Inhibition of acylcoenzyme A:cholesterol acyltransferase activity in CaCo-2 cells results in intracellular triglyceride accumulation

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Abstract The activity of acylcoenzyme A:cholesterol acyltransferase (ACAT) in CaCo-2 cells was inhibited by the ACAT inhibitor, 58-035. The inhibitory effect of this acylamide was specific for cholesterol esterification catalyzed by ACAT; the rates of triglyceride, phospholipid, and cholesterol synthesis were not inhibited by this agent. Cholesteryl esters were depleted in CaCo-2 cells 24 hr after inhibition of ACAT activity, whereas the unesterified cholesterol content increased by 56% after 96 hr. Moreover, inhibiting ACAT activity with 58-035 resulted in a time-dependent 2.5-fold increase in intracellular triglycerides. This accumulation of triglycerides in CaCo-2 cells was associated with a 37% increase in triglyceride synthesis by 96 hr in the presence of 58-035. Triglyceride-rich lipoprotein secretion ($d < 1.006$ g/ml) was not affected by inhibiting ACAT activity for up to 6 hr. However, triglyceride-rich lipoprotein secretion was significantly decreased in CaCo-2 cells that were preincubated with 58-035 for 24 to 96 hr. Lipoproteins of density < 1.006 g/ml that were isolated from CaCo-2 cells incubated with the ACAT inhibitor were deficient in cholesteryl esters and triglycerides compared to lipoproteins isolated from control cells. The data suggest that triglycerides accumulate in CaCo-2 cells in which ACAT activity has been inhibited by 58-035. This accumulation of triglycerides is associated with a modest increase in triglyceride synthesis and a decrease in triglyceride secretion. Altering intracellular cholesterol pools by regulating ACAT activity in the gut could result in the decrease of triglyceride transport and/or the secretion of triglyceride-rich lipoprotein particles of abnormal composition.—**Kam, N. T. P., E. Albright, S. N. Mathur, and F. J. Field.** Inhibition of acylcoenzyme A:cholesterol acyltransferase activity in CaCo-2 cells results in intracellular triglyceride accumulation. *J. Lipid Res.* 1989. **30:** 371–377.

Supplementary key words cholesterol metabolism • acylcoenzyme A:cholesterol acyltransferase (ACAT) • lipoprotein production • triglyceride

The esterification of cholesterol in the small intestinal absorptive cell is catalyzed by the microsomal enzyme, acylcoenzyme A:cholesterol acyltransferase, or ACAT (1–3). There is evidence that suggests that this enzyme plays a key role in regulating the transport of cholesterol from the intestinal lumen into the lymph. Heider, Pickens, and Kelly (4) showed a decrease in cholesterol absorption by inhibiting intestinal ACAT in cholesterol-fed rabbits. Bennett Clark and Tercyak (5) infused an ACAT inhibitor into the duodenums of lymph-fistula rats and demonstrated a decrease in the secretion of cholesteryl esters in lymph chylomicrons and very low density lipoproteins. Thus, regulating ACAT activity in the intestine may be a way to decrease cholesterol absorption or to modify triglyceride-rich lipoprotein composition.

Not all cholesterol leaving the absorptive cell is esterified. Approximately 50% of cholesterol is unesterified and makes up part of the surface of the lipoprotein along with phospholipids and apolipoproteins (6). The hydrophobic cholesteryl esters and triglycerides form the core of the particle. Regulating the cholesterol requirements in an enterocyte during triglyceride transport, therefore, is not a simple matter. In order for the synthesis and secretion of lipoproteins to occur there must be pools of cholesterol, both free and esterified, which are readily available for transporting triglycerides. ACAT regulates these cholesterol pools within the enterocyte, and changes in the activity of this enzyme could affect lipoprotein assembly or secretion, or cause the secretion of a lipoprotein particle that has an abnormal lipid composition.

CaCo-2 cells, a human intestinal cell line that has many of the morphological and biochemical properties of small intestinal absorptive cells, have been shown to secrete triglyceride-rich lipoproteins in response to fatty acids (7, 8). These cells also secrete the apolipoproteins B-100 and B-48, A-I, A-IV, E, and C-III (9, 10). In a recent study, the regulation of ACAT activity in CaCo-2 cells by exogenous micellar cholesterol has been described (11). In the present study, CaCo-2 cells were used to investigate the regulation of triglyceride transport. The data show

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase.

