Differential Modulation of ACAT1 and ACAT2 Transcription and Activity by Long Chain Free Fatty Acids in Cultured Cells†

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ABSTRACT: Fatty acyl CoA and cholesterol are the substrates for cholesteryl ester synthesis by acyl coenzyme A:cholesterol acyltransferase (ACAT). Two ACAT genes have been identified; ACAT1 is expressed ubiquitously while ACAT2 is primarily expressed in intestine and liver. We tested effects of different free fatty acids (FFAs) on ACAT1 and ACAT2 expression and activity in HepG2 human hepatocytes and THP1 human macrophages. Incubation of oleic acid, arachidonic acid, or eicosapentaenoic acid, but not 25-hydroxycholesterol, induced ACAT1 mRNA levels 1.5−2-fold in HepG2, with no affect on ACAT2 mRNA. FFA had no affect on ACAT1 mRNA in THP1 cells. To determine if FFAs affect ACAT1 or ACAT2 posttranscriptionally, cells were labeled with [^{3}H]cholesterol in the presence of the different FFAs for 1−5 h. Both HepG2 and THP1 cells showed the greatest cholesteryl ester production with oleic acid. This was also confirmed by the observation that more [^{3}H]oleic acid incorporated into CE compared to [^{3}H]eicosapentaenoic acid, even though there was no difference in the total uptake of these FFAs. In ACAT-deficient SRD4, CHO cells stably transfected with human ACAT1 or ACAT2, ACAT1 expressing cells showed a strong preference for oleic acid while ACAT2 expressing cells utilized unsaturated FFAs. Acyl CoA substrate specificity was further tested in microsomes isolated from these cells as well as HepG2 and THP1. THP1 and ACAT1 cells utilized oleoyl CoA preferentially. In contrast, HepG2 and ACAT2 microsomes utilized linolenoyl CoA as well. We conclude that FFAs increase ACAT1 mRNA levels in a cell specific manner, and furthermore that the ACAT reactions exhibit differential FFA utilization.

Fatty acyl CoA[1] and cholesterol are the two substrates for cholesteryl ester (CE) synthesis via the ACAT reaction. Sterol esterification prevents excess cholesterol accumulation and helps regulate optimal fluidity of the plasma membrane (for review, see ref *1*). CE formation is also involved in synthesis of VLDL and chylomicrons in liver and intestine, respectively (*2*−*4*). Furthermore, the accumulation of excess CE in macrophages and smooth muscle cells is associated with atherosclerosis (*5*).

Two ACAT enzymes have been described. ACAT1 is ubiquitous, while ACAT2, which is homologous to ACAT1, is expressed primarily in the liver and intestine of humans and animals (*6*−*8*). Northern blot analysis of human mRNA detects four ACAT1 transcripts (*1*) and one ACAT2 transcript (*6*). The significance of four ACAT1 mRNA species and whether they encode for identical proteins is not known. However, one of the ACAT1 transcripts is produced from a promoter located in a different chromosome to that of the ACAT1 coding sequences (*9*). This indicates that these ACAT transcripts may be regulated differently at the transcriptional level. In keeping with this, ACAT1 mRNA levels are increased in macrophages compared to monocytes (*10, 11*) and in mouse liver in response to a high fat, high cholesterol diet (*12*). There is no data available to indicate transcriptional regulation of ACAT2.

Free fatty acids (FFAs) are converted to fatty acyl CoA within cells and used for the ACAT reaction and other lipogenic processes. However, it is not known whether FFAs influence ACAT regulation. In nonhuman primates, changes in dietary fatty acids have been associated with changes in lipoprotein lipid compositions (*13*). It is possible that FFAs regulate ACAT and contribute to this process. Thus, in this report, we tested the effect of FFAs on ACAT1 and ACAT2 regulation. In addition, we investigated the substrate specificity of these enzymes for various FFAs in intact cells and isolated microsomes.

EXPERIMENTAL PROCEDURES

Materials. [1,2-^{3}H(N)]Cholesterol, [4-^{14}C]cholesterol, [9,10-^{3}H(N)]oleic acid, and [α-^{32}P]deoxycytidine 5′-triphosphate (3000 Ci/mmol) were purchased from Dupont NEM, Wilmington, DE. [5,6,8,9,11,12,14,15,17,18-^{3}H]eicosapentaenoic acid (EPA) was purchased from ICN Pharmaceuticals, Inc.,

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[1] Abbreviation: 25-OH, 25-hydroxycholesterol; ACAT, Acyl coenzyme A:cholesterol acyltransferase; AA, arachidonic acid; CE, cholesteryl ester; CoA, coenzyme A; EPA, eicosapentaenoic acid; FFA, free fatty acid; OA, oleic acid; PMA, phorbol 12-myristate 13-acetate.

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Irving, CA. 25-hydroxycholesterol, free fatty acids and their CoA derivatives (lithium salt), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. Cholesteryl oleate was obtained from Nu-Chek Prep, Inc., Elysian MN. Human low-density lipoprotein (LDL) was isolated by sequential ultracentrifugation (*14*). Dulbecco's modified Eagle's medium (DMEM), F12, RPMI, DMEM: F12 mixture (1:1), glutamine, penicillin, and streptomycin, neomycin sulfate (G418), and lipofectamine were purchased from Gibco Laboratories, Grand Island, NY. Triton WR1339 and ACAT inhibitor Dup 128 were kindly provided by Dr. Jeffrey Billheimer, Dupont Pharmaceutical Co., Wilmington, DE.

