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EFFECTS OF NB-598, A POTENT SQUALENE EPOXIDASE INHIBITOR, ON THE APICAL MEMBRANE UPTAKE OF CHOLESTEROL AND BASOLATERAL MEMBRANE SECRETION OF LIPIDS IN CACO-2 CELLS

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Abstract—Caco-2 cells grown on membrane filters were used as a model to study the effects of NB-598, an inhibitor of squalene epoxidase, on cholesterol absorption from the intestinal epithelia. NB-598 (10 μM) inhibited the synthesis of sterol and sterol ester from [^{14}C]acetate without affecting the synthesis of other lipids such as phospholipids (PL), free fatty acids (FFA) and triacylglycerol (TG). When labeled lipid was apically loaded as a micellar lipid solution into Caco-2 cell cultures, NB-598 reduced basolaterally secreted radioactivity in cholesterol, cholesterol ester, PL and TG. Furthermore, NB-598 suppressed the basolateral secretion of apolipoprotein (apo) B. When microsomes prepared from control Caco-2 cells were incubated with 10 μM NB-598, acyl CoA:cholesterol acyltransferase (ACAT) activity was inhibited slightly. After incubating Caco-2 cells with 10 μM NB-598, a slight reduction in cellular ACAT activity was also observed. These results suggest that suppression of the secretion of particles containing apo B and reduction of cellular ACAT activity in the intestinal epithelia are part of the mechanism of the cholesterol-lowering effect of NB-598.

NB-598 [(*E*)-*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3′-bithiophen-5-yl)methoxy]benzenemethanamine hydrochloride] is a potent mammalian squalene epoxidase inhibitor [1]. We previously reported that NB-598 (10 mg/kg) decreases serum levels of cholesterol and triacylglycerol (TG†) in dogs by 34 and 59%, respectively [2]. The serum cholesterol pool is regulated by three factors: (1) absorption of exogenous cholesterol from the diet, (2) endogenous cholesterol synthesis, and (3) excretion of cholesterol and/or its metabolites, the bile acids [3]. In humans, approximately 40% of serum cholesterol is dietary in origin [3]. In contrast, in dogs about 85% of serum cholesterol is derived from dietary cholesterol [3]. Therefore, questions arise as to why NB-598 markedly decreases serum cholesterol levels in dogs. We have already found that NB-598 slightly increases the fecal excretion of cholesterol in dogs [2]. Therefore, NB-598 is thought to suppress the absorption of dietary cholesterol.

We have reported that an inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, simvastatin [4], decreases the absorption of cholesterol in cholesterol-diet-fed rabbits [5, 6]. According to Ishida *et al.* [7], this mechanism is related to a reduction in microsomal acyl CoA:cholesterol acyltransferase (ACAT) activity in the intestinal mucosa. They concluded that endogenous cholesterol synthesis in the intestinal mucosa may be related to the regulation of microsomal ACAT activity [7]. Kam *et al.* [8] reported that another HMG-CoA reductase inhibitor, lovastatin, suppresses the basolateral secretion of TG, free cholesterol and cholesterol ester in human colon carcinoma Caco-2 cells on membrane filters. They suggested that the efficacy of lovastatin was independent of the inhibitory action of the drug on HMG-CoA reductase and was related to its inhibitory action on ACAT activity [8].

Caco-2 cells grown on membrane filters have been reported to exhibit physiological, morphological and biochemical characteristics of small intestinal epithelia [9]. Therefore, we examined the effects of NB-598 on lipid uptake and lipoprotein secretion in Caco-2 cells grown on membrane filters. Moreover, we studied the effects of NB-598 on ACAT activity in Caco-2 cells. L-654,969, an HMG-CoA reductase inhibitor (the open-acid form of simvastatin) [4], and melinamide (an ACAT inhibitor) [10] were used as reference drugs.

MATERIALS AND METHODS

Materials. NB-598 was synthesized in our laboratories. L-654,969 was prepared at Merck Research Laboratories (Rahway, NJ, U.S.A.). Melinamide (Artes®) was obtained from Sumitomo Pharmaceutical (Osaka, Japan) and was purified in

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† Abbreviations: TG, triacylglycerol; PL, phospholipids; FFA, free fatty acids; apo, apolipoprotein; ACAT, acyl CoA:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; DMEM, Dulbecco's modified Eagle's minimum essential medium; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; PBS, phosphate-buffered saline; and ELISA, enzyme-linked immunosorbent assay.

our laboratories. [2-^{14}C]Acetic acid, sodium salt (56.2 mCi/mmol) was obtained from Amersham (Bucks, U.K.). [9,10-^{3}H(N)]Oleic acid (7.4 Ci/mmol), [1-^{14}C]oleoyl CoA (57.8 mCi/mmol) and [1,2-^{3}H(N)]cholesterol (41.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Human low density lipoprotein (LDL) and purified human apolipoprotein (apo) A-I were obtained from Sigma (St. Louis, MO, U.S.A.). Human high density lipoprotein (HDL) was obtained from Protogen (Laufelfingen, Switzerland). Standard serum for human apo B was obtained from Daiichi Pure Chemicals (Tokyo, Japan). Mouse monoclonal antibody, MAB-012, which recognizes both human apo B100 and apo B48, and another mouse monoclonal antibody, AB-AI-1, which recognizes human apo A-I, were obtained from Canadian Bioclinical (Ontario, Canada). Horseradish peroxidase conjugated goat anti-mouse IgG was purchased from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals used were standard high purity materials.