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that inhibiting ACAT activity with the acylamide 58-035 (12) results in triglyceride accumulation within CaCo-2 cells. This is associated with a modest increase in triglyceride synthesis and a decrease in triglyceride-rich lipoprotein secretion.

MATERIALS AND METHODS

[2-^{3}H]Glycerol, [1-^{14}C]oleoyl coenzyme A, sodium [^{14}C] acetate, [1,2-^{3}H]cholesterol, and [9,10-^{3}H]oleic acid were purchased from New England Nuclear (Boston, MA). Oleic acid, sodium acetate, oleoyl coenzyme A, cholesterol, and fatty acid-free bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). The ACAT inhibitor, 58-035, was generously provided by Sandoz, Inc. (East Hanover, NJ). All other chemicals were reagent grade.

Cell culture

CaCo-2 cells were grown as previously described (11). The cells were used for experimentation 4 days after confluency and during active dome formation. For long-term incubation with 58-035, i.e., 24–120 hr, 58-035 (25 μg), dissolved in 10 μl of dimethyl sulfoxide, was added per ml of Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) containing 20% fetal calf serum. The medium was changed daily. For short-term incubation with 58-035 (less than 24 hr), the medium was changed to Medium-199 Earle's (Gibco, Grand Island, NY) containing 10 mM HEPES, pH 7.4. After the incubations, all experiments were performed in the M199 medium without fetal calf serum. Viability was assessed by trypan blue exclusion and light microscopy morphology (11).

Oleic acid–albumin preparation

A stock solution of oleic acid in 90% ethanol was kept under nitrogen at 4°C. The necessary aliquot of stock solution was taken and the sodium salt was prepared with excess NaOH. After the solvent was evaporated completely under nitrogen, the fatty acid was dissolved in 1.5 ml of hot distilled water and added rapidly to a small amount of M199 containing 10 mM HEPES, pH 7.4, and the appropriate amount of albumin to maintain a fatty acid: albumin ratio of 3:1. The volume was then adjusted with more M199 so that the final concentration of oleic acid was 250 μM.

Measurement of lipid synthesis

Intracellular lipid synthesis was estimated by measuring the incorporation of labeled oleate into cholesteryl oleate, triglyceride, and phospholipid as described previously (11).

The rate of cholesterol synthesis was measured using labeled acetate as substrate (13).

Lipoprotein production

The methodology used to estimate lipoprotein production was modified from the methodology described by Davis and Boogaerts (14) in rat hepatocytes. CaCo-2 cells were incubated with oleic acid (250 μM) attached to albumin (3:1, mol/mol) and [^{3}H]glycerol, 500 mCi/mmol (20 μM, 10 μCi/dish) in M199, 10 mM HEPES, pH 7.4, without fetal calf serum. At the times indicated, the medium was collected and cellular debris was removed by centrifugation at 2,000 rpm for 10 min. Two ml of human plasma, which had been heated to 60°C for 10 min, was added as carrier. The density was adjusted when necessary to 1.006 g/ml and the triglyceride-rich lipoproteins of density less than 1.006 g/ml were isolated by ultracentrifugation in a Ti-50 rotor at 140,000 *g* for 18 hr. Lipids from the cells and the isolated lipoproteins were extracted with chloroform–methanol 2:1 (v/v). The water phase of the lipoprotein extract was washed once with chloroform. The combined chloroform phases were washed three times with methanol–0.04 N HCl 1:1 (v/v) to remove free glycerol. The chloroform phases from cells and lipoproteins were dried under nitrogen and the lipids were separated by thin-layer chromatography using hexanes–diethyl ether–methanol–acetic acid 85:15:1:1 (v/v) as the developing solvent. The lipids were visualized by iodine vapors and scraped into 4 ml of liquid scintillation fluid and counted. The efficiency of ^{3}H counting was 0.50. It was determined that 80% of the glycerol label was incorporated into lipoprotein triglyceride with the remainder in phospholipids. The incorporation of labeled glycerol into total lipoprotein lipids was used as a measure of lipoprotein secretion.

