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Effect of lovastatin on acyl-CoA:cholesterol *O*-acyltransferase (ACAT) activity and the basolateral-membrane secretion of newly synthesized lipids by CaCo-2 cells

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Lovastatin, a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, was used to study the regulation of cholesterol metabolism and the basolateral-membrane secretion of triacylglycerol and cholesterol in the human intestinal cell line CaCo-2. At 0.1 μg/ml, lovastatin decreased ^3H_2O incorporation into cholesterol by 71%. In membranes prepared from cells incubated with lovastatin for 18 h, HMG-CoA reductase activity was induced 4–8-fold. Mevalonolactone prevented this induction. In intact cells, lovastatin (10 μg/ml) decreased cholesterol esterification by 50%. The reductase inhibitor decreased membrane acyl-CoA:cholesterol *O*-acyltransferase (ACAT) activity by 50% at 5 μg/ml. ACAT inhibition by lovastatin was not reversed by adding excess of cholesterol or fatty acyl-CoA to the assay. Lovastatin, in the presence or absence of mevalonolactone, decreased the basolateral secretion of newly synthesized cholesteryl esters and triacylglycerols. Lovastatin also inhibited the esterification of absorbed cholesterol and the secretion of this newly synthesized cholesteryl ester. Lovastatin is a potent inhibitor of cholesterol synthesis in CaCo-2 cells. Moreover, it is a direct inhibitor of ACAT activity, independently of its effect on HMG-CoA reductase and cholesterol synthesis.

INTRODUCTION

The intestinal absorptive cell synthesizes and secretes triacylglycerol-rich lipoproteins in response to an influx of long-chain fatty acids [1]. Because unesterified cholesterol is a necessary surface component for the lipoprotein particle, sufficient amounts of this sterol must be readily available to the cell for normal triacylglycerol transport to occur. There is some indirect evidence to suggest that unesterified cholesterol may be limiting during the active synthesis and secretion of triacylglycerol-rich lipoproteins by the intestine. Gebhard & Prigge [2] observed that HMG-CoA reductase activities were increased in mucosal biopsies obtained from isolated intestinal segments of dogs which had been perfused with various fatty acids. In addition, Stange & Dietschy [3] found an increase in the rates of cholesterol synthesis in intestines of rats fed on corn oil compared with rates observed in intestines of animals fed on normal chow. The results from both studies suggest that an increase in newly synthesized cholesterol is necessary to meet the increased demands for cholesterol during triacylglycerol transport. Other studies, however, have been less convincing. Bochenek & Rodgers [4] failed to observe a regulation of cholesterol synthesis in intestines of rats after the ingestion of diets enriched in safflower oil or tripalmitin oil. In another study, diets supplemented with menhaden oil or cocoa-butter oil, if anything, decreased HMG-CoA reductase activities in intestines of rabbits [5]. Lastly, neither HMG-CoA reductase nor ACAT activities in the human intestinal cell line CaCo-2 were altered by stimulating triacylglycerol-rich lipoprotein synthesis by adding 250 μM-oleic acid to the culture medium [6]. Therefore, in contrast with the results of the previous studies, the results of the present studies would imply that sufficient amounts of unesterified cholesterol are available within the intestinal cell, thereby making it unnecessary to increase the rate of newly synthesized cholesterol for the transport of triacylglycerols.

Lovastatin, a potent competitive inhibitor of the rate-controlling enzyme of cholesterol synthesis, HMG-CoA reductase, is being widely used in the treatment of hyper-cholesterolaemia in man [7,8]. In patients taking this drug for that purpose, Freeman *et al.* [9] demonstrated that HMG-CoA reductase activity in the small intestine of these individuals was decreased by approx. 50%. It is therefore possible that, under conditions of fat absorption, unesterified cholesterol may be limiting in the intestine of patients taking this drug. This could, in turn, alter triacylglycerol transport. In the present study, lovastatin was used as a tool to investigate the regulation of cholesterol metabolism and triacylglycerol transport in the human intestinal cell line CaCo-2. The results suggest that lovastatin is a potent inhibitor of cholesterol synthesis in these cells. Moreover, independently of its action on HMG-CoA reductase activity, lovastatin is also an inhibitor of cholesterol esterification and ACAT activity. Associated with its inhibition of ACAT activity, lovastatin causes a decrease in the basolateral-membrane secretion of newly synthesized cholesteryl esters and triacylglycerols in CaCo-2 cells.

METHODS

Materials

[9,10-^{3}H]Oleic acid, ^3H_2O, [4-^{14}C]cholesterol, [1-^{14}C]oleoyl-CoA, [5-^{3}H]mevalonolactone, 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA and [1,2-^{3}H]cholesterol were purchased from New England Nuclear (Boston, MA, U.S.A.). Oleic acid, oleoyl-CoA, progesterone, egg phosphatidylcholine, mevalonolactone, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HMG-CoA was from P-L Biochemicals (Milwaukee, WI, U.S.A.). Lovastatin was generously given by

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ACAT, acyl-CoA:cholesterol *O*-acyltransferase; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; DMSO, dimethyl sulphoxide.