Cells. HepG2 human hepatocytes (*15*) and THP1 human monocyte/macrophages were grown as described previously (*16*). Differentiation of THP1 cells was induced with phorbol 12-myristate 13-acetate (PMA, 150 ng/mL) for 3 days (*17*). SRD4 CHO cells lacking ACAT activity (*18*) were maintained in culture in F12 medium containing 10% lipoprotein deficient serum (LPDS).

Stable Transfection of SRD4 Cells with ACAT1 and ACAT2. Human ACAT1 cDNA was incorporated into the mammalian expression vector pCR3.1 (Invitrogen) under control of the CMV promoter. The ACAT1 cDNA contained a five bp 5′ untranslated region (UTR), a 1650 bp coding sequence, and one bp 3′ UTR. Vector pCR3.1 was also used to express a human ACAT2 cDNA using *Eco*R1 and *Not*1 restriction sites. The ACAT2 cDNA contained a 51 bp 5′ UTR, a 1566 coding sequence, and a 420 bp 3′ UTR (*6*). ACAT1 mutant SRD4 cells were stably transfected with ACAT1 or ACAT2 vectors using lipofectamine according to the manufacturer. Cells were incubated in growth medium containing 400 μg/mL G418 for two weeks to select cells, and subsequently maintained in culture in growth medium containing 300 μg/mL G418. G418 was removed from growth medium 2 days prior to experiments.

Northern Blot Analysis. Cells were incubated with or without various FFAs (0.3 mM) bound to albumin (1% BSA, FFA:BSA molar ratios of 2:1) or 25-hydroxycholesterol (10 μg/mL) for 12 h at 37 °C. Total RNA was isolated, separated by 1.2% denaturing agarose/formaldehyde electrophoresis, and transferred to Duralon UV membranes as described (*16*). Blots were hybridized with cDNA probes for both ACAT1 and ACAT2 cDNA probes (*6*) in QuickHyb (Stratagene) and washed twice with 2× sodium chloride/sodium citrate buffer (SSC), 1% SDS for 15 min at room temperature, followed by 0.1× SSC, 0.1% SDS for 30 min at 60 °C. ACAT1 and ACAT2 radioactivity was measured by phosphoimaging and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Competitive Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). DNA fragments containing 408 or 219 bp of human GAPDH cDNA flanked, respectively, by 25 bp of ACAT1 or β-actin cDNA sequence were generated by PCR, subcloned into pBS:KS, and transcribed in vitro by T7 RNA polymerase (Promega). Serial dilutions of these RNA standards were added to 3 μg of cellular RNA and reverse transcribed (Superscript II, GibcoBRL). PCR was performed using this cDNA and ACAT1 or β-actin specific oligos (A1, CGGAATATCAAACAGGAGCCCTTC; A2, CATTCCAAAGAACATGAAGAGCACG; β1, GAGCTGCCTGACGGCCAGGTC; β2 CACATCTGCTGGAAGGTGGACAG, respectively). The products were resolved on a 1.5% agarose gel with 0.5 μg/mL ethidium bromide and staining intensities were measured using the Kodak 2D imaging system. The cell and standard-derived product intensities were graphed and the intersection of the two lines was the relative RNA concentration (*19*).

Incorporation of [^{3}H]Cholesterol, [^{3}H]OA, or [^{3}H]EPA into Cholesteryl Esters in Cells. Two methods were used to determine FFA effects on the ACAT reaction. (Method 1) Cells were incubated with DMEM:F12 medium containing 1% BSA with 100 μg/mL LDL for 12 h at 37 °C. The medium was replaced with DMEM:F12, 1% BSA and [^{3}H]-cholesterol-containing liposomes to label cellular cholesterol for 4 h (*20*). Cells were incubated with DMEM containing 1% BSA with or without 0.3 mM FFA for 1−5 h at 37 °C. Data are expressed either as a ratio of CE to cholesterol radioactivity or percent changes of CE formation compared to control cells. (Method 2) Similar experiments were performed as method 1 except incubation with FFA was substituted with 0.3 mM [^{3}H]OA (4762 dpm/nmol) or [^{3}H]-EPA (1061 dpm/nmol) for 5 h at 37 °C. At the end of experiments, cellular lipids were extracted and separated as described (*15*). Protein content of intact cells was determined using a kit from Bio-Rad (Hercules, CA).

Measurement of Cholesteryl Ester Hydrolysis in HepG2 and ThP1 Cells. Cells were incubated with DMEM:F12 medium containing 1% BSA with 100 μg/mL LDL in the presence of 0.3 mM [^{3}H]OA or [^{3}H]EPA to promote the cholesteryl ester formation. After 5 h, incubation medium was replaced with a fresh DMEM medium containing 1% BSA with 10 μM Dup128 ACAT inhibitor to prevent re-esterification of released [^{3}H]fatty acids. Cells were incubated for different time periods up to 24 h at 37 °C to determine the hydrolysis of the preformed, labeled cholesteryl esters. At the end of the experiments, lipids were extracted and the amount of cholesteryl esters was determined as described.

