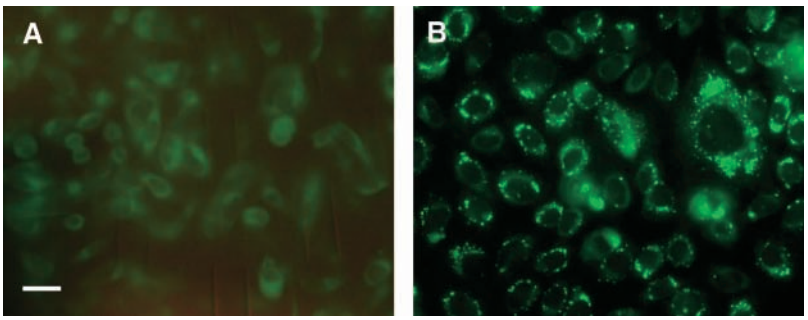

Fig. 1. Fluorescence microscopy after incubation of cells with NBD-cholesterol. AC29 cells (A) and AC29 cells stably transfected with ACAT2 (B) were incubated with 1 μg/ml NBD-cholesterol for 120 min. Cells were fixed with formaldehyde as described in Methods and viewed with green channel filters (488 nm excitation, 540 nm emission) using a 63× oil immersion objective. The bar in A = 9.5 μm.

TABLE 1. Esterification of NBD-cholesterol

Cell Type	Treatment	Free Cholesterol	Cholesteryl Ester
AC29	NBD	617 ± 22	–
	NBD + CP113	513 ± 24	–
ACAT1	NBD	566 ± 96	354 ± 53
	NBD + CP113	575 ± 43	–
ACAT2	NBD	671 ± 40	458 ± 19
	NBD + CP113	598 ± 37	–

AC29, ACAT1, and ACAT2 cells were incubated with 1 μg/ml NBD-cholesterol ± 2 μg/ml CP113 for 2 h. After incubation, cellular lipids were extracted and separated by high-performance TLC. The plates were scanned using a fluorescent plate reader, and peaks corresponding to free NBD-cholesterol and NBD-cholesteryl ester were quantitated as described in Methods. The quantitated values are expressed as arbitrary units. The data are expressed as means ± SD (n = 3).

from the bottom using a Tecan GENios fluorescent plate reader equipped with 485 nm excitation and 535 nm emission filters. After measurement, cellular protein was digested through incubation with 25 μl of 0.4 N NaOH for 2 h. Protein content was determined by the method of Lowry et al. (20). CE fluorescence was calculated by subtracting the background from the total fluorescence, and these values were normalized by cellular protein.

[^{14}C]Oleate incorporation into CEs

To compare measures of ACAT activity using fluorescence and [^{14}C]oleate incorporation into CEs, cells were seeded onto Falcon 12-well culture plates. When confluent, medium containing 1 μg/ml NBD-cholesterol and 1 μCi/ml [^{14}C]oleate was added, and cells were incubated for 6 h. To determine background fluorescence, cells were incubated with 1 μg/ml NBD-cholesterol, 1 μCi/ml [^{14}C]oleate, and 2 μg/ml CP113. Plates were washed two times with cold BSS and read on a fluorescent plate reader as described above. Cellular lipids were extracted through an overnight incubation with 1 ml of isopropanol. The isopropanol lipid extract was dried down under nitrogen, brought up in 50 μl of chloroform, and spotted onto a TLC plate. Lipids were separated using a solvent system of 140:60:2 hexane-ether-acetic acid. The CE band was scraped, and radioactivity was determined by scintillation counting. To solubilize cellular protein, 0.5 ml of 1 N NaOH was added to each well. A 100 μl aliquot was taken for protein determination by the method of Lowry et al. (20). Both fluorescence and radioactivity values were normalized by dividing by cellular protein.

ACAT assay

ACAT activity was assayed in either cell homogenates or microsomes. Microsomes were prepared from liver samples from wild-type, ACAT1$^{-/-}$ (8), and ACAT2$^{-/-}$ (9) mice as previously described (7). To prepare cell homogenates, cells were grown in 35 mm dishes, and when near confluence (>80%), the cell monolayer was scraped, suspended in BSS, and sonicated. An aliquot was taken for protein determination by the method of Lowry et al. (20), and 50 μg of whole cell or microsomal protein was used to determine ACAT activity as previously described (7). Briefly, protein was mixed with 1 mg of BSA and 50 nmol of free cholesterol in 45% (w/v) β-cyclodextrin and incubated for 30 min. Then, 30 nmol of [^{14}C]oleoyl-CoA was added and incubated for 10 min at 37°C in a shaking water bath. The reaction was stopped by the addition of 2:1 chloroform-methanol, phases were split, and an aliquot of the organic phase was subjected to TLC. The CE band was scraped and counted for ^{14}C radioactivity.