Lipoprotein (d < 1.006 g/ml) lipid mass

CaCo-2 cells were cultured in T-75 flasks. Five days prior to the experiment, 58-035 (25 μg/ml) was added to the culture medium and the medium was changed daily. Control flasks received the vehicle, dimethyl sulfoxide, only. On the day of the experiment, the medium was changed to M199, 10 mM HEPES, pH 7.4, containing oleic acid (250 μM) attached to albumin. After 18 hr, the medium was collected and centrifuged at 2,000 rpm for 10 min to remove cellular debris. The density was adjusted to 1.006 g/ml and the triglyceride-rich lipoproteins of d < 1.006 g/ml were isolated by ultracentrifugation for 30 hr at 140,000 *g* in a Ti-50 rotor. The cells were scraped from the flasks with a rubber scraper into phosphate-buffered saline. The lipids were extracted from the cells and the lipoprotein fraction with chloroform–methanol 2:1 (v/v). The chloroform phase was dried under nitrogen. Because of the small amount of lipoprotein cholesterol secreted per flask, lipids extracted from lipoproteins secreted by one flask of cells were analyzed for cholesterol only. Both phospholipids and triglycerides were analyzed

from the lipid extract of the lipoprotein fraction from cells in a single T-75 flask.

Enzyme assays

Acylcoenzyme A:cholesterol acyltransferase activity was measured as previously described; the specific activity of oleoyl CoA was 19,250 dpm/nmol (3). Total membranes were prepared from CaCo-2 cells by completely disrupting the cells by sonication in phosphate-buffered saline. The whole homogenate was centrifuged at 105,000 *g* for 1 hr. The membrane pellet was washed once by recentrifugation and used immediately for the measurement of ACAT activity.

Chemical analysis

Protein was determined according to the method of Lowry et al. (15). Cholesterol was measured by gas–liquid chromatography as described (16). Phospholipids and triglycerides were measured as described previously (17, 18).

RESULTS

Effect of 58-035 on ACAT activity in CaCo-2 cells

CaCo-2 cells were incubated with increasing concentrations of 58-035 for 1 hr. After removing the 58-035 and washing the cells, the rates of lipid synthesis were estimated by measuring the incorporation of labeled oleate into phospholipids, triglycerides, and cholesteryl esters. Compound 58-035 had no significant effect on the incorporation of oleate into triglycerides or phospholipids (data not shown). **Fig. 1** shows the effect of 58-035 on the incorporation of oleate into cholesteryl oleate. The acylamide caused a stepwise, concentration-dependent decrease in cholesteryl ester synthesis reaching a maximal effect by 15 μg/ml. The basal rate of cholesteryl ester synthesis in CaCo-2 cells incubated with the vehicle alone was 365 ± 17 pmol cholesteryl oleate formed/hr per dish.

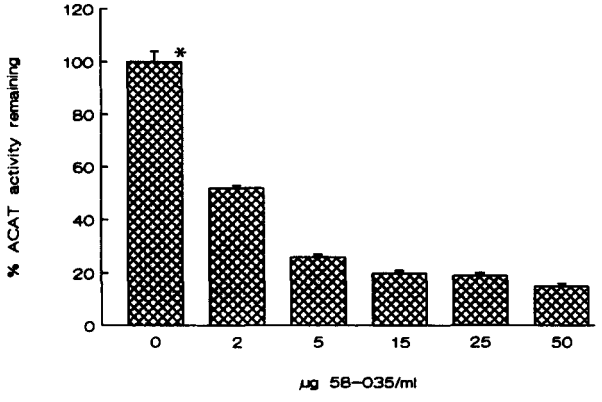

Fig. 1. Effect of 58-035 on [^{3}H]oleate incorporation into cholesteryl [^{3}H]oleate. CaCo-2 cells were cultured in 60-mm plastic petri dishes as described in Methods. On day 11 after plating, the medium was changed to M199 with 10 mM HEPES, pH 7.4, containing the given concentration of 58-035 dissolved in dimethyl sulfoxide. Control dishes received the vehicle alone. After 1 hr, the cells were washed twice with phosphate-buffered saline and the incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate was determined. The data represent the mean ± SE (n = 6 dishes at each concentration); *, $P < 0.001$ vs. all concentrations.

To confirm that 58-035 inhibited the activity of ACAT and not some other step in the pathway of oleate incorporation into cholesteryl oleate, total membranes were prepared from CaCo-2 cells after incubating them with or without 25 μg/ml of 58-035. Membrane ACAT activity was measured using labeled oleoyl CoA as substrate. ACAT activity in membranes prepared from cells incubated with 58-035 was decreased by 77% compared to activity in membranes prepared from control cells: 41.6 pmol cholesteryl oleate formed/mg per 4 min versus 181.5 ± 12.7 ($P < 0.001$), respectively.