In Vitro Microsomal ACAT Assay. Microsomes were isolated from cells by methods described by Chang et al. (*21*) and assayed for ACAT activity according to the method described by Billheimer et al. (*22*), using Triton WR1339 for incorporation of 20 μg of cholesterol and [^{3}H]cholesterol (1.5 μCi/sample) into microsomes. ACAT activation was initiated by addition of various fatty acyl CoA (0.1 mM) for 1 h at 37 °C. At the end of the experiments, lipids were extracted with chloroform: methanol (2:1) containing [^{14}C]-cholesterol and unlabeled cholesteryl oleate (10 μg/sample) and separated by thin-layer chromatography. The results were normalized based on protein concentrations of isolated microsomes.

Statistics. For statistical analysis, two-tailed t-tests were used to compare the effects of different FFAs.

RESULTS

ACAT1 and ACAT2 mRNA in HepG2 and THP1 Cells. ACAT1 and ACAT2 mRNA levels in HepG2 and THP1 cells were determined by northern blot analysis (Figure 1). Both THP1 and HepG2 cells showed four ACAT1 transcripts; two major bands at 3.8 and 4.3 kb and two minor bands of 4.8 and 6.5 kb. A single 2.2 kb ACAT2 mRNA band was observed only in HepG2 cells. When THP1 cells were differentiated into macrophages after 3 days of PMA

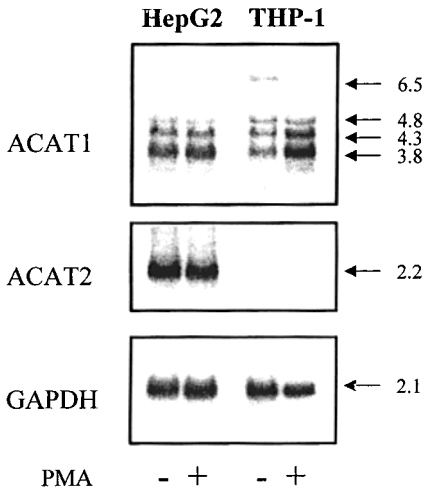

FIGURE 1: Expression of ACAT1 and ACAT2 mRNA in HepG2 and THP1 cells as measured by blot hybridization. Total RNA was isolated from HepG2 and THP1 cells that were preincubated with growth medium ±150 ng/mL phorbol ester (PMA). Total RNA (20 μg) was subjected to 1.2% agarose/formaldehyde electrophoresis and blot hybridization with ^{32}P-labeled probes. The size of individual bands was determined using a 0.24−9.5 kb RNA ladder standard (Gibco BRL). A typical blot is shown.

Table 1: Summary of ACAT mRNA Expression in HepG2 and THP-1 Cells as Determined by the Phosphoimager[a]

		ACAT1				ACAT2
cell types		3.8	4.3	4.8	6.5	2.2
HepG2	no FA	100	100	100	100	100
	stearic (18:0)	87	114	105	111	83
	oleic (18:1)	152*	148*	97	105	121
	arach. (20:4)	181*	162*	89	92	95
	EPA (20:5)	170*	156*	100	108	78
	25-OH	98	74	112	86	131
THP1	no FFA	100	100	100	100	ND
(differentiated)	stearic (18:0)	99	89	100	115	ND
	oleic (18:1)	67	87	107	93	ND
	arach. (20:4)	74	68	74	85	ND
	EPA (20:5)	92	75	108	90	ND
	25-OH	103	121	101	81	ND

[a] HepG2 and THP1 cells were incubated with indicated FFA or 25-hydroxycholesterol (25-OH) for 12 h and changes in mRNA levels of ACAT 1 and 2 were determined by Northern blot analysis as described in the Experimental Procedures. The results are expressed as average of three experiments and percent increase over control (no FFA) is shown. (*) Significant differences in ACAT expression between incubations with or without FFA ($p < 0.01$, $n = 3$). ND, not detected.

incubation, there was a 2−4-fold increase in 3.8 and 4.3 kb mRNA levels. Increased ACAT1 mRNA was also reflected in a 2-fold increase in ACAT activity in microsomes isolated from undifferentiated and differentiated THP1 cells (243.75 ± 41.28 vs 476.27 ± 13.11 dpm/mg protein/hr, respectively). There were no differences in ACAT1 or ACAT2 mRNA levels in HepG2 cells in the presence of PMA.

Effects of FFA on ACAT1 and ACAT2 mRNA Levels in HepG2 and THP1 Cells. To determine the effect of FFAs on ACAT mRNA levels, HepG2 and THP1 cells were incubated with 0.3 mM FFA for 12 h and mRNA levels were measured by blot hybridization (Table 1). There were 1.5−2-fold increases in ACAT1 mRNA levels in HepG2 cells incubated with oleic acid (OA), arachidonic acid (AA), and EPA. Stearic acid and 25-hydroxycholesterol had no effect. The changes in mRNA levels were only observed in the two major bands (3.8 and 4.3 kb bands). Similar FFA incubations with THP1 cells showed no significant changes in ACAT1 mRNA, indicating hepatocyte specific effects of FFAs on ACAT1 transcription. Unlike ACAT1, FFA incubations did not alter ACAT2 mRNA levels. The effect of FFAs on ACAT1 transcription in HepG2 cells was also examined with quantitative RT-PCR. Treatment of HepG2 cells with AA increased ACAT1 mRNA by 1.8-fold ($p < 0.01$), consistent with results observed in the northern blot analysis.