Inhibitor studies

AC29 cells stably expressing African green monkey ACAT1 or ACAT2 were used to test the effects of a variety of compounds on the activity of the two enzymes. Four compounds were synthesized by Dr. Chapman at the University of South Carolina, and we term

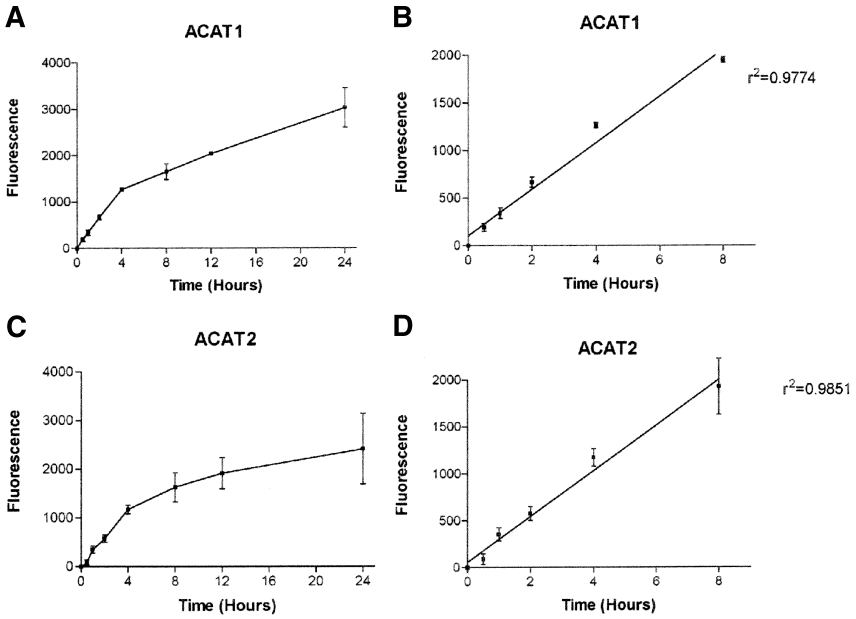

Fig. 2. Increase in cellular fluorescence over time. ACAT1 and ACAT2 cells were incubated with 1 μg/ml NBD-cholesterol for up to 48 h. At the indicated time points, plates were harvested and fluorescence was measured. Background fluorescence was determined from ACAT2 cells incubated with 1μg/ml NBD-cholesterol and 2 μg/ml CP113 and subtracted from each time point. A and C show data from the entire 48 h incubation for ACAT1 and ACAT2, respectively. B and D show data from the first 8 h of incubation for ACAT1 and ACAT2, respectively. Data are expressed as means ± SD (n = 3).

these compounds 1A, 1B, 1C, and 1D. Pyripyropene A (PPPA) was isolated by Dr. Satoshi Omura of the Kitasato Institute (21, 22). ACAT1 and ACAT2 cells were plated on the same 96-well plate and allowed to recover overnight. A stock solution of each compound was made to a concentration of 10 mg/ml in DMSO, and an equal volume of DMSO was added to all cells (0.05% final DMSO concentration). Cells were preincubated for 20 min with each compound over the concentration range 0.0001–20 μg/ml, with three wells for each concentration. After preincubation, the medium was removed and replaced with 100 μl of medium containing 1 μg/ml NBD-cholesterol and the same concentration of the compound as in the preincubation. Cells were incubated for 6 h, and the fluorescence was determined as described above. Three wells each of ACAT1 and ACAT2 cells were incubated in a similar manner in the presence of 2 μg/ml CP113 to determine the background fluorescence for each cell line. Fluorescence values were normalized to cellular protein mass and plotted versus the log of the concentration of compound. Prism 3 software was used for the curve fit of the data using a sigmoidal dose-response curve, and IC_{50} values were calculated from the curve fit.