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Dr. A. Alberts, of Merck, Sharp and Dohme (Rahway, NJ, U.S.A.). All other chemicals were reagent grade.

Cell culture

CaCo-2 cells were cultured as previously described [10]. For experiments performed on micropore filters, CaCo-2 cells were placed on fibronectin-treated nucleopore polycarbonate filters (Transwells-3412; 0.4 μm pore size; Costar, Cambridge, MA, U.S.A.) separating an upper and a lower well. Medium in the upper (1.5 ml) and lower (2.6 ml) wells was changed every other day. The cells were used 10 or 11 days after plating, and by this time were 5 or 6 days post-confluent. Because CaCo-2 cells grown on micropore filters or plastic remain polarized with their basolateral membranes abutting the growing surface and their apical membranes facing the growth medium, cells grown on permeable membranes are ideal for studying the polarized secretion of lipids. They form tight monolayers, as assessed by electron microscopy. Tight junctions were documented by freeze-fracture. To estimate 'tightness', transport of albumin from the top well to the bottom well was measured as described by Shasby & Shasby [11]. Cells were not used if more than 1% of the albumin was found in the lower well after 60 min. Cell viability was monitored by Trypan Blue exclusion, cell protein content and light microscopy. Lovastatin had no significant effect on CaCo-2-cell viability under the experimental conditions used.

Lipid synthesis

The rates of synthesis of cholesteryl ester, triacylglycerol and phospholipid were estimated by oleic acid incorporation into the respective lipid class as previously described [10]. Cholesterol synthesis was estimated by the rate of incorporation of ^3H_2O into cholesterol. CaCo-2 cells were incubated in 1 ml of serum-free medium containing 15 mCi of ^3H_2O. At the time indicated in the Figure legends, the cells were washed extensively in phosphate-buffered saline and scraped from the dish in 1 ml of 2 M-KOH. The dish was rinsed with 2 × 1 ml of methanol and combined with the alkali-solubilized cells. The lipids were saponified at 80 °C for 2 h in tightly capped tubes. The non-saponifiable fraction was extracted twice with hexanes. The hexane layer was washed once with 0.5 M-NaOH in water/ethanol (1:1, v/v). The aqueous layer was removed and the hexane layer was washed again with water/ethanol (1:1, v/v). The hexane layer was dried down under nitrogen and dissolved in a known volume of hexane. A sample was taken for determination of total radioactivity. The remainder was again dried and the residue plated on silica gel G t.l.c. plates and eluted in hexane/diethyl ether/acetic acid (70:30:1, by vol.). The plate was scanned with a Vanguard autoscanner and the band corresponding to cholesterol was scraped into liquid-scintillation fluid and counted for radioactivity.

In experiments using the Transwells, medium containing fetal-bovine serum was removed from the bottom well and was replaced with M199 (medium-199/Earle's; Gibco, Grand Island, NY, U.S.A.) containing 10 mM-Hepes, without fetal-bovine serum, pH 7.4. The medium from the top well was also removed and the cells were washed extensively with the M199 buffer. Lovastatin was added to 1.5 ml of M199 in 0.01 ml of dimethyl sulphoxide (DMSO). After incubation with the reductase inhibitor, the medium was removed and replaced by M199 containing 1 mM-taurocholate, 50 μM-cholesterol and 25 μM-[^{3}H]oleic acid, (sp. radioactivity 30000 d.p.m./nmol) [10]. Lovastatin or its vehicle was added back to the wells which had them previously. After 3 and 6 h, media from the upper and lower wells were removed and centrifuged at 2000 rev./min for 15 min to remove any intact cells or debris. The supernatants from the media were extracted with chloroform:methanol (2:1, v/v). The chloroform phase was evaporated to dryness under a stream of nitrogen. Chloroform (0.125 ml) was added and plated on silica gel G t.l.c. plates. The plates were eluted in hexanes/diethyl ether/methanol/acetic acid (85:15:1:1, by vol.). Bands corresponding to cholesteryl esters, triacylglycerols and phospholipids were scraped from the plates and counted for radioactivity. The specific radioactivity of the added labelled oleate was used to calculate the secretion of individual lipids into the lower well. The true specific radioactivities of the lipids within the cell are unknown. The calculated rates of secretion are therefore rough estimates, and only qualitative comparisons can be made.

Cholesterol uptake, esterification and basolateral secretion

CaCo-2 cells were cultured on micropore filters as described above. After preincubation with or without lovastatin, [^{14}C]cholesterol (50 μM; sp. radioactivity 5000 d.p.m./nmol), solubilized in M199 containing 1 mM-taurocholate and 30 μM-mono-olein, was added to the apical side [10]. Lovastatin was added back to the cultures which had it previously. At the end of the incubation, the lower media and cells were analysed as described for labelled oleate incorporation, except that bands corresponding to unesterified and esterified cholesterol were scraped from the plates and counted for radioactivity.