OA and EPA Stimulate ACAT Activity Differently. To determine whether FFA effects on increased ACAT transcription were correlated with ACAT activity, THP1 and HepG2 cells were preincubated with LDL as an exogenous cholesterol source, followed by incubation with OA or EPA for 1−5 h to allow conversion of [^{3}H]cholesterol to CE (Figure 2). Sequential incubations of LDL followed by FFAs were used to prevent potential FFA effects on stimulating LDL receptor mediated uptake that might also affect the rate of CE formation (*15*). In both cell lines, incubation of OA stimulated CE formation in a near linear fashion over 5 h incubation time. In contrast, although EPA stimulated ACAT1 transcription to similar levels as OA in HepG2 cells, CE formation induced by EPA was significantly reduced, indicating different specificities of FFA utilization by ACAT in HepG2 cells. Similarly, CE formation stimulated by OA in THP1 cells was 50% higher than EPA after 5 h incubation.

It is possible that differences in levels of CE formation attributed to effects of FFAs on ACAT activity could be due to differences in CE hydrolysis and not cholesterol esterification, as both processes occur concomitantly during incubation. Therefore, to assess CE hydrolysis, the experiments described above were continued for another 24 h in fresh medium, without LDL or FFA, in the presence of an ACAT inhibitor. Differences in hydrolysis did not account for higher CE formation in the presence of OA as compared to EPA (data not shown); cholesteryl esters formed with [^{3}H]-EPA were hydrolyzed at 27.6 ± 0.24%/24 h (mean ± SD) compared to 48.2 ± 1.20%/24 h for [^{3}H]OA.

Since CE hydrolysis did not explain differential CE formation by OA vs EPA, we questioned if differences in cell uptake of OA and EPA could contribute to differences in CE formation. Studies were performed with [^{3}H]OA or [^{3}H]EPA to determine total FFA uptake and their incorporation into cholesteryl esters. There were no significant differences in uptake of these FFAs in HepG2 and THP1 cells (Table 2). Again, in these experiments nearly 2-fold more OA was incorporated into CE compared to EPA in both cell lines, consistent with experiments shown in Figure 2.

Effects of Various FFA on ACAT Activity in HepG2 and THP-1 Cells. We next tested FFA preferences for ACAT1 and ACAT2 in inducing CE formation in intact cells (Figure 3). Incubation with OA resulted in the highest levels of CE synthesis in both HepG2 and THP1 cells, while stearic acid had no effect. Although there was a measurable increase in CE formation with other polyunsaturated FFAs, this was lower compared to cells that were incubated with OA.

FFA Specificity of ACAT1 and ACAT2. To examine the FFA specificity of the individual ACAT enzymes in the absence of transcriptional effects, CHO SRD4 cells that lack ACAT activity (*18*) were stably transfected with human ACAT1 or ACAT2 with an identical CMV promoter (Figure

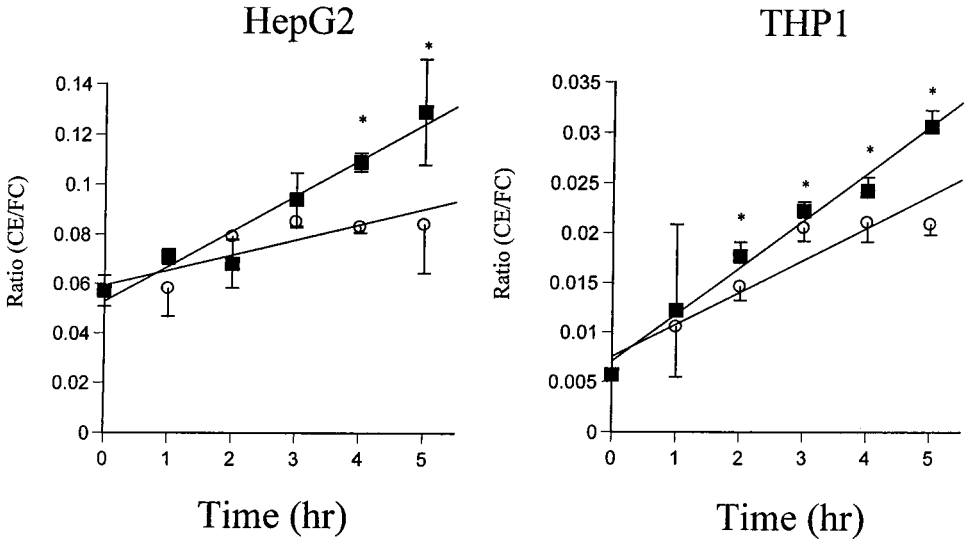

FIGURE 2: Time course of CE formation induced by OA or EPA in HepG2 and THP1 cells. Cells were incubated with DMEM/F12 medium containing 1% BSA in the presence of 100 μg/mL LDL for 12 h at 37 °C. After washing cells, medium was replaced with DMEM/F12 medium containing [^{3}H]cholesterol-containing liposomes (1 μCi/mL) for 4 h to label cellular cholesterol, followed by incubation with 0.3 mM OA (closed squares) or EPA (open circles) for indicated time points. At the end of experiments, cells were washed and cellular lipids were extracted overnight with 2-propanol containing a known amount of [^{14}C]cholesterol as an internal standard. Cholesterol and cholesteryl esters were separated by TLC as described and values were corrected by the recovery of [^{14}C]cholesterol and cell protein content. The results shown are expressed as average ratios of CE/cholesterol of triplicate dishes ± SD of a representative experiment. (*) Significant differences between OA vs EPA ($p < 0.01$).