Effect of 58-035 on cholesterol synthesis in CaCo-2 cells

To investigate whether 58-035 regulated the rate of intracellular cholesterol synthesis, CaCo-2 cells were incubated for 1 hr with 25 μg/ml of 58-035. After removing the acylamide and extensively washing the cells, the rate of incorporation of labeled acetate into unesterified cholesterol was determined. Compared to control cells, 58-035 did not affect cholesterol synthesis: 2.98 nmol of acetate incorporated/dish per hr versus 3.00.

Effect of 58-035 on lipid content in CaCo-2 cells

CaCo-2 cells were incubated with 25 μg/ml of 58-035 for 0 to 120 hr. At the end of the incubation period, the cells were extensively washed, scraped from the dish in phosphate-buffered saline, pH 7.4, and lipids were extracted in chloroform–methanol. **Table 1** shows these results. After 24 hr in the presence of 58-035, no cholesteryl esters could be detected in CaCo-2 cells. In contrast, unesterified cholesterol content increased significantly. By 96 hr, the free cholesterol content of the cells had increased by 56%. Intracellular phospholipids were not changed by the acylamide. Triglycerides, however, accumulated in CaCo-2 cells in which ACAT activity had been inhibited. This accumulation of triglyceride was time-dependent reaching a 2.5-fold increase by 96 hr.

To determine whether the increase in triglyceride content observed in CaCo-2 cells after long-term incubation with 58-035 was due to an increase in triglyceride synthesis, labeled oleate incorporation into cellular lipids was determined in CaCo-2 cells that had been incubated with the acylamide for 0 to 120 hr. As shown in **Fig. 2,** rates of cholesteryl ester synthesis were decreased by 83% by 24 hr and remained low throughout the experiment. Phospholipid synthesis rates were essentially unchanged by 58-035. There was a modest increase in triglyceride synthesis. By 48 hr, the rate of triglyceride synthesis had in-

TABLE 1. Effect of 58-035 on the lipid content of CaCo-2 cells

Hours with 58-035	Cholesterol		Triglyceride	Phospholipid
	Free	Ester		
	μg/mg cell protein			
0	18.2 ± 0.3[a]	1.81 ± 0.4	52 ± 1[a]	487 ± 7
24	24.2 ± 0.7	nd	73 ± 3	520 ± 15
48	23.8 ± 0.8	nd	86 ± 1	523 ± 12
72	27.9 ± 1.0	nd	108 ± 6	626 ± 19
96	28.4 ± 1.1	nd	127 ± 7	570 ± 9
120	27.7 ± 0.4	nd	127 ± 5	510 ± 33

CaCo-2 cells were cultured as described in Methods for the long-term incubation with 58-035. Compound 58-035 (25 μg/ml) was added in dimethyl sulfoxide at the indicated times. At the end of the incubation, lipids were extracted from the cells and analyzed as described. The data represent the mean ± SE; n = 3 dishes at each time point; nd, not detected.

[a] $P < 0.005$ vs. other time points.

creased by 25% reaching a maximum increase of 37% by 96 hr.

Effect of 58-035 on triglyceride-rich lipoprotein secretion in CaCo-2 cells

CaCo-2 cells were incubated for 2 hr in the presence or absence of 25 μg/ml of 58-035. At the end of this incubation period, cells were washed and incubated for 4 hr with [^{3}H]glycerol and oleic acid (250 μM) attached to albumin. Compound 58-035 was added back to the dishes that had the acylamide initially. At the end of each incubation period, the incorporation of glycerol into cellular triglycerides and lipoproteins of d < 1.006 g/ml was determined. **Fig. 3** shows that the presence of 58-035 did not affect the incorporation of glycerol into cellular triglycerides or secreted triglyceride-rich lipoproteins.

Fig. 4, however, shows data from an experiment in which CaCo-2 cells were incubated with 58-035 for 120 hr prior to determining lipoprotein secretion. In contrast to the results of the short-term experiment, there was a significant decrease in the rate of triglyceride-rich lipoprotein production in CaCo-2 cells incubated with the ACAT inhibitor. The incorporation of glycerol into cellular triglycerides was also increased in these cells.