RESULTS

The localization of the fluorescent signal of NBD-cholesterol was compared in AC29 cells, which do not express ACAT, and AC29 cells stably expressing ACAT2 (ACAT2 cells). Cells were incubated with 1 μg/ml NBD-cholesterol for 120 min and then viewed by fluorescence microscopy. In AC29 cells, the fluorescent signal was weak and diffuse, indicative of membrane localization (**Fig. 1A**), whereas in ACAT2 cells, there was a strong fluorescent signal localized to cellular lipid droplets (Fig. 1B). Similar results were seen with ACAT1 cells (data not shown). These results agree with the known property of NBD-cholesterol to be weakly fluorescent in a more polar environment (cell membrane) and strongly fluorescent in a nonpolar environment (neutral lipid droplet).

To demonstrate that NBD-cholesterol is esterified by ACAT, AC29, ACAT1, and ACAT2 cells were incubated with 1 μg/ml NBD-cholesterol with or without the ACAT inhibitor CP113. In preliminary studies, it was determined that 1 μg/ml NBD-cholesterol produced maximum fluorescence in ACAT-expressing cells (data not shown). After incubation, cellular lipids were extracted and separated by high-performance TLC, and fluorescent bands were scanned and quantified. In AC29 cells, only one fluorescent band was visible, corresponding to free NBD-cholesterol (**Table 1**). When ACAT1 and ACAT2 cells were incubated with NBD-cholesterol, two distinct peaks were visible, corresponding to free and esterified NBD-cholesterol, indicating that the fluorescent compound was esterified by these cells. The addition of the ACAT inhibitor CP113 eliminated the esterified NBD-cholesterol band, further demonstrating that it was a product of ACAT activity.

Preliminary experiments were carried out to determine the background fluorescence in cells attributable to free NBD-cholesterol. The total fluorescence within cells is the sum of free and esterified NBD-cholesterol, and the fluorescence attributable to esterified NBD-cholesterol can be calculated by subtracting the background fluorescence from the total fluorescence. Two methods to determine the background fluorescence were compared. NBD-cholesterol was incubated with AC29 cells (with no ACAT activity) or with ACAT2 cells and the ACAT inhibitor CP113. Similar low amounts of fluorescence were obtained using the two methods (data not shown). In addition, the low fluorescence levels in AC29 cells were the same with or without the addition of CP113, demonstrating no measurable ACAT activity in these cells (data not shown). Therefore, either cells that do not express ACAT (AC29 cells) or cells stably transfected with ACAT after treatment with the ACAT inhibitor CP113 can be used to determine background fluorescence.

Figure 2 shows the increase in fluorescence over time in ACAT1 and ACAT2 cells incubated with NBD-cholesterol. Because the background fluorescence attributable to free