Table 2: Uptake of [^{3}H]Oleic Acid or [^{3}H]EPA in HepG2 and THP1 Cells and Their Incorporation into Cholesteryl Esters[a]

cell types		total FFA uptake (nmol FFA/ mg cell protein)	nmol FFA incorporated into cholesteryl esters
HepG2	18.1	12.36 ± 25.3	3.14 ± 0.18 *
	20:5	120.90 ± 12.0	1.86 ± 0.64 *
THP1	18.1	104.2 ± 18.8	2.61 ± 0.60 *
	20:5	139.2 ± 20.2	1.17 ± 0.06 *

[a] Cells were incubated with 0.3 mM [^{3}H]oleic acid (18:1) or [^{3}H]EPA (20:5) for 5 h as described in the Experimental Procedures. Total FFA uptake was determined by measuring radioactivity in cells before various lipids were isolated with TLC. FFA incorporation into cholesteryl esters is shown. ND, not detected. (*) significant difference between oleic acid vs EPA ($p < 0.01$).

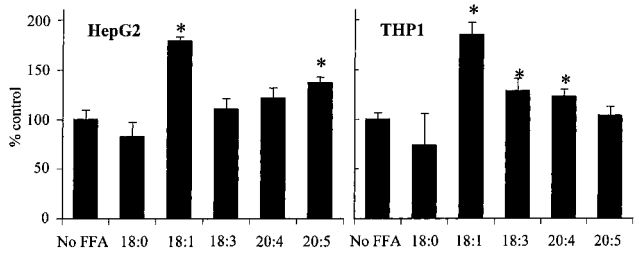

FIGURE 3: Effect of FFAs on esterification of [^{3}H]cholesterol in HepG2 and THP1 cells. Cells were labeled with [^{3}H]cholesterol-containing liposomes and incubated with DMEM containing 1% BSA with or without 0.3 mM FFA for 1−5 h at 37 °C to allow [^{3}H]cholesterol incorporation into cholesteryl ester as described in the Experimental Procedures. Actual values for esterified cholesterol in controls (no FFAs) were 15 987 and 9452 dpm/mg cell protein for HepG2 and THP1 cells, respectively. Results were normalized based on protein content and expressed as the mean of triplicate dishes of a representative experiment. (*) Significant differences in ACAT activity from control (no FA) ($p < 0.01$); 18:0, stearic acid; 18:1, OA; 18:3, linolenic acid; 20:4, AA; 20:5, EPA.

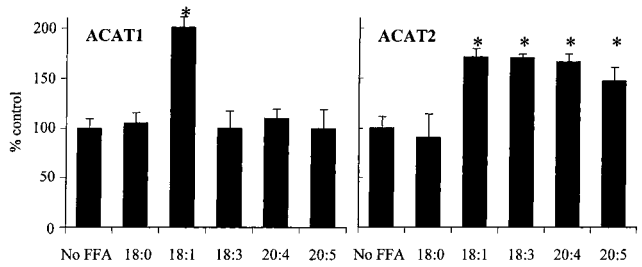

FIGURE 4: FFA substrate specificity in ACAT1 and ACAT2 cells. Cells were labeled with [^{3}H]cholesterol-containing liposomes and incubated with DMEM:F12 containing 1% BSA with or without indicated FFA (0.3 mM) for 5 h at 37 °C to assess [^{3}H]cholesterol incorporation into cholesteryl ester as described in the Experimental Procedures. Actual values for esterified cholesterol in controls were 20 048 and 38 562 dpm/mg cell protein for ACAT1 and ACAT2 cells, respectively. (*) Significant differences in ACAT activity from control (no FFAs) ($p < 0.01$); 18:0, stearic acid; 18:1, OA; 18:3, linolenic acid; 20:4, AA; 20:5, EPA.

4). In ACAT1 cells, OA stimulated high levels of CE formation, while other FFAs did not increase CE formation (compared to control incubation without FFAs), consistent with results observed in THP1 and HepG2 cells. A saturated FFA, stearic acid, failed to promote CE synthesis by either ACAT1 or ACAT2, suggesting that stearic acid was not readily accessible to either ACAT protein. In ACAT2 cells, polyunsaturated FFAs such as AA and EPA increased CE formation to levels similar to that of OA. The time course of CE formation in response to OA and EPA incubation showed that OA progressively increased CE formation by ACAT1 with time (Figure 5). This effect was specific to OA since EPA had no effect. In contrast, both OA and EPA stimulated CE formation in ACAT2 cells. Interestingly, ACAT1 cells consistently showed reduced CE to cholesterol ratios compared to ACAT2 cells (note different *y*-axis scales in Figure 5). Although mechanisms behind this difference are not clear, this could be due to differential access to labeled cholesterol by ACAT1 and ACAT2 enzymes. These