Another experiment was done to investigate when 58-035 exerted its effect on triglyceride-rich lipoprotein production. CaCo-2 cells were incubated with the acylamide or its vehicle, dimethyl sulfoxide, for 24 to 120 hr. At the end of the incubation period, labeled glycerol and oleic acid (250 μM) were added for 4 hr. The incorporation of glycerol into cell triglycerides and lipoproteins of d < 1.006 g/ml was determined. **Table 2** shows the data from this experiment. The inhibitory effect of 58-035 on lipoprotein secretion was evident by 24 hr. The inhibition ranged from 61% after 24 hr to 45% after 72 hr. Triglyceride-rich lipoprotein secretion decreased in control cells during the experiment for reasons that are not clear. The rate of labeled glycerol incorporated into cellular triglyceride was modestly increased by 58-035, suggesting that cells incubated with the acylamide had higher rates of triglyceride synthesis compared to control cells.

Effect of 58-035 on triglyceride-rich lipoprotein lipid mass

CaCo-2 cells were cultured in T-75 flasks. Five days before the experiment, half the flasks received 25 μg/ml of 58-035. The other half received the vehicle, dimethyl sulfoxide (1%, v/v). At the end of 5 days, the cells were washed and 250 μM oleic acid attached to albumin in M199, 10 mM HEPES, pH 7.4, was added to each flask to stimulate triglyceride-rich lipoprotein production. The ACAT inhibitor was added back to the flasks that previously contained the acylamide. After 18 hr, the medium was collected; the density was adjusted to 1.006 g/ml; and lipoproteins of d < 1.006 g/ml were isolated by ultracentrifugation for 30 hr. **Table 3** shows the lipid analyses of

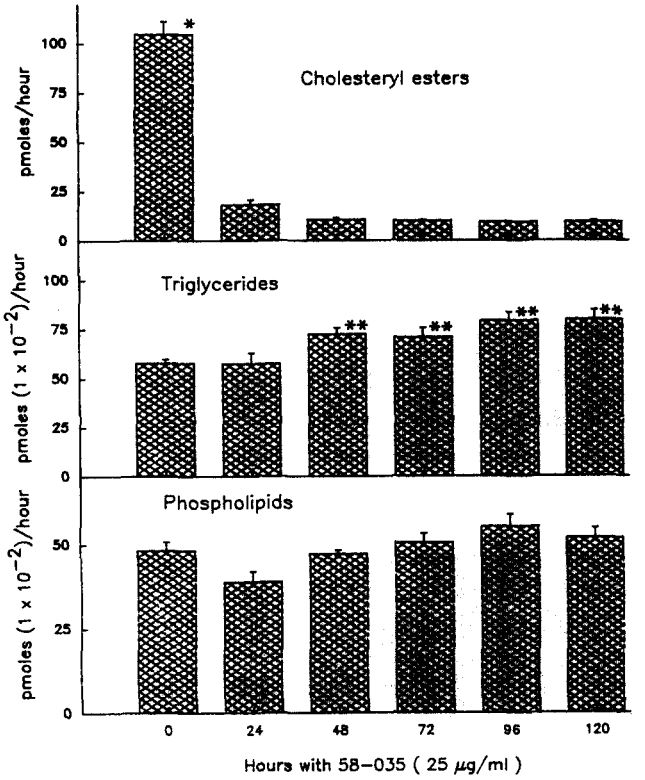

Fig. 2. Effect of long-term incubation of 58-035 on [^{3}H]oleate incorporation into cellular lipids. CaCo-2 cells were cultured in 60-mm plastic petri dishes in DMEM containing 20% fetal calf serum. At the time indicated, 58-035 (25 μg/ml) was added and the medium was changed daily until the day of the experiment. After the incubation period, the cells were washed twice with phosphate-buffered saline and [^{3}H]oleate incorporation into cholesteryl esters, triglycerides, and phospholipids was determined as described in Methods. The data are given as the means ± SE (n = 3 dishes at each time point); *, $P < 0.001$ vs. all hours; **, $P < 0.05$ vs. control.