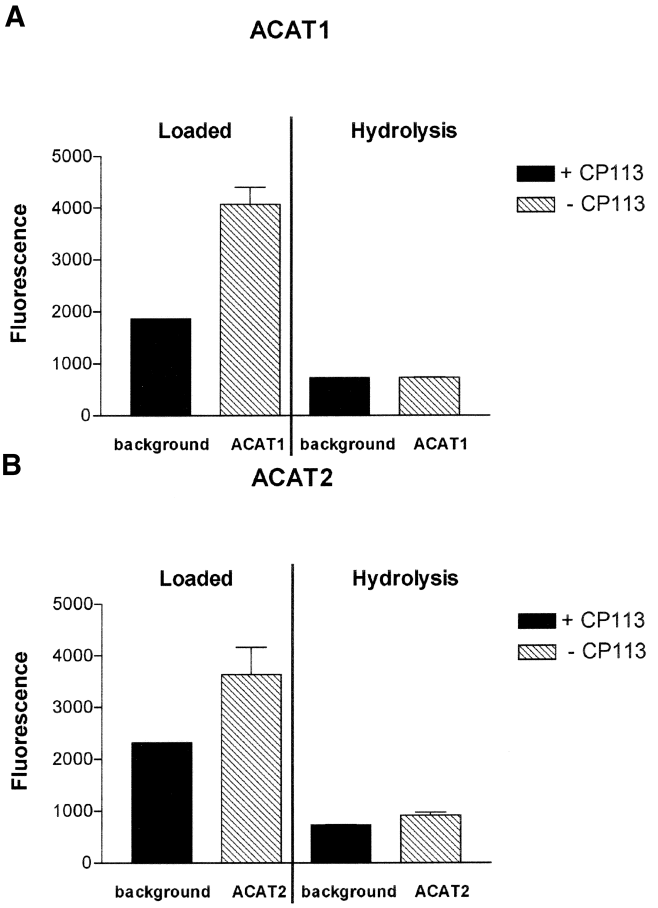

Fig. 3. Hydrolysis of NBD-cholesteryl ester (CE). ACAT1 and ACAT2 cells were first loaded with NBD-CE through incubation with 1 μg/ml NBD-cholesterol for 6 h. Background indicates cells in which ACAT was inhibited during the NBD-cholesterol incubation, and ACAT indicates cells with active ACAT. To determine the fluorescent background, ACAT1 (A) and ACAT2 (B) cells were incubated with 1 μg/ml NBD-cholesterol and 2 μg/ml CP113 for 6 h. After the loading period, cells were washed two times with balanced salt solution (BSS) and the fluorescence was measured. Hydrolysis medium consisting of 10% FBS and 2 μg/ml CP113 was then added and incubated for 18 h. After the hydrolysis period, cells were washed two times with BSS and the fluorescence was measured. Data are expressed as means ± SD (n = 3).

NBD-cholesterol was subtracted, the resulting fluorescence values represent esterified NBD-cholesterol and the extent of the increase with time is an indicator of ACAT activity. Although this increase was not linear over 48 h (Fig. 2A, C), it was linear through the first 8 h for both ACAT1 and ACAT2 cells (Fig. 2B, D). Based on these data, further incubations with NBD-cholesterol were done for 6 h maximum to remain within the range of linearity.

To determine if esterified NBD-cholesterol could be hydrolyzed, ACAT1 or ACAT2 cells were first incubated with NBD-cholesterol for 6 h to load the cells with NBD-CEs. To determine background attributable to free NBD-cholesterol, ACAT1 or ACAT2 cells were incubated with NBD-cholesterol and CP113. After the loading period, the fluorescence was 2.2-fold higher in ACAT1 cells and 1.8-fold higher in ACAT2 cells than in the cells in which ACAT was inhibited (**Fig. 3**), demonstrating the increase in fluorescence when NBD-cholesterol is esterified. Both groups of cells were then incubated with hydrolysis medium consisting of 10% FBS and CP113 for 18 h. In cells loaded with NBD-CEs, the fluorescence decreased during the hydrolysis period, indicating that the cells hydrolyzed NBD-CEs. In addition, the fluorescence in both sets of cells decreased below the free NBD-cholesterol background from the loading period and the fluorescence in the medium increased (data not shown), indicating that cells effluxed NBD-cholesterol. Thus, the results shown in Table 1 and Fig. 3 demonstrate that NBD-cholesterol undergoes the esterification and hydrolysis cycle typical of native cholesterol in cells expressing ACAT1 or ACAT2.

The fluorescence measure of ACAT activity was correlated with two independent measures of ACAT activity, i.e., [^{14}C]oleate incorporation into CEs in live cells and a broken-cell assay using incorporation of [^{14}C]oleate from radioactive oleoyl-CoA into CEs. For this study, AC29 cells and six stable cell lines with different levels of ACAT2 activity were incubated with both NBD-cholesterol and [^{14}C]oleate. In these dishes, both fluorescence and [^{14}C]oleate incorporation in CEs was measured. In addition, parallel dishes from each cell line were harvested for the broken-cell ACAT assay. The fluorescence values correlated well with both [^{14}C]oleate incorporation into CE (r = 0.94) (**Fig. 4A**) and ACAT activity measured in the broken cell assay (r = 0.94) (Fig. 4B), indicating that the increase in fluorescence upon esterification of NDB-cholesterol is an accurate measure of cellular ACAT activity.

The fluorescent ACAT assay was then used to identify ACAT1- and ACAT2-specific inhibitors. AC29 cells stably transfected with either ACAT1 or ACAT2 were used to separately compare the effects of various compounds on the two enzymes. PPPA selectively inhibited ACAT2 without affecting cell integrity, demonstrating that the compound is fully available to the enzyme within live cells (**Fig. 5**, **Table 2**). By contrast, ACAT1 was not significantly inhibited at any of the concentrations examined (0.0001–20 μg/ml). The ACAT2-specific effect of PPPA was confirmed by comparing the responses in microsomes isolated from liver samples from ACAT1$^{-/-}$, ACAT2$^{-/-}$, and wild-type mice as well as with microsomes from AC29 cells stably expressing human ACAT1 or ACAT2 (**Table 3**). The ACAT IC_{50} values for wild-type mice and ACAT1$^{-/-}$ mice were identical at 0.11 μM, whereas activity in ACAT2$^{-/-}$ mice was not inhibited at any of the concentrations used. A similar specificity of PPPA was also demonstrated using