Cell culture. Caco-2 cells (No. HTB37: a human colon carcinoma cell line) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were grown in flasks containing medium A [Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 1% (w/v) non-essential amino acids, penicillin G (50 μg/mL), streptomycin (50 μg/mL) and 20% (v/v) fetal bovine serum] in a humidified incubator [5% (v/v) CO_2] at 37°. For the maintenance of stock cultures, a nearly confluent cell layer was dissociated with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA, and the cells in medium A were replated at a concentration of approximately 2×10^4 cells/cm^2 into new flasks. For the experiments, medium A was changed to medium B [10 mM Hepes-buffered DMEM (pH 7.4) containing 1% (w/v) non-essential amino acids, penicillin G (50 μg/mL), streptomycin (50 μg/mL) and 1.5% (w/v) bovine serum albumin (BSA, essentially fatty acid-free)].

Lipid synthesis. The synthesis of sterol, phospholipids (PL), free fatty acids (FFA), TG, sterol ester and squalene was estimated by [^{14}C]acetate incorporation into the respective lipid classes as previously described [11]. On day 0, Caco-2 cells were seeded in 1 mL of medium A at a concentration of 2×10^5 cells/3.8-cm^2 plastic dish (12-well plate, Corning Glassworks, Corning, NY, U.S.A.). The medium was changed every 2 or 3 days. On day 14, medium A was replaced with medium B. The cells were preincubated for the indicated time with each drug dissolved in dimethyl sulfoxide (Me_2SO) [final concentration, 0.1% (v/v)], and were then labeled with 1 μCi of [^{14}C]acetate (1 mM) for 2 hr. The same amount of Me_2SO was added to the control. After separating the medium, the cells were lysed in 0.2 M NaOH aq. Aliquots of this cell lysate were used for protein determination. For the determination of sterol and squalene synthesis, one-third of the cell lysate was heated at 75° for 1 hr in the presence of ethanolic KOH [final concentration 20% (w/v) KOH], and non-saponifiable lipids were extracted with hexane. The non-saponifiable lipids were separated on silica gel G thin-layer chromatography (TLC, Art 5583, E. Merck, Darmstat, Germany) using hexane:diethyl ether:acetic acid (85:15:1, by vol.) as a solvent system. To measure the synthesis of other lipids, one-third of the cell lysate was neutralized immediately with 1 M HCl and the lipids were extracted by the method of Folch *et al.* [12]. PL, FFA and TG were separated on TLC using hexane:diethyl ether:acetic acid:methanol (85:15:1:1, by vol.) as a solvent system. Another solvent system (petroleum ethyl:diethyl ether:acetic acid, 95:5:0.5, by vol.) was used for the separation of sterol ester and squalene. The lipids were visualized on TLC by exposure to I_2 vapor. Radioactivity on TLC was detected using a radiochromato-scanner (LB-282, Berthold, Wildbad, Germany) and was counted with a liquid scintillation counter (TRI-CARB 2000CA, Packard Instrument, Meriden, CT, U.S.A.).

Cell culture on membrane filters. The cultivation of Caco-2 cells on membrane filters was performed according to the method of Traber *et al.* [9], with some modifications. For the experiments, 5×10^5 cells in 1.5 mL of medium A were plated onto filters (Cell Culture Insert®, 0.45-μm pores, 4.9 cm^2, Becton Dickinson, Lincoln Park, NJ, U.S.A.), and each filter was placed in a dish (6-well plate, diameter: 35 mm, Becton Dickinson). After 2 or 3 days, the medium was removed. One milliliter of medium A was added to the upper (apical) side of each filter, and 2 mL of medium A was added to the lower (basolateral) side. The medium was changed every 2 or 3 days. Fourteen or 15 days after plating, the cells were used for the experiment; they were 7 or 8 days post-confluent by this time. Transepithelial resistance was monitored with a Millicell-ERS apparatus (Millipore, Bedford, MA, U.S.A.), as described [13]. Cells were not used if their resistance was less than 300 ohm × cm^2 on the day of the experiment.

Apical uptake and basolateral secretion of lipids. On the day of the experiment, the monolayer of Caco-2 cells on each filter was washed twice with medium B. Fifty micromoles of [^{14}C]cholesterol-micelle in medium B (1 mL) containing 400 μM oleic acid and 800 μM taurocholate was added to the apical side of the chambers, and 2 mL of medium B was added to the basolateral side. Each compound dissolved in Me_2SO was added to both sides. In the control, Me_2SO alone was added to both sides. The final concentration of Me_2SO did not exceed 0.2% (v/v). After incubation, the lipids that were secreted to the basolateral side and that accumulated in the cells were extracted by the methods of Folch *et al.* [12] and analyzed as described in the secretion on lipid synthesis. In some experiments, oleic acid was radiolabeled in the lipid micelle.

Preparation of microsomes from the cells. Microsomes were prepared according to the method of Field and Salome [14], with some modifications. Caco-2 cells were sonicated with a Branson sonifier (Sonifier 185, Branson, Danbury, CT, U.S.A.) in cold buffer A [0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4 and 0.03 M EDTA (pH 7.2)]. After centrifugation (10,000 *g*, 20 min), the supernatant was recentrifuged at 105,000 *g* for 60 min. The sediment was washed and resuspended in buffer A.

The washed microsomes were used for the ACAT assay.