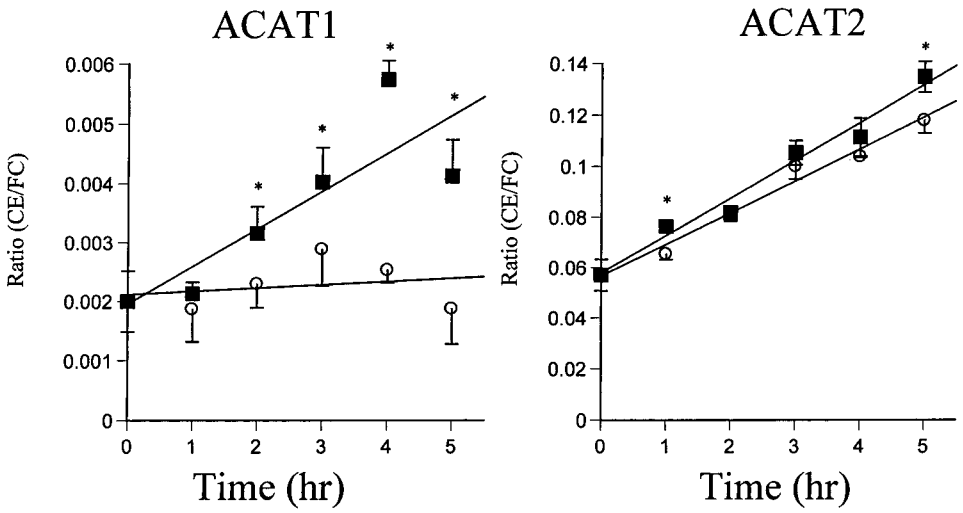

FIGURE 5: Time course of CE formation induced by OA or EPA in ACAT1 and ACAT2 transfected cells. Cells were labeled with [^{3}H]-cholesterol-containing liposomes and incubated with DMEM:F12 containing 1% BSA with or without 0.3 mM OA (closed squares) or EPA (open circles) for 1−5 h at 37 °C to allow [^{3}H]cholesterol incorporation into cholesteryl ester as described in the Experimental Procedures. The results are expressed as average ratios of CE/cholesterol of triplicate dishes ± SD of a representative experiment. (*) Significant differences between OA vs EPA ($p < 0.01$).

Table 3: Uptake of [^{3}H]Oleic Acid or [^{3}H]EPA in ACAT1 or ACAT2 Transfected Cells and Their Incorporation into Various Lipid Classes[a]

cell types		total FFA uptake (nmol FFA/ mg cell protein)	nmol FFA incorporated into cholesteryl esters
CHO SRD4	18.1	95.68 ± 21.3	ND
	20:5	89.11 ± 3.65	ND
CHO ACAT1	18.1	93.82 ± 3.50	3.08 ± 0.27 *
	20:5	87.82 ± 8.20	0.92 ± 0.25 *
CHO ACAT2	18.1	82.26 ± 2.38	4.01 ± 0.50
	20:5	74.82 ± 10.42	3.56 ± 0.91

[a] Cells were incubated with 0.3 mM [^{3}H]oleic acid (18:1) or [^{3}H]EPA (20:5) for 5 h as described in the Experimental Procedures. Total FFA uptake was determined by measuring radioactivity in cells before various lipids were isolated with TLC. FFA incorporation into cholesteryl esters is shown. ND, not detected. (*) significant difference between oleic acid vs EPA ($p < 0.01$).

Table 4: Cholesterol Esterification by Different Fatty Acyl CoA in Isolated Microsomes[a]

	synthesis of [^{3}H]cholesteryl esters (dpm/mg of protein/h)				
cell type	control	18:1	18:3	20:4	20:5
HepG2	136.45	3932.98	2894.29	1431.46	1384.75
	74.28	3308.82	2426.81	1789.70	1483.12
THP-1	114.57	2666.99	690.26	424.63	502.69
	59.24	2636.24	610.34	359.30	412.68
CHO-ACAT1	504.26	6592.26	963.44	648.11	588.43
	516.53	6326.74	664.56	359.30	630.53
CHO-ACAT2	197.09	4164.44	2790.57	918.19	648.27
	157.41	4052.23	2819.41	714.15	595.88

[a] ACAT activity was assayed in isolated microsomes from the different cell types as described in the Experimental Procedures. Control indicates samples that were extracted immediately after preincubation with cholesterol. The results are expressed as dpm [^{3}H]cholesteryl esters/ mg cell protein/hr of duplicate incubations of a representative experiment. Values from incubations performed in duplicate are shown; 18:1, oleoyl CoA; 18:3, linolenoyl CoA; 20:4, arachidonyl CoA; 20:5 eicosapentaenoyl CoA.

results suggest fundamental differences between ACAT1 and ACAT2 in their response to different FFAs.

Uptake of [^{3}H]OA or [^{3}H]EPA were also tested in these cell lines (Table 3). Total FFA uptake was similar in these cells. Control SRD4 CHO cells without ACAT transfection showed no detectable CE formation. Incorporation of [^{3}H]-OA into CE was nearly 3-fold higher than EPA in ACAT1 cells, while ACAT2 showed similar utilization of [^{3}H]OA or [^{3}H]EPA.