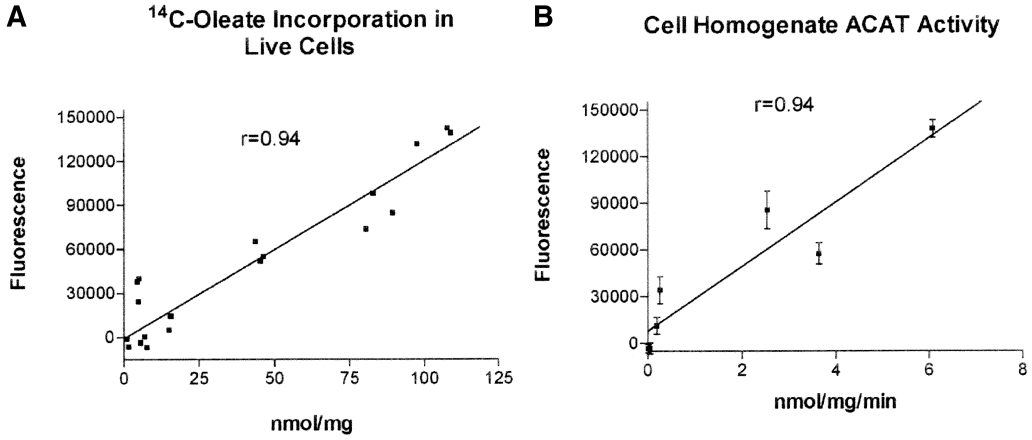

Fig. 4. Correlation of different measures of ACAT activity. AC29 and six cell lines stably expressing different levels of ACAT2 activity were seeded onto 12-well culture plates. A: To compare the fluorescence measure of ACAT activity with [^{14}C]oleate incorporation into CEs, cells were incubated with 1 μg/ml NBD-cholesterol and 1 μCi/ml [^{14}C]oleate for 6 h. Fluorescence was measured, and background fluorescence determined using AC29 cells was subtracted. Cellular lipids were then extracted and separated by TLC, and the CE bands were counted for ^{14}C radioactivity as described in Methods. Each cell line was tested in triplicate, and each point represents an individual well. B: To compare the fluorescent measure of ACAT activity with the broken-cell assay, a parallel set of 12-well plates was seeded as described above and incubated with base medium alone (10% FBS). After the 6 h incubation, cells were harvested and ACAT activity was measured using [^{14}C]oleoyl-CoA incorporation into CEs as described in Methods. For each cell line, cells were pooled to produce sufficient amounts of protein, and ACAT activity was measured in duplicate for each pooled cell line. Data are expressed as means ± SD (n = 2).

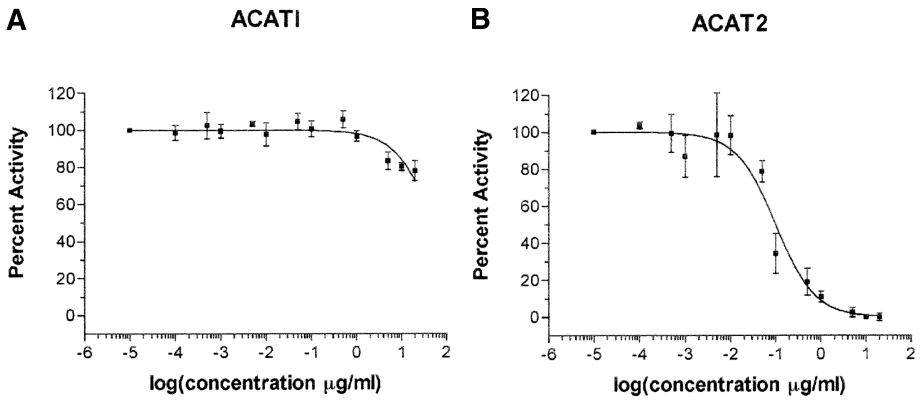

Fig. 5. Selective inhibition of ACAT2 with pyripyropene A (PPPA). ACAT1 (A) and ACAT2 (B) cells were seeded onto a 96-well plate and incubated with 1 μg/ml NBD-cholesterol and 0.0001–20 μg/ml PPPA for 6 h as described in Methods. To determine background, ACAT1 and ACAT2 cells were incubated with 1 μg/ml NBD-cholesterol and 2 μg/ml CP113. Fluorescence values were plotted versus the log of the concentration of PPPA, and Prism 3 for Macintosh software was used to generate a curve fit with a sigmoidal dose-response curve. IC_{50} values were calculated from the curve fit using Prism software. The fluorescence values for ACAT1 and ACAT2 cells without inhibitor added (100% activity) are 34,348 and 28,522, respectively. Data are expressed as means ± SD (n = 3).

microsomes prepared from the transfected cells. ACAT activity in human ACAT1 cell microsomes was not affected, whereas in human ACAT2 cells, activity was inhibited, with an IC_{50} of 0.18 μM (Table 3).