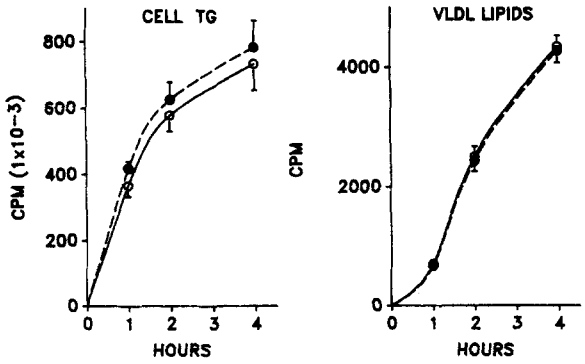

Fig. 3. Effect of short-term incubation of 58-035 on [^{3}H]glycerol incorporation into cellular triglycerides and lipids of lipoproteins of d < 1.006 g/ml. CaCo-2 cells were cultured in 60-mm plastic petri dishes in DMEM containing 20% fetal calf serum. On the day of the experiment, the medium was changed to M199 with 10 mM HEPES, pH 7.4, with or without 25 μg/ml of 58-035. After a 2-hour preincubation, cells were washed and incubated for 4 hr with [^{3}H]glycerol and 250 μM oleic acid as described in Methods. Compound 58-035 was added back to the dishes which had the inhibitor initially. At the times indicated, the incorporation of labeled glycerol into cellular triglycerides and lipids of lipoproteins of d < 1.006 g/ml (VLDL) was determined. The data points are means ± SE (n = 4 dishes at each time point per experiment) (one representative experiment of three experiments); (○) control; (●) 58-035.

this lipoprotein fraction. In triglyceride-rich lipoproteins secreted by CaCo-2 cells incubated with 58-035, the cholesteryl ester content was significantly decreased by 73%. The unesterified cholesterol mass tended to be higher in lipoproteins secreted by cells incubated with 58-035 compared to that observed in lipoproteins secreted by control cells. The difference, however, did not reach statistical significance. Lipoprotein phospholipids were unchanged by 58-035. Moreover, there was a significant decrease in the mass of triglycerides in lipoproteins secreted by cells in which ACAT activity had been inhibited.

DISCUSSION

The acylamide, 58-035, has been shown to be a potent inhibitor of ACAT activity (12). The present study has clearly demonstrated the inhibition of ACAT activity by 58-035 in the human intestinal cell line CaCo-2. Compound 58-035 was specific for the cholesterol acyltransferase as both the triglyceride and phospholipid acyltransferases were not inhibited. At concentrations of the acylamide that resulted in maximal inhibition of ACAT activity, 15–20% of the total intracellular cholesterol esterifying activity remained. As determined by the activity that remained after measuring ACAT activity in membranes prepared from CaCo-2 cells incubated with 58-035, the residual esterifying activity was also coenzyme A-dependent. Heider, Pickens, and Kelly (4) observed this in rabbit intestinal microsomes using a related acylamide 57-118. This suggests that there is either another coenzyme A-dependent enzyme in the intestine that catalyzes the esterification of cholesterol and is not inhibited by 58-035, or there is a portion of the enzyme that is not accessible to the acylamide. Because there are no data to suggest that another ACAT-like enzyme exists within the intestine, and since it is believed that ACAT is an integral membrane protein that is tightly bound to the membrane (19), it is most likely that 58-035 does not reach all of the enzyme.

Although 58-035 has been used to study the role of intestinal ACAT in cholesterol absorption (4, 5), it has been used most effectively as a tool to investigate cholesterol metabolism and cell surface lipoprotein receptor activity by modifying intracellular cholesterol pools (20, 21). In CaCo-2 cells, 58-035 had a profound effect on cholesteryl ester mass. After 24 hr in the presence of 58-035, there were no measurable cholesteryl esters remaining in CaCo-2 cells. Conversely, the unesterified cholesterol content significantly increased. Because the rate of cholesterol synthesis was not inhibited by the acylamide and unesterified cholesterol is available to the cells via the medium, it is not difficult to explain an increase in the intracellular free cholesterol content under conditions in which 80–85% of the cell's cholesterol esterifying activity has been inhibited. This effect of ACAT inhibition does not hold true for all cells, however, and the reasons for this difference are not clear. For example, HepG2 and J774 cells do not accumulate unesterified cholesterol when incubated with 58-035 and lipoproteins (20, 21), whereas Fu5AH, smooth muscle cells, and the Chinese hamster ovary cell, 25-RA, do (12, 22).