Enzyme assays

HMG-CoA reductase activity was measured as described in [12]. Specific radioactivity of the substrate was 23000 d.p.m./nmol. ACAT activity was measured as described previously [13], with oleoyl-CoA of specific radioactivity 25500 d.p.m./nmol. In one experiment, liposomes containing cholesterol solubilized in egg phosphatidylcholine (2:1, mol/mol) were prepared as previously described [14], and added to the assays before determination of ACAT activity. Activities were measured in total membrane preparations [6].

Chemical analysis

Protein was determined by the method of Lowry *et al.* [15]. Cholesterol was determined by g.l.c. with cholestane as an internal standard [16].

Statistical analysis

Student's unpaired *t* test was used to determine significance.

RESULTS

Effect of lovastatin on cholesterol synthesis

To investigate the effect of lovastatin on the rate of cholesterol synthesis, CaCo-2 cells were preincubated for 20 min in serum-free medium containing increasing concentrations of the inhibitor. ^3H_2O was added, and the incorporation of water into cholesterol was determined. Results in Fig. 1(*a*) show that lovastatin was a potent inhibitor of cholesterol synthesis in CaCo-2 cells. At a concentration of 0.1 μg/ml, the rate of cholesterol synthesis was decreased by 71%. Maximal inhibition occurred between concentrations of 0.2 and 0.4 μg/ml.

The effect of lovastatin on the rate of cholesterol synthesis in CaCo-2 cells was rapid (Fig. 1*b*). A decrease in water incorporation into cholesterol by lovastatin was apparent within minutes after adding the inhibitor to the culture medium.

Effect of lovastatin on HMG-CoA reductase activity

Total membranes were prepared from CaCo-2 cells grown in medium containing fetal-calf serum for 11 days. Before measurement of HMG-CoA reductase activity, membranes were preincubated for 15 min with increasing concentrations of

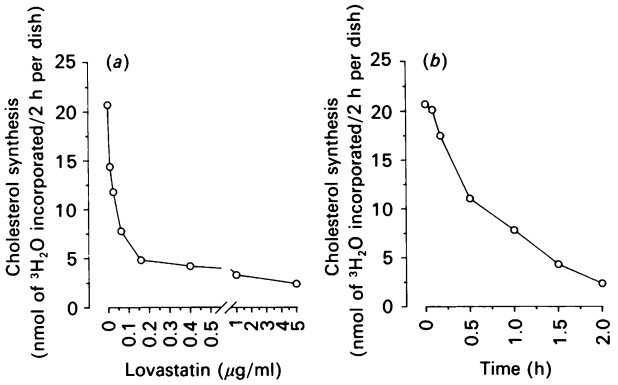

Fig. 1. Effect of lovastatin on ^3H_2O incorporation into cholesterol in CaCo-2 cells

(*a*) CaCo-2 cells were preincubated for 20 min in 1 ml of serum-free medium containing increasing concentrations of lovastatin in 0.01 ml of DMSO. Control cells received the same amount of DMSO but without the inhibitor. The cells were washed three times with phosphate-buffered saline, and 1 ml of serum-free medium was added containing 15 mCi of ^3H_2O. Lovastatin was added back to the dishes which had it originally. After 2 h, the medium was removed and cells were extensively washed and scraped from the dish. Lipids were extracted, saponified and separated by t.l.c. as described in the Methods section. (*b*) CaCo-2 cells were incubated for 2 h in 1 ml of serum-free medium containing 15 mCi of ^3H_2O. At the indicated time, lovastatin (5 μg/ml) was added. The data represent means ± S.E.M. of 3–5 dishes at each concentration or time point. Error bars are absent because they are smaller than the data points.

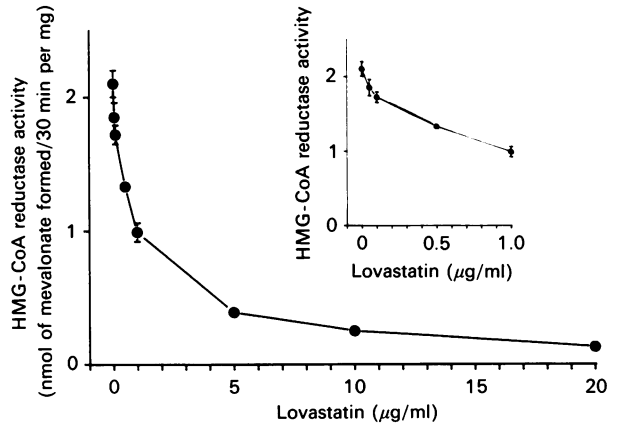

Fig. 2. Effect of lovastatin on HMG-CoA reductase activity in CaCo-2 cell membranes

Total membranes were prepared from CaCo-2 cells grown for 11 days in medium containing fetal-calf serum. Lovastatin was added to the membranes in DMSO (1 %, v/v). Control membranes received the same amount of DMSO but without the inhibitor. After 15 min, HMG-CoA reductase activity was determined. The insert shows the effect of lovastatin on HMG-CoA reductase activity at concentrations less than 1 μg of the drug/ml.

lovastatin. Reductase activity was then determined by adding the labelled substrate and cofactors (Fig. 2). At a lovastatin concentration of 1 μg/ml, HMG-CoA reductase activity was decreased by 50%. At higher concentrations of the inhibitor, further inhibition of reductase activity was observed.