We have identified four compounds, here designated 1A, 1B, 1C, and 1D (**Fig. 6**), that inhibit ACAT1 more efficiently than ACAT2, with IC_{50} values 66- to 187-fold lower for ACAT1 than for ACAT2 (Table 2). These compounds are derivatives of a Warner-Lambert compound (here termed compound 1A) in which the R functional group was modified (Fig. 6). Derivatives 1B, 1C, and 1D demonstrated ACAT1 inhibitory activity similar to that of 1A, with a slight increase (2- to 3-fold) in selectivity. By contrast, numerous ACAT inhibitors that have been made by various pharmaceutical companies were tested and found to inhibit both enzymes to approximately the same extent. Representative data for CP113, a compound originally studied by Pfizer, is shown in Table 2. No specificity for ACAT1 versus ACAT2 was found; similarly, we found no specificity among the Parke, Davis compounds PD-138142-15 and PD-156759 and the DuPont compounds XD-793-11 and DuP128.

DISCUSSION

A new, relatively rapid fluorescence-based method using NBD-cholesterol in intact cells was developed to measure ACAT activity. The assay capitalizes on the property of NBD-cholesterol to increase fluorescence output when the hydrophobicity of its environment increases. Stably transfected cell lines expressing only ACAT1 or ACAT2 were developed for use in the assay, as it has been demonstrated that ACAT-transfected cells form an abundance of lipid droplets (7, 23, 24). The data indicated that lipid droplet formation in both ACAT1- and ACAT2-transfected cells provided the requisite environment for a fluorescence increase proportional to ACAT activity. Thus, the assay in these cell lines proved useful for the identification of compounds that specifically inhibit activities of either ACAT1 or ACAT2. For both ACAT enzymes, compounds capable of selective inhibition by more than 2 orders of magnitude were identified. The fact that either enzyme can be selectively inhibited is a further demonstration of the uniqueness in the structure and function of these enzymes. The availability of selective inhibitors should facilitate the study of the roles of these two enzymes, particularly in tissues that express both ACAT1 and ACAT2.

The data provided here document the uniqueness of ACAT1 and ACAT2. In cell transfection studies, we have

TABLE 2. Differential effects of inhibitors on ACAT1 and ACAT2

Compound	ACAT1 IC_{50}	ACAT2 IC_{50}	Fold Difference
	nM	*nM*	
1A	4.2	275	66
1B	10.3	1,500	146
1C	3.6	530	148
1D	3.2	600	187
PPPA	ND	190	>200
CP113	0.003	0.012	4

ACAT1 and ACAT2 cells were seeded onto a 96-well plate and incubated with 1μg/ml NBD-cholesterol and 0.0001–20 μg/ml of each compound for 6 h as described in Methods and the legend to Fig. 5. ND indicates that activity was not affected by the concentration used and that an IC_{50} could not be determined. Note that the fold difference between the ACAT1 and ACAT2 IC_{50} values for pyripyropene A (PPPA) is a conservative, low-end estimation, because ACAT1 was not affected by the highest concentration used and an IC_{50} could not be determined. Each compound was tested in separate experiments. The fluorescence values for ACAT1 and ACAT2 cells, respectively, without inhibitor added (100% activity) for each compound are as follows: 1A, 12,198 and 8,939; 1B, 21,200 and 12,420; 1C, 8,588 and 9,160; 1D, 26,826 and 15,470; PPPA, 34,348 and 28,522; and CP113, 12,904 and 14,219.