Effects on ACAT activity of NB-598 addition to microsomes. Microsomal ACAT activity was determined according to the method described by Field and Mathur [15], with some modifications. The cholesterol in the microsomal fraction and [1-^{14}C]-oleoyl CoA were used as a substrate. The reaction mixture (0.2 mL) consisted of 0.3 mg microsomal protein, 0.25% BSA and 40 μM [1-^{14}C]oleoyl CoA in buffer A. Each compound dissolved in Me_2SO was added to the reaction mixture. The final concentration of Me_2SO was 1% (v/v). After preincubation for 15 min at 37°, the enzyme assay was started by adding [^{14}C]oleoyl CoA to the reaction mixture. After 5 min of incubation, the reaction was terminated by adding 4 mL of chloroform:methanol (2:1, v/v). Cholesteryl oleate was separated on TLC. The area corresponding to cholesteryl oleate was scraped into a vial, and the radioactivity was counted as described above.

Effects on ACAT activity of incubating cells with NB-598. Caco-2 cells were grown in a 58-cm^2 plastic dish with medium A for 13 days. The cells were washed with medium B, and then cultured with medium B including cholesterol-micelle and each compound. The compound was dissolved in Me_2SO, and the final concentration of Me_2SO was 0.1% (v/v). After 18 hr of incubation, the cells were washed extensively with phosphate-buffered saline (PBS) to remove the compound. Microsomes were prepared as described above. The reaction mixture (0.2 mL) consisted of 0.1 mg microsomes, 0.25% BSA and 40 μM [1-^{14}C]oleoyl CoA in buffer A. To avoid the effects of endogenous cholesterol, liposome (2 mol of cholesterol:1 mol of phosphatidylcholine) [15] was added to the reaction mixture. The microsomes were preincubated for 1 hr with or without exogenous cholesterol, and ACAT activity was determined as described above.

Effects on secretion of apo B and apo A-I. Apolipoprotein secretion was studied under the same conditions as those of the experiments on lipid uptake and secretion. After 18 hr of incubation, apo B in the medium was determined using enzyme-linked immunosorbent assay (ELISA) according to the method of Young *et al.* [16], with some modifications. Microtiter plates (96-well, Becton Dickinson) were coated with human LDL (5 μg/mL in PBS) at 4° for 18 hr. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (washing buffer). Residual binding sites on the plates were blocked by incubation with BSA [3% in PBS (w/v)] at 23° for 1 hr. The wells were then washed with the washing buffer. Fifty microliters of samples and standard human serum were pipetted into the wells, and 50 μL of mouse monoclonal antibody (MAB-012; diluted 30,000-fold in 3% BSA–PBS) was added. The microtiter plates were then allowed to stand at 4° for 18 hr. After washing the wells, 0.1 mL of horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 3000-fold in 1% BSA–PBS) was added and the plate was incubated at 23° for 1 hr. After washing the wells, 0.1 mL of 0.1 M citrate-phosphate buffer (pH 3.8) containing 1 mg/mL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was added, and the plates were kept for 30 min at room temperature. The reaction was stopped by adding 50 μL of 0.2% sodium azide. The concentration of apo B was determined by measuring absorbance (415 nm). Apo A-I was measured using similar procedures except for a few points. For the apo A-I ELISA assay, the plate was coated with human HDL (2 μg/well) instead of LDL, and the mouse monoclonal antibody (AB-AI-1), which recognizes human apo A-I, was used. Human apo A-I was used to prepare the standard curve.

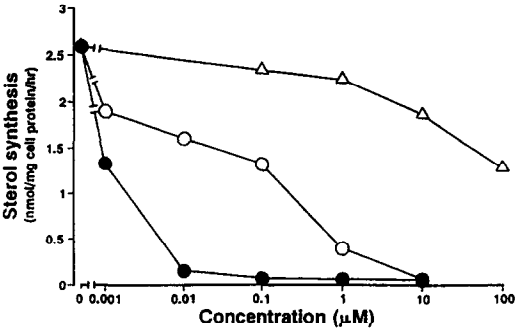

Fig. 1. Effects of NB-598, L-654,969 and melinamide on [^{14}C]acetate incorporation into sterol in Caco-2 cells. A monolayer of Caco-2 cells in a 3.8-cm^2 well was preincubated with each compound at each concentration for 1 hr in 1 mL of medium B. [^{14}C]Acetate (1 mM; specific radioactivity 2060 dpm/nmol) was then added and the medium was incubated for 2 hr. After incubation, the medium was removed and the cells were washed extensively with PBS. Cellular lipids were quantified as described in Materials and Methods. Each value is the mean of duplicate or triplicate determinations. Key: (●) NB-598, (○) L-654,969, and (△) melinamide. The control value for sterol synthesis was 2.6 ± 0.02 nmol/mg cell protein/hr.

Determination of protein. Protein concentrations were determined according to the method of Lowry *et al.* [17], using BSA as a standard.

Data analysis. The data from these studies were statistically analyzed using the Mann-Whitney U-test. Variations in the mean values are expressed as standard deviations (SD) in the figures and tables.

RESULTS

Effects on sterol synthesis. To investigate the effects of NB-598 on sterol synthesis, Caco-2 cells were preincubated with inhibitor for 1 hr, and the incorporation of [^{14}C]acetate into sterol was determined. NB-598 inhibited sterol synthesis with an IC_{50} value of 1 nM (Fig. 1). The HMG-CoA reductase inhibitor L-654,969 also inhibited sterol synthesis with an IC_{50} value of 44 nM. Both NB-598 and L-654,969 completely inhibited sterol synthesis at 10 μM. An ACAT inhibitor, melinamide, slightly reduced sterol synthesis even at this concentration.