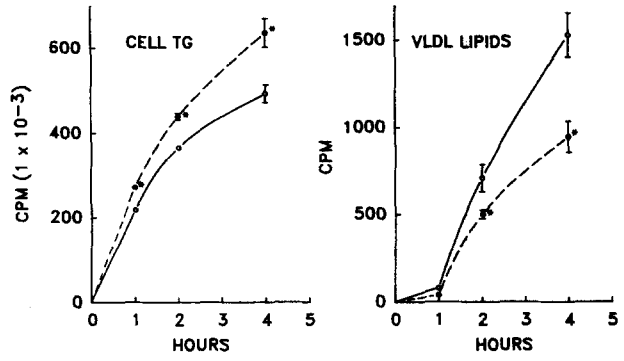

Fig. 4. Effect of long-term incubation of 58-035 on [^{3}H]glycerol incorporation into cellular triglycerides and lipids of lipoproteins of d < 1.006 g/ml. CaCo-2 cells were grown in 60-mm plastic petri dishes in DMEM containing 20% fetal calf serum. Five days prior to the experiment, 25 μg/ml of 58-035 was added to the culture media. Control dishes received the vehicle alone. On the day of the experiment, the medium was changed to M199 with 10 mM HEPES, pH 7.4. The incorporation of labeled glycerol into cellular triglycerides and lipids of lipoproteins of d < 1.006 g/ml (VLDL) was determined for the time indicated as described for Fig. 3 and in Methods; n = 3 dishes for each time point per experiment (one representative experiment of three experiments); (○) control; (●) 58-035; *, $P < 0.02$ vs. control.

TABLE 2. Effect of 58-035 on [^{3}H]glycerol incorporation into cellular triglycerides and lipoproteins of d < 1.006 g/ml

Hours with 58-035	Lipoproteins (d < 1.006 g/ml)		Cell Triglycerides	
	Control	58-035	Control	58-035
	cpm/dish		*cpm* × 10^{-3}*/dish*	
24	4339 ± 334	1686 ± 86	845 ± 6	955 ± 10
48	2475 ± 102	1040 ± 132	857 ± 33	956 ± 11
72	1857 ± 65	1026 ± 113	774 ± 5	957 ± 11
96	1445 ± 97	757 ± 43	791 ± 4	987 ± 19
120	1550 ± 72	786 ± 63	808 ± 2	964 ± 6

CaCo-2 cells were grown in 60-mm petri dishes as described in Methods. On the indicated day, 58-035 (25 μg/ml) or its vehicle, dimethyl sulfoxide, was added and the medium was changed daily. On the day of the experiment, the cells were washed and [^{3}H]glycerol, 20 μM, 10 μCi/dish, was added with 250 μM oleic acid attached to albumin (3:1, mol:mol). At the end of a 4-hr incubation, the medium was collected and lipoproteins of d < 1.006 g/ml were isolated. The cells were washed two times. The lipids were extracted from the cells and lipoproteins and separated by thin-layer chromatography. The data represent the mean ± SE (n = 3 dishes at each time point). One representative experiment is shown out of three separate experiments done.

CaCo-2 cells accumulated triglycerides in the presence of 58-035. This has not been noted previously. Long-term incubation of CaCo-2 cells with 58-035 resulted in a stepwise, time-dependent increase in triglyceride content. Triglycerides increased by 40% in 24 hr and by 144% in 96 hr. The rate of triglyceride synthesis, as estimated by labeled oleate incorporation into triglycerides, was not changed by 58-035 after 24 hr. By 48 hr, the rate of triglycleride synthesis had increased 25%, reaching a maximum of 37% by 96 hr. It is unlikely that this small change in triglyceride synthesis can alone account for the 2.5-fold increase in triglyceride mass observed in CaCo-2 cells incubated with 58-035. If, however, this increase in triglyceride synthesis were to be combined with a decrease in triglyceride transport, this would be a more likely explanation for the accumulation of triglycerides in CaCo-2 cells incubated with the ACAT inhibitor. Short-term inhibition of ACAT activity by 58-035 (6 hr) did not affect production of lipoproteins of d < 1.006 g/ml, suggesting that the acute regulation of ACAT, per se, does not regulate triglyceride-rich lipoprotein production in CaCo-2 cells. When CaCo-2 cells were incubated for 24 to 96 hr with 58-035, however, a substantial reduction in lipoprotein secretion occurred. The major difference between the short-term and long-term incubation with the acylamide was the alteration which occurred in the cholesterol content of CaCo-2 cells over the long-term incubation. After 6 hr, 58-035 did not significantly change the free or esterified cholesterol content (data not shown). In contrast, after 24 hr, there was a 33% increase in unesterified cholesterol and complete absence of cholesteryl ester content in cells incubated with 58-035. In a lymph-fistula rat model, in which 58-035, safflower oil, and cholesterol were infused into the duodenums of these animals, Bennett Clark and Tercyak (5) did not observe a change in triglyceride transport over a 6-hr period. This agrees with our short-term incubation of CaCo-2 cells with 58-035 and suggests that the down-regulation of ACAT activity, by itself, is not regulatory for triglyceride transport. What does appear to be necessary is an alteration in intracellular cholesterol pools as caused by 58-035 which interferes with the normal assembly and/or secretion of the lipoprotein particle. Although cholesteryl esters only constitute 1–2% of the core lipid (6), they may be important for the orderly synthesis and secretion of triglyceride-rich lipoproteins by CaCo-2 cells.