Because the ACAT reaction utilizes acyl CoA derivatives of FFAs, the acyl CoA specificity of ACAT1 and ACAT2 was further tested in microsomes isolated from HepG2, THP1, ACAT1, and ACAT2 cells (Table 4). Microsomes from THP1 cells, which have only ACAT1, used oleoyl CoA preferentially with a 30-fold increase in CE formation over background, while other fatty acyl CoA showed only 10−25% increases compared to that of oleoyl CoA. In all cases, CE formation was almost totally inhibited by the ACAT inhibitor Dup 128 (data not shown). HepG2 microsomes also showed the highest CE formation with oleoyl CoA compared to other CoA. However, linolenoyl CoA and arachidonyl CoA still showed 25 and 15-fold higher CE synthesis over background, respectively. Microsomes isolated from ACAT1 cells showed strong substrate preference for oleoyl CoA, consistent with results in THP1 cells. Unlike ACAT1, however, ACAT2 also used linolenoyl CoA to form CE (70% of oleoyl CoA), further indicating a broader FFA specificity for ACAT2 as observed in HepG2 cells. Although AA and EPA stimulated ACAT2-mediated CE formation in cultured cells similar to OA, their derivatives, arachidonyl CoA and eicosapentaenoyl CoA, had less of an effect in microsomes. Nevertheless, ACAT activity was still substantially enhanced compared to control levels, and to experiments without acyl CoA incubation.

DISCUSSION

In the current studies, we tested the effect of FFAs on ACAT1 and ACAT2 mRNA expression in HepG2 hepatocytes and THP1 macrophages. Northern blot analysis showed that four ACAT1 transcripts were expressed in both cell lines,

while ACAT2 mRNA was present only in HepG2 cells. The expression of ACAT1 mRNA was altered in several ways; differentiation of THP1 cells into macrophages produced a 2−4-fold increase in ACAT1 transcription, consistent with previous reports (*10*, *23*). In HepG2 cells, incubation of OA, AA, and EPA induced nearly a 2-fold increase in ACAT1 mRNA. However, no effect on ACAT1 mRNA was observed in THP1 cells. These cell specific effects of ACAT1 mRNA levels, likely due to differential transcription initiation and/or alteration in mRNA stability, are in concert with the observation that increases in ACAT1 mRNA levels occur in livers of mice fed a high fat, high cholesterol diet (*12*).

Changes in ACAT1 mRNA expression, either by differentiation of THP1 cells into macrophages or OA, AA, or EPA incubation in HepG2 cells, were observed only in the major 3.8 and 4.3 kb bands. Similarly, only these two transcripts are modulated in THP1 cells by vitamin D_3 or 9-*cis*-retinoic acid (*11*). Presumably, FFAs might only affect transcription from sequences proximal to ACAT1 gene (*9*). Unlike ACAT1, there were no changes in ACAT2 mRNA levels in HepG2 cells with FFA incubation, indicating a differential regulatory mechanism for ACAT1 and ACAT2 despite the structural and functional similarities of the ACAT proteins.

In HepG2 cells, OA also increased ACAT1 activity, while AA and EPA had less of an effect on CE formation despite significant increases in ACAT1 mRNA. By contrast, monocyte differentiation produced concordant changes in ACAT1 mRNA levels and activity. The discrepancy in FFA effects on ACAT1 mRNA levels vs ACAT activity was not due to different rates of CE hydrolysis. In fact, CE hydrolysis was more pronounced in cells that were incubated with OA compared to EPA. Although we have not determined the mechanism behind this, it is possible that CE derived from different FFAs are compartmentalized differently leading to different accessibility to hydrolases. In cells of the liver and intestines, one pool might be used for CE storage, while another is selectively used for lipoprotein synthesis.

Since nearly equal amounts of OA or EPA were taken up by cells, potential mechanisms that lead to varying CE formation by these FFAs are differences in delivery to the ACAT enzymes or differences in FFA affinity for each ACAT. Furthermore, ACAT1 and ACAT2 catalytic domains are likely oriented to opposite sides of membranes (*24*). This could also influence FFA access to the two enzymes.

The modulation at ACAT enzymes due to different FFA affinity was further confirmed in the ACAT deficient CHO cells (SRD4 cells) stably transfected with ACAT1 or ACAT2. We found that ACAT1 cells preferentially utilized OA, while ACAT2 cells utilized AA or EPA to similar levels as OA to promote CE formation. Of note, despite the presence of both ACAT genes in HepG2 cells, HepG2 cells did not show significant utilization of AA and EPA, in keeping with the concept that ACAT1 is the dominant enzyme for CE formation and storage in hepatocytes (*25*, *26*).

In microsomal ACAT assays of ACAT1 and ACAT2 cells, FFA substrate specificity was reflected in those of intact cells. The incubation of oleoyl CoA showed the highest CE formation by ACAT1, while ACAT2 used several acyl CoA species equally well. In keeping with our observations, Cases et al. (*7*) showed that ACAT1 utilized oleoyl CoA preferentially, while ACAT2 was relatively less specific for various fatty acyl CoA. Interestingly, even though ACAT1 appeared to be the dominant form in intact HepG2 cells (*25*, *26*), there was substantial CE formation by linolenoyl CoA in microsomes of HepG2 cells. This ACAT1 predominance may be due to different compartmentalization that allows greater accessibility to cholesterol. In keeping with this, our studies showed that ACAT1 and ACAT2 demonstrate similar esterification activity when provided with saturating amounts of free cholesterol in microsomes.