Effects on the synthesis of other lipids. The effects of NB-598 on lipid synthesis from [^{14}C]acetate were investigated after short (1 hr) and long (18 hr) preincubation times. As shown in Table 1, 10 μM

Table 1. Effects of NB-598, L-654,969 and melinamide on the incorporation of [^{14}C]acetate into lipids in Caco-2 cells

Compound	pmol/mg cell protein/hr (% of control)					
	Sterol	PL	FFA	TG	SE	SQ
1-hr Preincubation						
Control	1150 ± 50 (100)	1500 ± 127 (100)	1370 ± 22 (100)	1860 ± 78 (100)	149 ± 10 (100)	78 ± 10 (100)
NB-598 (10 μM)	52 ± 12* (4.5)	1570 ± 184 (105)	1460 ± 78 (106)	1660 ± 123 (89)	103 ± 16* (69)	1370 ± 83* (1760)
L-654,969 (10 μM)	37 ± 3* (3.2)	1640 ± 217 (110)	1490 ± 78* (108)	2050 ± 116 (110)	120 ± 17* (81)	1 ± 1* (1.3)
Melinamide (10 μM)	743 ± 34* (64)	1540 ± 137 (103)	1700 ± 99* (124)	2150 ± 105* (115)	29 ± 5* (20)	23 ± 3* (29)
18-hr Preincubation						
Control	870 ± 96 (100)	1620 ± 143 (100)	1520 ± 43 (100)	1600 ± 58 (100)	101 ± 9 (100)	53 ± 7 (100)
NB-598 (10 μM)	52 ± 5* (5.9)	1900 ± 126 (117)	1490 ± 81 (98)	1580 ± 63 (99)	23 ± 4* (22)	2450 ± 100* (4640)
L-654,969 (10 μM)	109 ± 11* (13)	1730 ± 116 (107)	1420 ± 60 (93)	2090 ± 95* (131)	41 ± 4* (40)	3 ± 2* (5)
Melinamide (10 μM)	693 ± 75* (80)	1670 ± 124 (102)	1720 ± 56* (113)	2090 ± 72* (131)	27 ± 4* (27)	20 ± 2* (38)

A monolayer for Caco-2 cells in a 3.8-cm^2 well was preincubated with a 10 μM concentration of each compound for 1 or 18 hr in 1 mL of medium B. [^{14}C]Acetate (1 mM; specific radioactivity 2240 dpm/nmol) was then added, and the medium was incubated for 2 hr. After incubation, cellular sterol, phospholipids (PL), free fatty acids (FFA), triacylglycerol (TG), sterol ester (SE) and squalene (SQ) were quantified as described in Materials and Methods. For sterol determination, the cellular lipids were saponified. Each value is the mean ± SD for 6 different wells.

* Significantly different from the value of each control, $P < 0.01$.

NB-598 did not decrease the synthesis of PL, FFA and TG at either preincubation time. L-654,969 and melinamide also did not suppress the synthesis of these lipids. After 18 hr of incubation, NB-598 inhibited sterol synthesis by 94%. L-654,969 and melinamide inhibited this parameter by 87 and 20%, respectively. NB-598 markedly increased intracellular levels of squalene after both 1 and 18 hr of preincubation, whereas decreases occurred with L-654,969 and melinamide. NB-598 reduced sterol ester synthesis to a greater extent after 18 hr (78%) than after 1 hr of preincubation (31%), suggesting that the cellular sterol pool was depleted by 18 hr of preincubation with NB-598. A similar tendency was observed with L-654,969 treatment. Melinamide inhibited sterol ester synthesis by 80% after 1 hr of preincubation and by 73% after 18 hr of preincubation. With respect to lipid synthesis in Caco-2 cells, NB-598 and L-654,969 inhibited sterol synthesis and melinamide mainly suppressed sterol esterification.

Culture conditions of Caco-2 cells grown on membrane filters. To investigate the effects of NB-598 on cholesterol absorption, Caco-2 cells were grown on membrane filters that separate an upper and a lower well. First, optimum conditions for the basolateral secretion of lipoprotein were examined. As already reported [18], the basolateral secretion of apo B reached maximum levels after 14 or 15 days of cultivation (data not shown). At this time, the amount of apo B secreted to the basolateral side by Caco-2 cells was three times greater than the amount secreted to the apical side (data not shown). Caco-2 cells were found to secrete apo B in a polarized manner. Therefore, cells cultivated for 14 or 15 days were used in subsequent experiments.

Second, the optimum lipid composition of micelles for the basolateral secretion of cholesterol and cholesterol ester was examined, using [^{3}H]cholesterol as a tracer. When lipid micelle consisting of 50 μM [^{3}H]cholesterol, 400 μM oleic acid and 800 μM taurocholate was apically loaded into Caco-2 cell cultures, the highest radioactivity was found in the cholesterol and cholesterol ester in the basolateral medium (data not shown). The time course of secretion was then examined using this micelle. Cellular radioactivity increased time dependently and reached saturation levels after 8 hr of incubation (data not shown). Basolateral radioactivity was difficult to detect within 3 hr of incubation, because radioactive cholesterol is diluted by intracellular cholesterol. The radioactive cholesterol and cholesterol ester, which were secreted to the basolateral side, were increased time dependently for at least 24 hr (data not shown). Therefore, we used this micelle and a relatively long incubation time (18 hr) in the following experiments.