It has been demonstrated in other cells that, when HMG-CoA reductase activity is competitively inhibited, the amount of

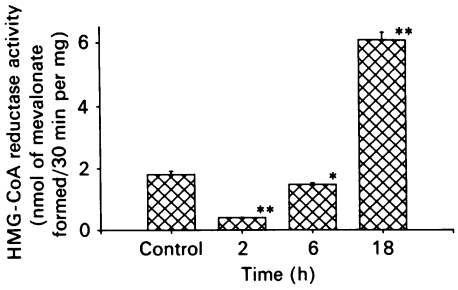

Fig. 3. HMG-CoA reductase activity in membranes prepared from CaCo-2 cells incubated with lovastatin

CaCo-2 cells were incubated for 18 h in 1 ml of serum-free medium. At the indicated time, lovastatin (5 μg/ml) was added. After incubation for 2, 6 and 18 h with the inhibitor, the cells were washed three times in cold phosphate-buffered saline. The cells were scraped from the dish and sonicated at 4 °C in a buffer containing 0.1 M-sucrose, 0.05 M-KCl, 0.04 M-KH_2PO_4 and 0.03 M-EDTA, pH 7.4. The whole homogenate was centrifuged at 105000 *g* for 1 h. The membrane pellet was washed twice by dilution with the above buffer and re-centrifugation. The resulting pellet was used to measure the activity of HMG-CoA reductase. The data represent means ± S.E.M. of triplicate determinations on three individual dishes: $*P < 0.05$ and $**P < 0.001$ versus control.

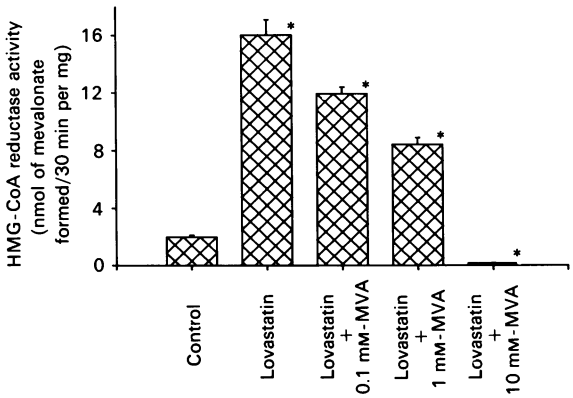

Fig. 4. HMG-CoA reductase activity in membranes prepared from CaCo-2 cells incubated with lovastatin with or without mevalonate

CaCo-2 cells were incubated for 18 h in 1 ml of serum-free medium containing 5 μg of lovastatin/ml with or without the indicated concentrations of mevalonolactone. At the end of 18 h, total membranes were prepared and washed as described in Fig. 3, and HMG-CoA reductase activities were determined. The data represent means ± S.E.M. of triplicate assays for at least three individual dishes in each group: $*P < 0.001$ versus control. Abbreviation: MVA, mevalonolactone.

intracellular enzyme increases. On removal of the inhibitor, the induction of enzyme protein is unmasked, giving a large increase in the activity of HMG-CoA reductase [17,18]. To investigate the effect of lovastatin on HMG-CoA reductase activity in CaCo-2 cells incubated with the inhibitor, total membranes were prepared from cells which had been incubated for 2, 6 and 18 h with 5 μg of lovastatin/ml. In an attempt to remove the inhibitor completely, the membrane preparations were diluted and re-centrifuged several times. As shown in Fig. 3, HMG-CoA reductase activities in membranes prepared from CaCo-2 cells which were incubated with lovastatin for 2 and 6 h were decreased compared with the activity observed in membranes prepared from control cells. In contrast, the activity of HMG-CoA

reductase in membranes prepared from cells incubated for 18 h with lovastatin was increased approx. 3.5-fold over the activity in control membranes.

To determine if the addition of mevalonate would prevent the induction of reductase by lovastatin, CaCo-2 cells were incubated for 18 h in the presence of 5 μg of lovastatin/ml and increasing concentrations of mevalonolactone. Total membranes were then prepared and HMG-CoA reductase activity was determined. As shown in Fig. 4, the addition of mevalonolactone significantly inhibited the induction of reductase protein, as estimated by enzyme activity in CaCo-2 cells incubated with lovastatin. At a mevalonolactone concentration of 10 mM, HMG-CoA reductase activity was decreased below that observed in membranes prepared from control cells.

Effect of lovastatin on cellular lipid synthesis

To investigate the regulation of triacylglycerol transport by lovastatin, it was first necessary to determine its effect on lipid synthesis in CaCo-2 cells. Cells were preincubated for 30 min with increasing concentrations of lovastatin. Labelled oleate was then added, and the rate of incorporation of the fatty acid into cholesteryl ester, triacylglycerol and phospholipid was determined over the next 30 min. As indicated by the data shown in Fig. 5, lovastatin had no significant effect on the incorporation of oleate into triacylglycerols or phospholipids at any of the concentrations tested. The incorporation of oleate into cholesteryl oleate, however, was significantly decreased. At a lovastatin concentration of 10 μg/ml, the rate of cholesterol esterification was decreased by over 50%, and at 25 μg/ml it was decreased by 80%.