TABLE 3. Effect of PPPA on microsomal ACAT activity

Microsome Source	ACAT IC_{50}	Inhibition at 5 μM PPPA
	μM	%
Wild-type mice	0.11	93
$ACAT1^{-/-}$ mice	0.11	95
$ACAT2^{-/-}$ mice	ND	10
Human ACAT1 cells	ND	8
Human ACAT2 cells	0.18	96

Microsomes were isolated from liver samples from wild-type, $ACAT1^{-/-}$, or $ACAT2^{-/-}$ mice or cells stably expressing human ACAT1 or ACAT2. ACAT activity was assayed with the addition of PPPA over a concentration range of 0.5–5 μM (0.26–2.6 μg/ml). Dose-response curves and IC_{50} values were generated using Prism 3 for Macintosh software as described in the legend to Table 2. ND indicates that activity was not affected by the concentrations used and that an IC_{50} could not be determined. The ACAT activity (nmol/mg/min) of each type of microsome without PPPA addition (100% activity) was as follows: wild-type mice, 1.01; $ACAT1^{-/-}$ mice, 0.68; $ACAT2^{-/-}$ mice, 0.021; human ACAT1 cells, 0.24; and human ACAT2 cells, 0.29.

noted that activity levels of ACAT2 are consistently much higher than those of ACAT1, suggesting differences in the behavior of the two enzymes. We studied the membrane topology of both highly homologous enzymes and found that some regions of either enzyme were on opposite sides of the membrane, again suggesting that the enzymes have unique structures that probably relate to functional uniqueness (6). For ACAT1 versus ACAT2, we found a different tissue localization, with ACAT2 mRNA being confined to liver and intestine (2). The cellular localization of enzyme protein within these tissues also was unique (7), with ACAT2 being present only in hepatocytes and enterocytes. The findings in the present study again suggest uniqueness of function for ACAT1 and ACAT2. The fact that we were able to identify compounds specific for ACAT1 versus ACAT2 also defines the two enzymes as distinct enzyme proteins. The ability to separately inhibit either enzyme with specific chemicals promises the opportunity to more accurately define the physiological function of the two enzymes in the future.

Previously, the sesquiterpene-like compound PPPA had been shown to be an effective ACAT inhibitor using rat liver microsomes (22) and to selectively inhibit ACAT2 in assays comparing microsomal activity from insect cells transfected with baculoviral constructs of either ACAT1 or ACAT2 (25). We have extended these observations to mammalian cells and have shown that PPPA can effectively transverse the plasma membrane and inhibit ACAT2