Effects on the cellular uptake, esterification and basolateral secretion of [^{3}H]*cholesterol.* The effects of NB-598 on cellular cholesterol uptake, esterification

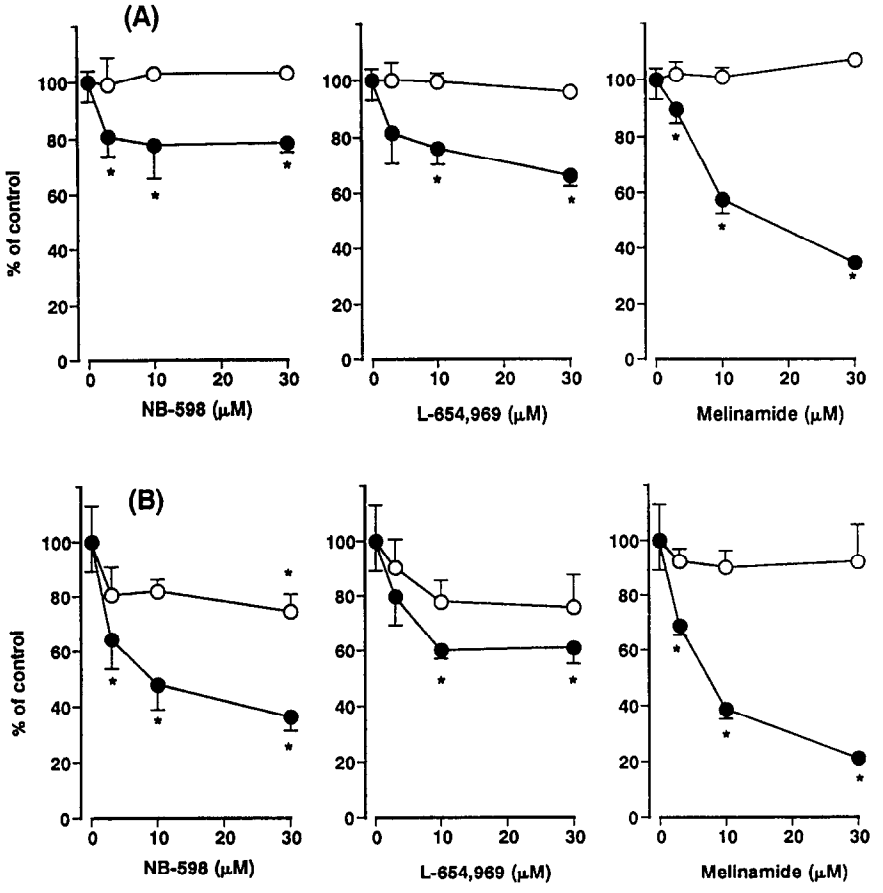

Fig. 2. Effects of NB-598, L-654,969 and melinamide on the uptake, esterification and secretion of [^{3}H]cholesterol by Caco-2 cells. Fifty micromoles of [^{3}H]cholesterol (specific radioactivity 220 dpm/pmol) solubilized in medium B (1 mL) containing 400 μM oleic acid and 800 μM taurocholate was added to the apical side of the chamber and 2 mL of medium B was added to the basolateral side. Compound was added to both sides of the chamber as a Me_2SO solution. In the control chamber, Me_2SO was added to both sides. After 18 hr of incubation, the [^{3}H]cholesterol and [^{3}H]cholesterol ester in the cells and the basolateral medium were quantified as described in Materials and Methods. Each value is the mean ± SD for 3–5 different wells. Key: (A) cells, and (B) basolateral medium; (○) free cholesterol, and (●) cholesterol ester. The control values for cellular free cholesterol (FC) and cholesterol ester (CE) were 8.04 ± 0.32 and 0.48 ± 0.034 nmol/mg cell protein/18 hr, respectively. The values for basolateral FC and CE were 77.6 ± 10 and 17.7 ± 1.9 pmol/mg cell protein/18 hr, respectively. An asterisk (*) indicates significantly different from the value of each control, $P < 0.05$.

and basolateral secretion were examined. Lipid micelle, in which cholesterol was tritium-labeled, was added to the apical side of the chambers, and NB-598 was added to both sides. As shown in Fig. 2A, NB-598 even at a concentration of 30 μM did not affect the amount of [^{3}H]cholesterol. In contrast, NB-598 reduced cellular [^{3}H]cholesterol ester by about 20%. As shown in Fig. 2B, NB-598 reduced the amount of radioactive cholesterol in the basolateral medium by about 20%. NB-598 remarkably reduced radioactive cholesterol ester in the basolateral medium in a concentration-dependent manner. A similar tendency was also observed with L-654,969 treatment (Fig. 2). Melinamide did not affect the amount of [^{3}H]cholesterol in cells and the basolateral medium. However, melinamide remarkably reduced [^{3}H]cholesterol ester in cells and the basolateral medium.