Effect of lovastatin on ACAT activity

A decrease in the rate of cholesterol esterification in CaCo-2 cells by lovastatin was unexpected. Because the incorporation of oleate into triacylglycerols and phospholipids was not affected by the inhibitor, a general effect on oleate uptake or esterification could not explain the results. It did not appear likely that within 1 h lovastatin could deplete a cholesterol pool utilized by ACAT, particularly when it is thought that newly synthesized cholesterol contributes very little to the ACAT pool in the intestine [3]. To test this, cells were incubated with 25 μg of lovastatin/ml in the

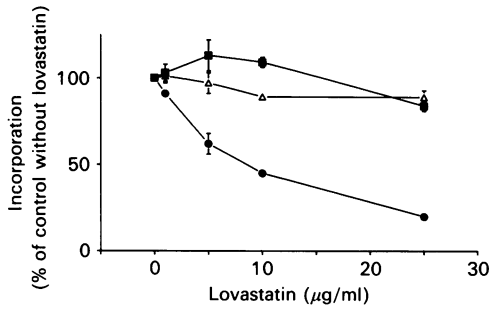

Fig. 5. Effect of lovastatin on [^{3}H]oleate incorporation into CaCo-2 cell lipids

CaCo-2 cells were preincubated for 30 min in 1 ml of serum-free medium containing the indicated concentrations of lovastatin. The cells were then washed, and 1 ml of serum-free medium containing 50 μM-[^{3}H]oleic acid (sp. radioactivity 5.5 d.p.m./pmol) attached to albumin was added. Lovastatin was added back to the dishes which had it originally. After 30 min, the cells were washed twice with phosphate-buffered saline. The cells were scraped from the dish, sonicated, and lipids were extracted as described in the Methods section. The lipids were separated by t.l.c. The data represent means ± S.E.M. from 6–9 dishes at each concentration. Control values for cholesteryl esters, triacylglycerols and phospholipids were 150 ± 15 pmol/dish, 3.0 ± 0.3 nmol/dish and 1.6 ± 0.2 nmol/dish respectively. Symbols: ●, cholesteryl esters; △, triacylglycerols; ■, phospholipids.

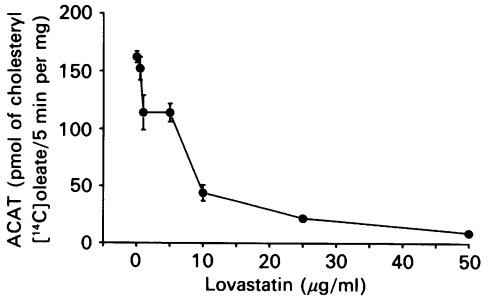

Fig. 6. Effect of lovastatin on ACAT activity in CaCo-2 cell membranes

The experiment was performed as described in Fig. 2. ACAT activity was determined after a 15 min preincubation with the inhibitor. The data represent means ± S.E.M. of assays done in triplicate.

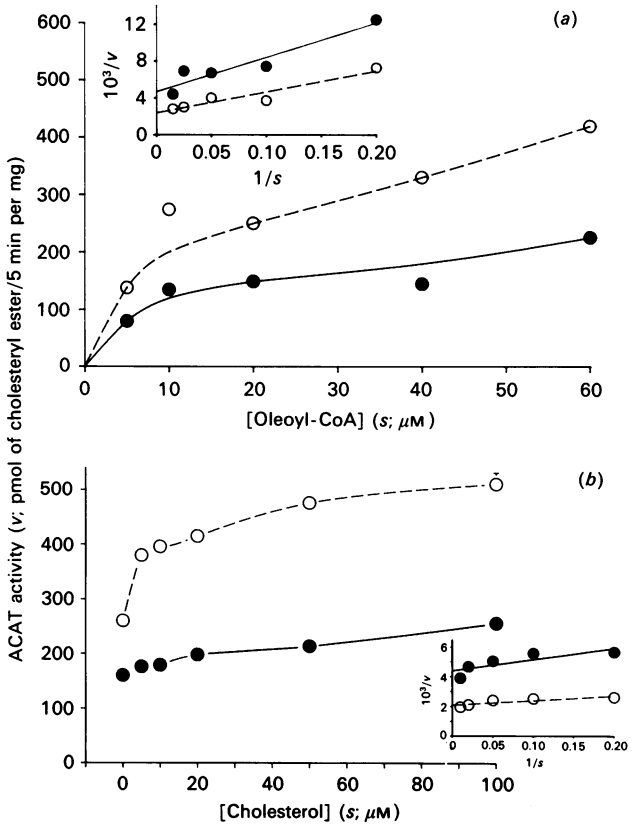

Fig. 7. Effect of lovastatin on ACAT activity in CaCo-2 cell membranes

Lovastatin (5 μg/ml) was added to total membranes prepared from CaCo-2 cells grown for 11 days in medium containing fetal-calf serum. Lovastatin was added to the membranes in DMSO (1%, v/v). Control membranes received the same amount of DMSO but without the inhibitor. (*a*) The reaction was started by adding increasing concentrations of labelled oleoyl-CoA. (*b*) Cholesterol, solubilized in egg phosphatidylcholine liposomes (2 mol of cholesterol: 1 mol of phosphatidylcholine) was added in increasing concentrations. After 1 h of preincubation, ACAT activity was measured. The data represent means ± S.E.M. of triplicate incubations and assays performed in triplicate. Key: ●, lovastatin; ○, control.