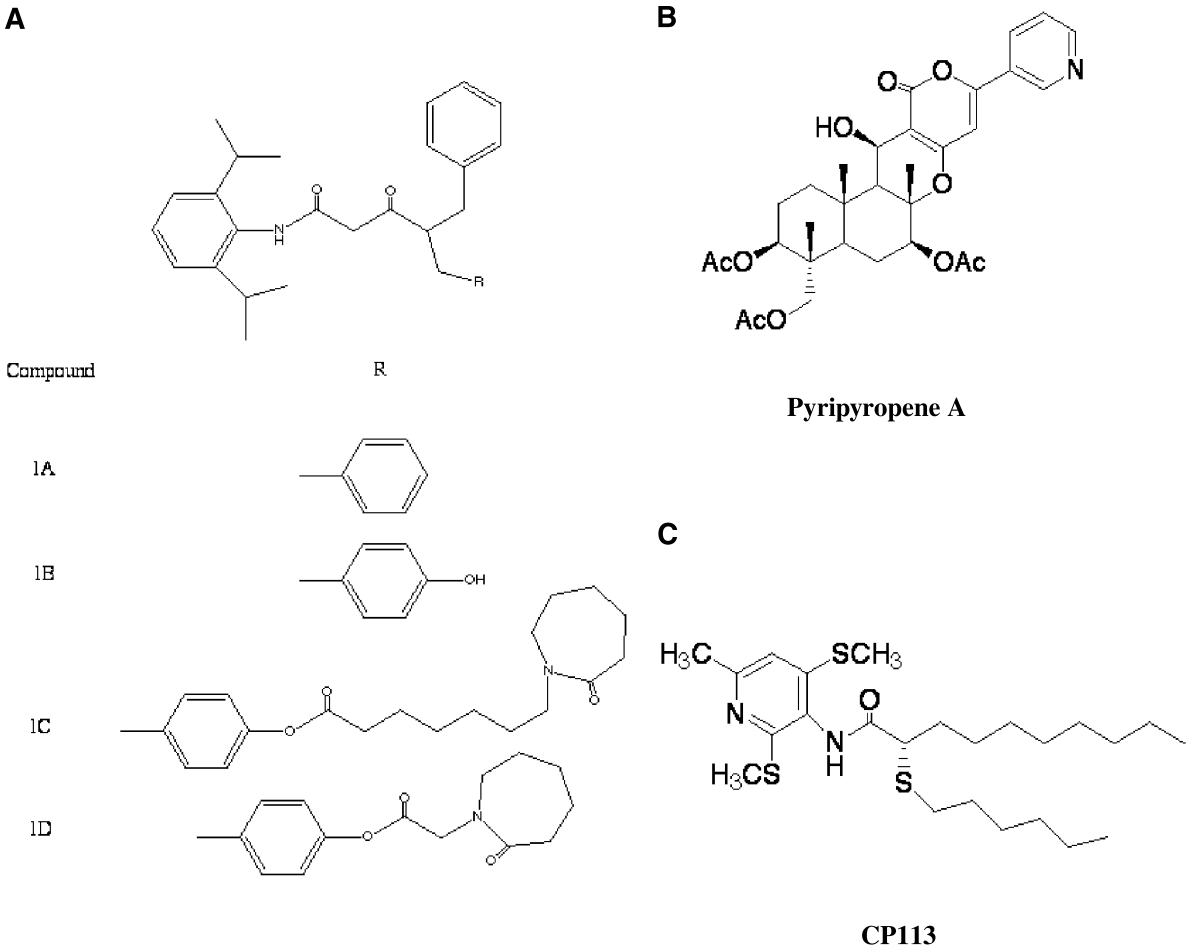

Fig. 6. Structures of ACAT1- and ACAT2-specific inhibitors. A: ACAT1-specific inhibitors based on a Warner-Lambert compound (1A) that differ by the indicated R groups. B: The ACAT2-specific inhibitor PPPA. C: Pfizer compound CP113.

while having little if any effect on ACAT1. It should be noted that these studies were carried out in cells expressing African green monkey ACAT1 or ACAT2, which has a high degree of homology with the human enzymes (98.4% for ACAT1 and 96% for ACAT2). We have additionally shown that the inhibition of ACAT activity by PPPA occurs in microsomes from ACAT1$^{-/-}$ and wild-type mice but not from ACAT2$^{-/-}$ mice (Table 3), further documenting its ACAT2 specificity. Additionally, we show that in microsomes isolated from human ACAT1- and ACAT2-expressing cells, inhibition occurs only when ACAT2 is transfected (Table 3), again showing the high sensitivity and specificity of PPPA for ACAT2 and that the results from cells with African green monkey ACAT1 and ACAT2 apply to the human ACAT enzymes. Given the likelihood that ACAT2 is the enzyme associated with lipoprotein CE secretion, the importance of PPPA as a candidate compound to inhibit this enzyme in vivo and limit atherogenic hypercholesterolemia is high, because such treatment might confer the same benefit to reduce atherosclerosis as was found with ACAT2 gene deletion (13).

The fluorescence of the cholesterol analog NBD-cholesterol is dependent upon the polarity of its environment, which allowed the development of a relatively high-throughput ACAT assay. We have shown that this assay is useful in identifying ACAT1- and ACAT2-specific inhibitors. Free NBD-cholesterol localizes in cellular membranes and is weakly fluorescent in this polar environment (17–19) (Fig. 1A). When NBD-cholesterol is esterified by ACAT, NBD-CE localizes in the nonpolar neutral lipid droplets and is strongly fluorescent (17–19) (Fig. 1B). We have demonstrated that NBD-cholesterol can be esterified by cells expressing either ACAT1 or ACAT2 and that NBD-CEs can subsequently be hydrolyzed with the resulting free NBD-cholesterol effluxed from the cell (Fig. 3). It has been reported previously that AC29 cells have CE hydrolysis activity (24). These results are in agreement with other reports that NBD-cholesterol traffics into cells and is processed in a similar manner to cholesterol (14–16). The ability of NBD-cholesterol to undergo a CE cycle made it an attractive choice for use as a cholesterol marker, and its ability to increase in fluorescence upon esterification was used as a measure of ACAT activity. The fluorescent measure of ACAT activity correlated well with two standard ACAT assays, [^{14}C]oleate incorporation into CEs in live cells and [^{14}C]oleoyl-CoA incorporation into CEs using cell homogenates (Fig. 4). An advantage of the new assay is that it is less laborious and time-consuming than procedures using radioactive substrates. The assay uses cells seeded into 96-well plates and consists of a 6 h incubation with NBD-cholesterol followed by measurement on a fluorescent plate reader, a process that takes only minutes. Therefore, including the time for the NBD-cholesterol media additions, in ~8 h, literally hundreds of samples can be processed if multiple plates are used. In addition, by using intact cells stably transfected with ACAT1 or ACAT2, we were able to screen for compounds that can enter mammalian cells and specifically inhibit only one of the enzymes.

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