Effects on cellular incorporation of [^{3}H]*oleic acid and the basolateral secretion of radioactive lipids.* The micelle including [^{3}H]oleic acid was added to the apical side of the chambers, and the effects of NB-598 on PL, TG and sterol ester secretion were investigated. As shown in Fig. 3A, NB-598 (10 μM) slightly reduced the incorporation of [^{3}H]oleic acid into sterol ester (7%) and did not affect the incorporation of [^{3}H]oleic acid into TG and PL. On the contrary, NB-598 remarkably reduced the amount of radioactive TG, PL and sterol ester that was secreted basolaterally by 35, 41 and 46%, respectively (Fig. 3B). L-654,969 (10 μM) also reduced the radioactive basolateral TG, PL and sterol ester fraction by 23, 33 and 24%, respectively. Ten micromoles of NB-598 and L-654,969 inhibited sterol synthesis by about 90% after 18 hr of preincubation (Table 1). Therefore, the reduction

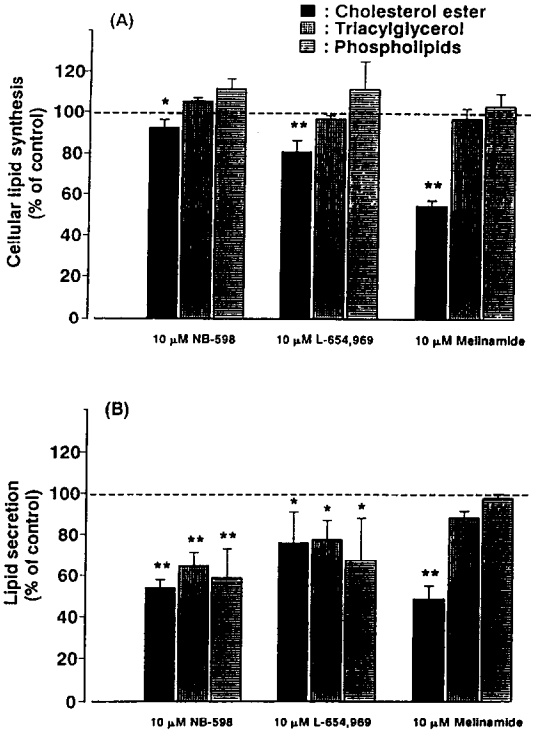

Fig. 3. Effects of NB-598, L-654,969 and melinamide on the incorporation of [^{3}H]oleic acid into Caco-2 cells and basolateral medium lipids. Fifty micromoles of cholesterol solubilized in medium B (1 mL) containing 400 μM [^{3}H]-oleic acid (specific radioactivity 20 dpm/pmol) and 800 μM taurocholate was added to the apical side of the chamber and 2 mL of medium B was added to the basolateral side. Compound was added to both sides of the chamber as a Me_2SO solution. After 18 hr of incubation, the lipids in the basolateral medium and in the cells were quantified as described in Materials and Methods. Each value is the mean ± SD for 4–6 different cultures (when missing, SD bars were too small to depict). (A) cells, and (B) basolateral medium. Control values for cellular cholesterol ester (CE), triacylglycerol (TG) and phospholipids (PL) were 1.07 ± 0.06, 25.5 ± 1.3 and 13.9 ± 2.6 nmol/mg cell protein/18 hr, respectively. The values for basolateral CE, TG and PL were 35.6 ± 4.7, 296 ± 39 and 111 ± 22 pmol/mg cell protein/18 hr, respectively. Key: Significantly different from the value of each control: (*) $P < 0.05$ and (**) $P < 0.01$.

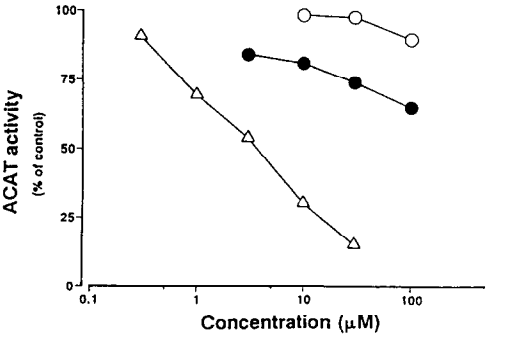

Fig. 4. Effects of the addition of NB-598, L-654,969 and melinamide on ACAT activity in microsomes. Microsomes were prepared from control Caco-2 cells grown in medium A, as described in Materials and Methods. Inhibitor was added to 0.3 mg of microsomes as a Me_2SO solution and preincubated for 15 min. The final concentration of Me_2SO was 1% (v/v). Enzyme assay was started by adding [^{14}C]-oleoyl coenzyme A to the reaction mixture. After 5 min of incubation, the reaction was terminated. ACAT activity was determined as described in Materials and Methods. Key: (●) NB-598, (○) L-654,969, and (△) melinamide. Values are the means of duplicate or triplicate determinations. In the control, 273 ± 1.8 pmol/mg microsomal protein/min of cholesteryl [^{14}C]oleate was formed.

in radioactivity in all of the lipid classes appeared to be caused by the inhibition of sterol synthesis. Melinamide, which had a minor effect on sterol synthesis (Table 1), only reduced radioactive cholesterol ester in cells and the basolateral medium, indicating that inhibition of ACAT did not affect TG and PL secretion (Fig. 3).

Effects on ACAT activity of NB-598 addition to microsomes. Since NB-598 decreased the basolateral secretion of cholesterol ester as potently as an ACAT inhibitor (Figs. 2B and 3B), the inhibition of ACAT activity was examined. Microsomes obtained from control Caco-2 cells were incubated with NB-598, and ACAT activity was measured. Under our assay conditions, melinamide potently inhibited microsomal ACAT activity with an IC_{50} value of 3.6 μM as reported [10] (Fig. 4). NB-598 slightly inhibited ACAT activity (19% at 10 μM), but its potency was far less than that of melinamide. Even at a concentration of 30 μM, L-654,969 did not inhibit ACAT activity.