absence or presence of increasing concentrations of mevalonolactone. The incorporation of labelled oleate into cholesteryl oleate was again used to estimate cholesteryl ester synthesis. Lovastatin decreased the rate of cholesterol esterification by 90%. The addition of mevalonolactone at concentrations of 0.1 mM, 1 mM and 10 mM did not reverse the inhibition of cholesterol esterification by lovastatin (results not shown).

To address the possibility that lovastatin was a direct inhibitor of ACAT activity, total membranes were prepared from CaCo-2 cells grown in medium containing fetal-calf serum. At 15 min before measurement of ACAT activity, lovastatin was added to the membranes in increasing concentrations. Fig. 6 shows these results. Lovastatin resulted in a step-wise decrease in ACAT activity, with a 50% decrease occurring at a lovastatin concentration between 5 and 10 μg/ml. The inhibitory effect on membrane ACAT activity was 5–10 times less potent than its effect on membrane HMG-CoA reductase activity (Fig. 2).

To determine if either substrate of ACAT, oleoyl-CoA or cholesterol, could compete with lovastatin for the enzyme, ACAT activity in CaCo-2 cell membranes was measured in the presence of 5 μg of lovastatin/ml and increasing concentrations of oleoyl-CoA or cholesterol (Fig. 7). At concentrations of oleoyl-CoA ranging from 5 to 60 μM, the inhibitory effect of lovastatin on ACAT activity was similar. The reciprocal plot suggests that no competition existed (Fig. 7*a*). To test whether cholesterol competed with lovastatin for this enzyme, cholesterol was initially added to the assay, solubilized in Triton WR-1339 [19]. The detergent, however, markedly interfered with the assay. To circumvent this problem, cholesterol was added in egg phosphatidylcholine liposomes. The liposomes alone without cholesterol had no effect on baseline ACAT activity. Under these conditions, added cholesterol did not compete with lovastatin for the enzyme (Fig. 7*b*).

Effect of lovastatin on lipid secretion

CaCo-2 cells were grown on polycarbonate micropore filters which separate an upper and a lower well. Lovastatin, at a concentration which would maximally inhibit both HMG-CoA reductase and ACAT activities (25 μg/ml), was added to the apical media. To circumvent the inhibition of HMG-CoA reductase, some of the wells also received 10 mM-mevalonolactone. Control cells received the vehicle alone. After a preincubation for 30 min, a clear micellar solution containing 1 mM-taurocholate, 50 μM-cholesterol and 25 μM-[^{3}H]oleic acid was added to the apical surface. Lovastatin or lovastatin plus mevalonolactone was added back to the cells which had them originally. The incorporation of labelled oleate into cholesteryl oleate, triacylglycerols and phospholipids was determined in cells, and in the apical and basal media. Fig. 8 shows results after 6 h. A 3 h time point provided the same information, but with 50% less label incorporated into the lipids. As expected from the previous data, lovastatin significantly inhibited the incorporation of oleate into cellular cholesteryl oleate. Likewise, the basal secretion of newly synthesized cholesteryl oleate was markedly decreased. Essentially no apical secretion of cholesteryl esters was observed. As observed above, the addition of mevalonolactone did not significantly increase the incorporation of oleate into cellular cholesteryl oleate. It also had no observable effect on the basal secretion of labelled cholesteryl esters.

Lovastatin modestly increased the rate of oleate incorporation into cellular triacylglycerols, an observation noted previously when ACAT is inhibited [20]. The basal secretion of newly synthesized triacylglycerols, however, was significantly decreased in CaCo-2 cells incubated with the inhibitor. The addition of mevalonolactone had no effect on the secretion of labelled triacylglycerols. Again, apical secretion of labelled triacylglycerols was a minor component compared with basal secretion, and was unaffected by lovastatin or mevalonolactone.