In summary, certain FFAs modulate ACAT1 mRNA levels in a cell-specific manner. FFAs may affect ACAT transcription or mRNA stability that leads to differential ACAT mRNA expression. Since there is no direct evidence that cholesterol alters ACAT1 transcription, changes in ACAT1 mRNA levels in certain tissues such as livers of mice fed a high fat, high cholesterol diet (*12*) may be induced by the FFA component of the diet. In contrast, neither FFA or cholesterol affected ACAT2 transcription, consistent with studies where ACAT2 mRNA was not altered in livers of nonhuman primates with diets varying in FFA and cholesterol content (*24*). Although different FFAs modulate ACAT1 mRNA levels in cell specific manner, this does not appear to correlate with ACAT activity. This implies that the effects of FFAs on ACAT proteins may have a significant role in regulating ACAT activity. Differences in FFA utilization may help clarify distinct roles for each ACAT, e.g., a specific role for ACAT2 in lipoprotein synthesis and secretion compared to ACAT1 in preventing the cytotoxic accumulation of free cholesterol. Furthermore, differential ACAT regulation by different FFAs could modify lipoprotein compositions, cholesterol storage in different tissues, and the progression of atherosclerosis (*27*).

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REFERENCES

1. Chang, T. Y., Chang, C. C., and Cheng, D. (1997) *Annu. Rev. Biochem. 66*, 613−638.
2. Musanti, R., Giorgini, L., Lovisolo, P. P., Pirillo, A., Chiari, A., and Ghiselli, G. (1996) *J. Lipid Res. 37*, 1−14.
3. Huff, M. W., Telford, D. E., Barrett, P. H., Billheimer, J. T., and Gillies, P. J. (1994) *Arterioscler. Thromb. 14*, 1498−1508.
4. Field, F. J., Kam, N. T., and Mathur, S. N. (1990) *Gastroenterology 99*, 539−551.
5. Tabas, I. (1995) *Curr. Opin. Lipidol. 6*, 260−268.
6. Oelkers, P., Behari, A., Cromley, D., Billheimer, J. T., and Sturley, S. L. (1998) *J. Biol. Chem. 273*, 26765−26771.
7. Cases, S., Novak, S., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Welch, C. B., Lusis, A. J., Spencer, T. A., Krause, B. R., Erickson, S. K., and Farese, R. V., Jr. (1998) *J. Biol. Chem. 273*, 26755−26764.
8. Anderson, R. A., Joyce, C., Davis, M., Reagan, J. W., Clark, M., Shelness, G. S., and Rudel, L. L. (1998) *J. Biol. Chem. 273*, 26747−26754.
9. Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S., Ma, Z. M., Chang, C. C., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) *J. Biol. Chem. 274*, 11060−11071.

10. Miyazaki, A., Sakashita, N., Lee, O., Takahashi, K., Horiuchi, S., Hakamata, H., Morganelli, P. M., Chang, C. C., and Chang, T. Y. (1998) *Arterioscler. Thromb. Vasc. Biol. 18*, 1568−1574.
11. Maung, K. K., Miyazaki, A., Nomiyama, H., Chang, C. C. Y., Chang, T. Y., and Horiuchi, S. (2001) *J. Lipid Res. 42*, 181−187.
12. Uelmen, P. J., Oka, K., Sullivan, M., Chang, C. C., Chang, T. Y., and Chan, L. (1995) *J. Biol. Chem. 270*, 26192−26201.
13. Thomas, M. J., Thornburg, T., Manning, J., Hooper, K., and Rudel, L. L. (1994) *Biochemistry 33*, 1828−1834.
14. Rumsey, S. C., Galeano, N. F., Arad, Y., and Deckelbaum, R. J. (1992) *J. Lipid Res. 33*, 1551−1561.
15. Rumsey, S. C., Galeano, N. F., Lipschitz, B., and Deckelbaum, R. J. (1995) *J. Biol. Chem. 270*, 10008−10016.
16. Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F., and Deckelbaum, R. J. (1998) *J. Biol. Chem. 273*, 25537−25540.
17. Auwerx, J. (1991) *Experientia 47*, 22−31.
18. Metherall, J. E., Ridgway, N. D., Dawson, P. A., Goldstein, J. L., and Brown, M. S. (1991) *J. Biol. Chem. 266*, 12734−12740.
19. Ross, R., Kleiz, R., and Reske-Kunz, A. B. (1995) *PCR Methods Appl. 4*, 371−375.
20. Tabas, I., Rosoff, W. J., and Boykow, G. C. (1988) *J. Biol. Chem. 263*, 1266−1272.
21. Chang, T. Y., Limanek, J. S., and Chang, C. C. Y. (1981) *Anal. Biochem. 116*, 298−302.
22. Billheimer, J. T., Tavani, D., and Nes, W. R. (1981) *Anal. Biochem. 111*, 331−335.
23. Wang, H., Germain, S. J., Benfield, P. P., and Gillies, P. J. (1996) *Arterioscler. Thromb. Vasc. Biol. 16*, 809−814.
24. Joyce, C., Skinner, K., Anderson, R. A., and Rudel, L. L. (1999) *Curr. Opin. Lipidol. 10*, 89−95.
25. Lee, O., Chang, C. C., Lee, W., and Chang, T. Y. (1998) *J. Lipid Res. 39*, 1722−1727.
26. Chang, C. C., Sakashita, N., Ornvold, K., Lee, O., Chang, E. T., Dong, R., Lin, S., Lee, C. Y., Strom, S. C., Kashyap, R., Fong, J. J., Farese, R. V., Patoiseau, J. F., Delhon, A., and Chang, T. Y. (2000) *J. Biol. Chem. 275*, 28083−28092.
27. Thomas, M. J. and Rudel, L. L. (1996) *J. Nutr. 126*, 1058S−1062S.

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