Effects on ACAT activity of incubating cells with NB-598. To further investigate the effects of NB-598 on ACAT activity, Caco-2 cells were incubated with NB-598 for 18 hr and microsomes were extracted. ACAT activity was measured in the presence of an excess amount of exogenous liposomal cholesterol to avoid the influence of endogenous microsomal cholesterol levels. As the liposomal cholesterol level increased, ACAT activity increased (data not shown). Liposomal cholesterol fully activated ACAT activity at concentrations of more than 400 μM (data not shown). As shown in Table 2, in the absence of exogenous liposomal cholesterol, NB-598 reduced ACAT activity by 31%. L-654,969 also reduced ACAT activity by 16%. NB-598 reduced ACAT activity by 22% even in the presence of a 600 μM concentration of liposomal cholesterol. However, ACAT activity in L-654,969-treated cells was similar to the control level in the presence of exogenous cholesterol. These results indicate that some of the inhibitory effects of NB-598 on ACAT activity may contribute to the reduction of labeled cholesterol ester in basolateral medium (Figs. 2B and 3B).

Effects on apolipoprotein secretion. Lipids in the basolateral medium were considered to be secreted as lipoproteins with apolipoproteins. Therefore, the effects of NB-598 on basolaterally secreted apolipoproteins were investigated in Caco-2 cells grown on membrane filters. Caco-2 has been reported

Table 2. ACAT activity in Caco-2 cells after treatment with NB-598 or L-654,969

Compound	Microsomal ACAT activity (pmol/mg microsomal protein/min)	
	− Cholesterol	+ Cholesterol
Control	193 ± 8.49 (100)*	399 ± 25.7 (100)
NB-598 (10 μM)	134 ± 5.35† (69)	313 ± 8.35† (78)
L-654,969 (10 μM)	162 ± 7.63† (84)	388 ± 11.2 (97)

Microsomes were prepared from Caco-2 cells treated with each inhibitor as described in Materials and Methods. ACAT activity was determined in the presence or absence of exogenous liposomal cholesterol as described in Materials and Methods. Values are the means ± SD of four different cultures. Plus cholesterol: 600 μM liposomal cholesterol was added.

* Percent of control.

† Significantly different from the value of each control, $P < 0.05$.

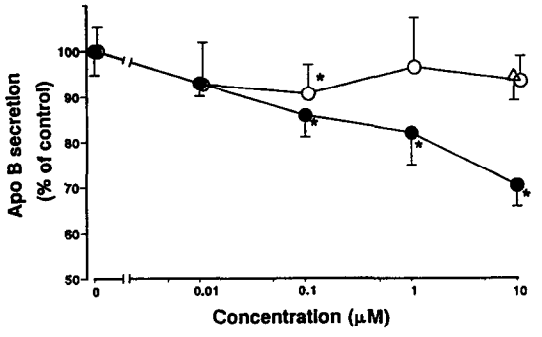

Fig. 5. Effects of NB-598, L-654,969 and melinamide on apo B secretion. Monolayers of Caco-2 cells grown on filters were washed with medium B, and 50 μM cholesterol solubilized in medium B (1 mL) containing 400 μM oleic acid and 800 μM taurocholate was added to the apical side of the chamber. Two milliliters of medium B was added to the basolateral side. After 18 hr of incubation, the apo B in the basolateral medium was determined by ELISA, as described in Materials and Methods. Key: (●) NB-598, (○) L-654,969, and (△) melinamide. Each value is the mean ± SD of 3–6 different cultures. In the control cells, apo B was secreted at a rate of 0.98 ± 0.05 μg protein/mg cell protein/18 hr. An asterisk (*) indicates significantly different from the control value, $P < 0.05$.

to secrete both apo B100 and apo B48 [9]. Since the adult human intestine predominantly secretes apo B48, we tried to determine the amount of apo B48 secreted from Caco-2 cells by ELISA. However, the standard deviations of the individual data were relatively large for quantification. Therefore, major apolipoproteins, the total amount of apo B (100 plus 48) and apo A-I, were evaluated in this study. As shown in Fig. 5, NB-598 was found to inhibit the basolateral secretion of apo B in a concentration-dependent manner. NB-598 at 10 μM inhibited apo B secretion by 30%, indicating that NB-598 reduced the number of newly secreted lipoprotein particles containing apo B. However, L-654,969 and melinamide at a concentration of 10 μM did not affect apo B secretion. Neither NB-598, L-654,969 nor melinamide affected the basolateral secretion of apo A-I up to the concentration of 10 μM (data not shown).

DISCUSSION

The small intestinal epithelia support the *de novo* synthesis of lipids and are a major production site of extracellular lipid transport proteins, particularly apo A-I, apo A-IV and apo B48. The enterocytes can absorb and metabolize lipids, and then secrete TG-rich lipoproteins into the circulation. As a model system for intestinal lipid absorption, cultures of intestinal epithelia sheets are impractical because the membrane organization is lost rapidly [19, 20]. Caco-2 cells have been reported to differentiate into enterocyte-like cells with microvilli and basolateral membranes separated by tight junctions when grown on plastic or permeable filters [21–23]. Caco-2 cells have also been reported to secrete TG-rich lipoproteins containing apo B100 and apo B48 such as very low density lipoprotein (VLDL) or LDL, and TG-poor lipoproteins containing apo A-I-like HDL to the basolateral side of the chambers [9, 24]. These culture systems have been reported to be a suitable model for small intestinal epithelial transport [9]. Therefore, to examine the effects of NB-598 on intestinal cholesterol absorption, its effects on the cellular uptake of cholesterol, esterification of cholesterol and basolateral secretion of lipoproteins were investigated using Caco-2 cells grown on membrane filters.