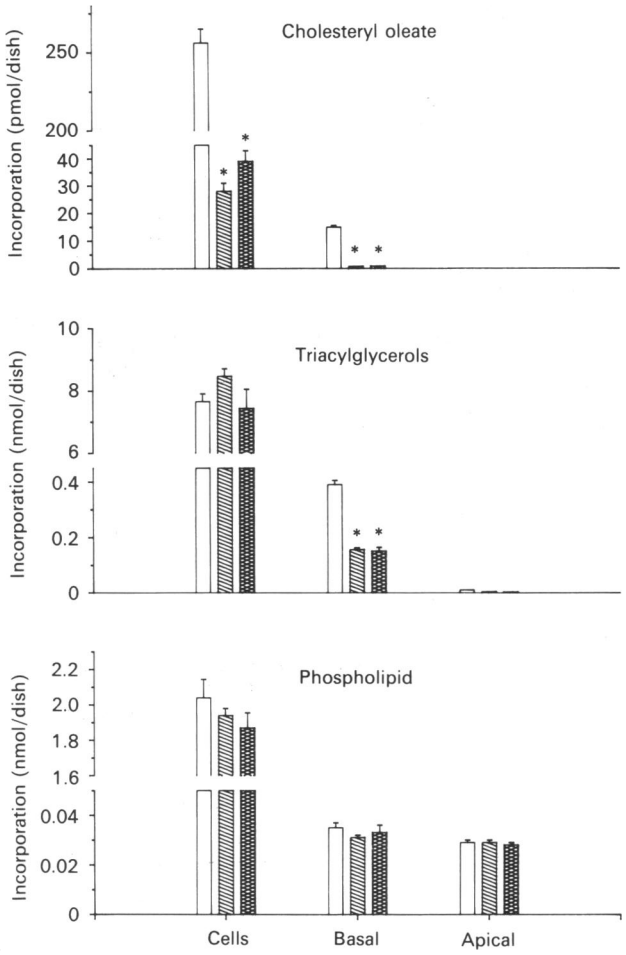

Fig. 8. Effect of lovastatin with or without mevalonolactone on the incorporation of [^{3}H]oleate into CaCo-2 cellular and media lipids

CaCo-2 cells were cultured on micropore polycarbonate filters separating an upper and a lower well. At the start of the experiment, the lower well was changed to serum-free medium. The cells were exhaustively washed, and 1.5 ml of serum-free medium containing 25 μg of lovastatin/ml without or with 10 mM-mevalonolactone and 1 mM-taurocholate, 50 μM-cholesterol and 25 μM-[^{3}H]oleic acid (sp. radioactivity 30 d.p.m./pmol) was added. After 3 and 6 h, the incorporation of [^{3}H]oleic acid into cholesteryl esters, triacylglycerols and phospholipids in cells and both apical and basal medium was determined as described in the Methods section. The data represent means ± S.E.M. of 4–8 sets of cells at the 6 h time point: $*P < 0.001$ versus control. Key: □, control; ▨, lovastatin; ■, lovastatin plus mevalonolactone.

The incorporation of oleate into phospholipids in cells and apical and basal media was unchanged by lovastatin or mevalonolactone. Unlike secretion of cholesteryl ester and triacylglycerols, which was basally oriented, phospholipid secretion lacked polarity.

Effect of lovastatin on cholesterol uptake, esterification and secretion

Because lovastatin inhibits ACAT activity and ACAT inhibitors have been shown to decrease cholesterol absorption [21,22], the effect of this inhibitor on cholesterol uptake, esterification and secretion was determined. CaCo-2 cells were grown on the filters separating upper and lower wells. Lovastatin

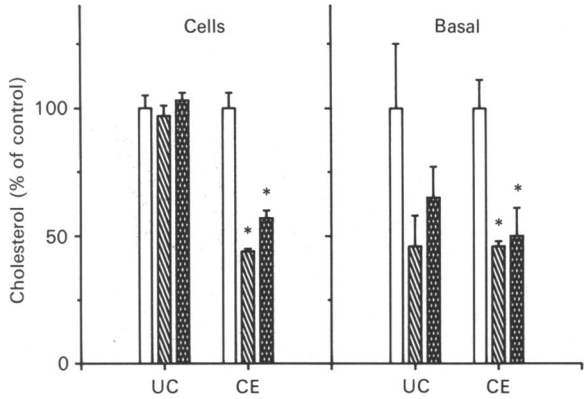

Fig. 9. Effect of lovastatin with or without mevalonolactone on the uptake, esterification and secretion of micellar [^{14}C]cholesterol by CaCo-2 cells

CaCo-2 cells were cultured on micropore filters as described in Fig. 8. Lovastatin (25 μg/ml) without or with 10 mM-mevalonolactone and a clear solution containing 1 mM-taurocholate, 50 μM-[^{14}C]-cholesterol (sp. radioactivity 5000 d.p.m./nmol) and 30 μM-mono-olein was added to the apical well. After 3 and 6 h, the amounts of [^{14}C]cholesterol and [^{14}C]cholesteryl ester associated with the cell and that released into the lower well were determined. The data represent means ± S.E.M. of 4–8 sets of cells from the 6 h incubation (*$P < 0.02$ versus control). The values for cells incubated with lovastatin or lovastatin plus mevalonolactone were expressed relative to control incubations which were designated as 100%. Control values for cellular UC (unesterified cholesterol) and CE (cholesteryl ester) were 2.71 ± 0.08 and 0.143 ± 0.015 nmol/dish respectively. Values for basal UC and CE were 124 ± 50 and 4 ± 0.2 pmol/dish respectively. Key: □, control; ▨, lovastatin; ■, lovastatin plus mevalonolactone.