It cannot be said with certainty that the effect of 58-035 on triglyceride-rich lipoprotein secretion is related solely to its inhibitory effect on ACAT activity. Compound 58-035 is lipophilic, and it is possible that the acylamide itself could displace the core lipids from the lipoprotein particle resulting in a decrease in triglyceride transport. Inhibiting ACAT activity with other agents such as CL 277,082 (23) or progesterone (24) may affect triglyceride-rich lipoprotein secretion differently.

The decrease in triglyceride mass that was observed in lipoproteins of d < 1.006 g/ml secreted by CaCo-2 cells incubated with 58-035 for 120 hr substantiated the results of experiments using radiolabeled glycerol to measure lipoprotein secretion. A decrease of 20% in triglyceride mass compared to the triglycerides found in lipoproteins secreted from control cells is not particularly striking, but the decrease was a consistent and significant finding. Mass measurements of secreted lipids in lipoproteins of d < 1.006 g/ml may not represent the composition of nascent lipoproteins as CaCo-2 cells have the capacity to hydrolyze triglyceride within the triglyceride-rich lipoprotein particle by 24 hr (personal observations). Although the amount of triglyceride mass that was secreted may be

TABLE 3. Effect of 58-035 on triglyceride-rich lipoprotein lipid mass in CaCo-2 cells

	Cholesterol			
	Free	Ester	Triglyceride	Phospholipid
	μg/flask			
Control	0.35 ± 0.03	0.71 ± 0.18	12.2 ± 0.8	1.49 ± 0.24
58-035	0.51 ± 0.06	0.19 ± 0.06[a]	9.7 ± 0.7[b]	1.40 ± 0.30

CaCo-2 cells were cultured in T-75 flasks for 11 days. For the last 5 days of culture 58-035 (25 μg/ml) was added. Control flasks received the vehicle without 58-035. On day 10, oleic acid, 250 μM, attached to albumin (3:1, mol:mol) was added for 18 hr to stimulate lipoprotein secretion. At the end of 18 hr, the medium was collected, lipoproteins of d < 1.006 g/ml were prepared as described in Methods, and the lipids were analyzed. The data given are the means ± SE (n = 6 flasks for cholesterol, 14 flasks for triglyceride, 4 flasks for phospholipids).

[a] $P < 0.02$ vs. control.

[b] $P < 0.05$ vs. control.

underestimated, the data suggest that a combination of a modest increase in the rate of triglyceride synthesis in association with a modest decrease in triglyceride transport could explain the accumulation of triglycerides in CaCo-2 cells incubated with 58-035. The acylamide also caused a marked decrease in the amount of cholesteryl esters that were transported in lipoproteins of d < 1.006 g/ml. This makes good sense and agrees with the observations of Bennett Clark and Tercyak (5).

We wish to thank Ms. Joan Dickman for the excellent assistance in the preparation of this manuscript. This work was supported by Atherosclerosis, Specialized Center of Research, grant HL 14230 from the National Heart, Lung, and Blood Institute, and DK 29706 from the National Institute of Diabetes and Digestive and Kidney Diseases. Dr. Kam is a recipient of a National Research Service Award, DK 08103 from the National Institute of Diabetes and Digestive and Kidney Diseases.

Manuscript received 4 April 1988, in revised form 16 June 1988, and in re-revised form 2 September 1989.

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