We previously reported that NB-598 reduces the secretion of cholesterol, cholesterol ester, TG and PL into culture medium in Hep G2 cells [25]. In the present studies, NB-598 was found to reduce the basolateral secretion of radiolabeled cholesterol and cholesterol ester using [^{3}H]cholesterol and also to decrease the basolateral secretion of radioactive TG, PL and sterol ester using [^{3}H]oleic acid as a precursor. Caco-2 cells have been reported to demonstrate the presence of LDL receptors [26]. As LDL receptors are thought to be regulated by the cellular sterol pool in mammalian cells, the LDL receptors of Caco-2 cells are also considered to bind, internalize and degrade LDL following the LDL receptor theory [27]. Therefore, it is possible that reuptake of the particle containing apo B by LDL receptors occurs. In our experiment, 10 μM NB-598 reduced the basolateral secretion of apo B but 10 μM L-654,969 did not (Fig. 5). Both NB-598 and L-654,969 inhibited sterol synthesis by about 90% (Table 1). If the reuptake of particles containing apo B occurred through induction of LDL receptors using sterol synthesis inhibitors, L-654,969 should reduce the amount of apo B found in the basolateral medium. However, L-654,969 had no such effect. We feel that the reduction in lipid secretion induced by NB-598 was associated with a decrease in the number of lipoprotein particles containing apo B irrespective of inhibition of cholesterol synthesis.

The lipoprotein core is occupied by hydrophobic

lipids such as TG and cholesterol ester. We have reported that Hep G2 cells treated with NB-598 secrete squalene into culture medium and that the squalene is found in lipoprotein fractions containing apo B [25]. Squalene is a very hydrophobic lipid-like cholesterol ester. Therefore, squalene is thought to be secreted from Hep G2 cells as a core lipid of lipoproteins. In Caco-2 cells, the accumulated squalene might compete with triacylglycerols and cholesterol esters for the core of the lipoprotein particle, thus decreasing their secretion and disrupting the normal assembly and secretion of the lipoprotein particle.

No reports have addressed the regulation of apo B synthesis in Caco-2 cells. In Hep G2 cells, apo B synthesis is not regulated by the level of transcription and translation, but by the degradation of newly synthesized apo B [28]. In our preliminary experiments using [^{35}S]methionine pulse-chase, it was found that suppression of apo B secretion depends on an enhancement of apo B intracellular degradation in Hep G2 cells treated with NB-598 [29].

L-654,969 reduced the basolateral secretion of lipids. However, it did not affect the basolateral secretion of apo B (Fig. 5). This suggests that L-654,969 did not affect the number of particles containing apo B. Our results show that lipoproteins with modified lipid composition were secreted after treatment with L-654,969. Similarly, Hep G2 cells treated with CS-514, another HMG-CoA reductase inhibitor, affected neither the synthesis nor secretion of apo B and apo A-I, but secreted cholesterol-free LDL- and HDL-like lipoproteins into the culture medium [30].

Melinamide affected the esterification of cellular cholesterol and the secretion of cholesterol ester. However, it did not affect the secretion of apolipoprotein. Another ACAT inhibitor, PD128042, reportedly inhibits the basolateral secretion of cholesterol ester in Caco-2 cells [31]. After treatment with melinamide, Caco-2 cells appeared to secrete cholesterol ester-free lipoproteins into the medium.

NB-598, L-654,969 and melinamide suppressed the basolateral secretion of cholesterol ester. However, their mechanisms of suppression may differ from each other. Melinamide is thought to reduce the secretion of cholesterol ester by inhibiting ACAT. L-654,969 may reduce this variable by inhibiting cholesterol synthesis, whereas NB-598 may reduce it by inhibiting cholesterol synthesis, inhibiting ACAT and decreasing apo B secretion.

The HMG-CoA reductase inhibitor lovastatin has been reported to suppress the secretion of cholesterol, cholesterol ester, TG and PL by inhibiting ACAT in Caco-2 cells [8]. L-654,969 did not affect ACAT activity (Fig. 4, Table 2), but reduced the basolateral secretion of all lipid classes (Figs. 2B and 3B). Therefore, in addition to the inhibition of ACAT, the inhibition of cholesterol synthesis may play a role in the decrease in lipid secretion seen in Caco-2 cells. Cholesterol ester secretion into lymph from the intestinal tract is thought to be related to ACAT activity [32, 33] in intestinal cells. However, additional mechanisms related to cholesterol synthesis and apo B secretion may be involved.

Approximately 85% of body cholesterol is reported to be derived from dietary cholesterol in dogs [3]. The administration of NB-598 to dogs for 2 weeks decreased serum cholesterol and chylomicron cholesterol levels by 34 and 42%, respectively [2]. In addition, serum and chylomicron TG levels decreased by 59 and 68%, respectively [2]. Therefore, the effect of NB-598 on intestinal lipid transport is thought to be one of the main reasons for the remarkable decrease in cholesterol and TG levels in dogs. We have reported that simvastatin decreases the absorption of cholesterol in cholesterol-diet-fed rabbits, but not in normal-diet-fed rabbits [5]. Long-term treatment with simvastatin down-regulated intestinal ACAT activity in cholesterol-fed rabbits. In the present study, L-654,969 did not down-regulate ACAT activity in Caco-2 cells. Down-regulation of ACAT activity by L-654,969 in Caco-2 cells may require long-term treatment and cholesterol loading.

In the present report, we have shown that NB-598 inhibited the absorption of cholesterol associated with a suppression of apo B secretion and ACAT activity in Caco-2 cells grown on filters. These effects may explain why NB-598 remarkably reduces serum cholesterol levels in dogs.

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