(25 μg/ml) with or without 10 mM-mevalonolactone was added to the apical surface for 30 min. The apical medium was then replaced with a clear micellar solution containing 1 mM-taurocholate, 30 μM-mono-olein and 50 μM of labelled cholesterol. Lovastatin, with or without mevalonolactone, was added back to the cells which had them previously. After 6 h, the amount of cellular labelled cholesterol and the amount secreted basolaterally were determined (Fig. 9). Lovastatin had no effect on the amount of labelled unesterified cholesterol associated with the cell. It did, however, decrease the amount of cholesteryl esters synthesized from the absorbed cholesterol by 60%. Cells exposed to lovastatin secreted 55% less labelled unesterified cholesterol compared with control cells, but variability within experiments and between experiments resulted in a change which was not statistically significant. Lovastatin did, however, significantly decrease the basal secretion of labelled cholesteryl esters by 50%. The addition of 10 mM-mevalonolactone to CaCo-2 cells containing lovastatin did not significantly affect any of the parameters measured in this experiment.

DISCUSSION

The intended purpose of this work was to investigate the regulation of triacylglycerol transport by inhibiting cholesterol synthesis in CaCo-2 cells. Lovastatin, a potent competitive inhibitor of HMG-CoA reductase, did indeed decrease the basolateral secretion of newly synthesized triacylglycerols and cholesteryl esters by CaCo-2 cells (Fig. 8). This regulatory effect, however, was independent of the drug's inhibitory action on HMG-CoA reductase. After addition of 10 mM-mevalonolactone, an amount sufficient to satisfy the cell's requirement for cholesterol or a non-sterol derivative of mevalonate, the observed decrease in triacylglycerol and cholesteryl ester transport by lovastatin was unaltered. We have recently shown that the ACAT inhibitor, 58-035, will decrease secretion of triacylglycerol-rich lipoprotein by CaCo-2 cells grown on an impermeable surface, resulting in the cellular accumulation of triacylglycerols [20]. This led us to suspect that lovastatin may also be affecting ACAT activity, a suspicion which was subsequently proved by the present studies. During the preparation of this manuscript, Kobayashi *et al.* [23] reported that simvastatin, a related HMG-CoA reductase inhibitor, prevented an increase in serum cholesterol by markedly decreasing VLDL and LDL in cholesterol-fed rabbits. Because ACAT inhibitors have been shown to decrease cholesterol absorption in cholesterol-fed rabbits and rats [21,22], those investigators proceeded to study the effect of simvastatin and other HMG-CoA reductase inhibitors on intestinal ACAT activity [24]. They found that simvastatin did inhibit intestinal ACAT in rabbits fed on cholesterol, and that this was associated with a significant decrease in cholesterol absorption [25,26]. The lactone forms of the agents appear to be necessary for ACAT inhibition, as the dihydroxy acid derivatives of these compounds lacked the inhibitory effect [24]. The present work supports those observations and extends them. Lovastatin inhibits ACAT activity in a human intestinal cell by a mechanism which does not involve competition with the enzyme for either of its substrates. By its inhibitory effect on ACAT, lovastatin decreases the basal secretion of newly synthesized cholesteryl ester and triacylglycerol.

The results suggest that cholesteryl ester formation or a small pool of intracellular cholesteryl ester is more important for normal triacylglycerol transport by the intestine than is the availability of newly synthesized cholesterol. Goh & Heimberg [27], however, have proposed that unesterified cholesterol is limiting during the active secretion of VLDL by the liver. Recently, Khan *et al.* [28] studied VLDL-lipid secretion in perfused livers of rats given lovastatin. They found that VLDL-lipid production was significantly decreased in animals ingesting lovastatin. Hepatic concentrations of unesterified cholesterol, however, were not altered by lovastatin, but cholesteryl ester content was decreased by 55%. Moreover, a close correlation between the secretion of VLDL triacylglycerol and hepatic cholesteryl ester content was observed. These investigators suggested that perhaps a metabolically active pool of cholesteryl ester was necessary for the synthesis and secretion of VLDL. Our data in CaCo-2 cells would agree in principle with that hypothesis. We would postulate further, however, that in that study by Khan *et al.* [28] lovastatin was regulating cholesteryl ester content and VLDL secretion by its inhibition of ACAT activity, and not by its effect on newly synthesized cholesterol.

Similar to other cell lines which have been studied, lovastatin or its analogue, compactin, is a potent inhibitor of both cholesterol synthesis and HMG-CoA reductase activity in CaCo-2 cells [17,18,29,30]. Likewise, when mevalonate production is suppressed and feedback inhibition is lost, overproduction of the enzyme occurs [18,31]. By addition of excess mevalonate to CaCo-2 cells incubated with lovastatin, the induction of reductase activity is prevented [18]. Thus the regulation of cholesterol synthesis and HMG-CoA reductase activity by lovastatin in CaCo-2 cells is similar to that observed in normal intestinal mucosa and in other non-malignant tissue.

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