Plasma membrane sphingomyelin and the regulation of HMG-CoA reductase activity and cholesterol biosynthesis in cell cultures[1]

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Abstract We have examined the mechanism of the inhibition of cholesterol synthesis in cells treated with exogenous sphingomyelinase. Treatment of rat intestinal epithelial cells (IEC-6), human skin fibroblasts (GM-43), and human hepatoma (HepG2) cells in culture with sphingomyelinase resulted in a concentration- and time-dependent inhibition of the activity of HMG-CoA reductase, a key regulatory enzyme in cholesterol biosynthesis. The following observations were obtained with IEC-6 cells. Free fatty acid synthesis or general cellular protein synthesis was unaffected by the addition of sphingomyelinase. Addition of sphingomyelinase to the in vitro reductase assay had no effect on activity, suggesting that an intact cell system is required for the action of sphingomyelinase. The products of sphingomyelin hydrolysis, e.g., ceramide and phosphocholine, had no effect on reductase activity. Sphingosine, a further product of ceramide metabolism, caused a stimulation of reductase activity. Examination of the incorporation of [^{3}H]acetate into the nonsaponifiable lipid fractions in the presence of sphingomyelinase showed no changes in the percent distribution of radioactivity in the post-mevalonate intermediates of the cholesterol biosynthetic pathway, but there was increased radioactivity associated with the polar sterol fraction. Pretreatment of cells with ketoconazole, a known inhibitor of oxysterol formation, prevented the inhibition of reductase activity by sphingomyelinase and decreased the incorporation of [^{3}H]acetate in the polar sterol fraction. Ketoconazole had no effect on exogenous sphingomyelinase activity in vitro in the presence or absence of cells. Endogenous sphingomyelinase activity was also unaffected by ketoconazole. Addition of inhibitors of endogenous sphingomyelinase activity, e.g., chlorpromazine, desipramine, and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), to the culture medium caused a dose-dependent stimulation of reductase activity. However, these agents had no effect on the inhibition of reductase activity by exogenous sphingomyelinase. Treatment of cells with small unilamellar vesicles of dioleyl phosphatidylcholine or high density lipoprotein$_3$ resulted in increased efflux of cholesterol and stimulation of reductase activity. Under similar conditions, the inhibitory effect of exogenous sphingomyelinase on reductase activity was prevented by incubation with small unilamellar vesicles of phosphatidylcholine or high density lipoprotein. These results support the hypothesis that alteration of the ratio of sphingomyelin:cholesterol in the plasma membrane plays a modulatory role on the flow of membrane cholesterol to a site where it may be converted to a putative regulatory molecule, possibly an oxysterol. —**Gupta, A. K., and H. Rudney.** Plasma membrane sphingomyelin and the regulation of HMG-CoA reductase activity and cholesterol biosynthesis in cell cultures. *J. Lipid Res.* 1991. **32:** 125–136.

Supplementary key words cholesterol • HMG-CoA reductase • oxysterols • small unilamellar vesicles • HDL$_3$ • ketoconazole • sphingomyelinase

It is recognized that the phospholipid composition of a particular membrane entity is a major determinant of the level of cholesterol associated with that membrane. Among phospholipids of mammalian cells, sphingomyelin stands out as the most reliable marker of this association (1, 2). Approximately 90% of cellular cholesterol and sphingomyelin is located in the plasma membrane with the remainder distributed in other cellular membrane fractions including endoplasmic reticulum, mitochondria, and Golgi (3–5). Slotte and Bierman (6) have shown that disturbance of this ratio by the addition of sphingomyelinase (SMase) to fibroblasts in culture resulted in a rapid release of cholesterol from the plasma membrane to an intracellular pool where it was re-esterified to cholesteryl ester via acyl CoA:cholesterol acyltransferase (ACAT) activity. Additionally, the endogenous synthesis of cholesterol was inhibited. We investigated the mechanism of the inhibition of cholesterol biosynthesis by

Abbreviations: LPDS, lipoprotein-deficient serum; SMase, sphingomyelinase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; SUV, small unilamellar vesicle; ACAT, acyl CoA:cholesteryl acyltransferase; LDL, low density lipoprotein; HDL, high density lipoprotein; FCS, fetal calf serum; CS, calf serum; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; MVA, mevalonic acid.

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SMase and we observed that the extent of inhibition of [^{3}H]acetate incorporation into cholesterol could be entirely accounted for by a corresponding decrease in HMG-CoA reductase activity, which appears to be related to the generation of a regulatory molecule, possibly a polar sterol. Our results also support the possibility of a connection between cellular SMase activity and the regulation of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Materials

(RS)-3-[^{14}C]HMG-CoA (57 mCi/mmol), (RS)-5-[^{3}H]mevalonolactone (24 Ci/mmol), [^{3}H]acetate (1.6 Ci/mmol), (4,8,12,13,17,21,-^{3}H]squalene (19.4 Ci/mmol), [methyl-^{3}H]choline chloride (60 Ci/mmol) were obtained from DuPont-New England Nuclear Corp. [^{3}H]Cholesterol (48 Ci/mmol) and [4-^{14}C]cholesterol (54 mCi/mmol) were obtained from Research Products Inc. [9,10 (n)-^{3}H]oleic acid (43 Ci/mmol) was obtained from Amersham Corporation. SMase, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), sphingomyelin, sphingosine, desipramine, chlorpromazine, and ceramide were obtained from Sigma Chemical Co. All cell culture supplies and serum were obtained either form MA Biologicals or Hyclone Laboratories. All other chemicals were obtained from local sources and were of highest purity grade available.

Cell culture

Rat intestinal epithelial cells (IEC-6 cells) obtained from American Type Culture Collection (CRL-1592) were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum or calf serum as described previously (7, 8). Prior to the addition of the SMase or other agents, cells were incubated for 24–48 h in medium supplemented with lipoprotein-deficient serum protein (2 mg/ml) as described in pertinent figure and table legends. All experiments were conducted using cells in the logarithmic phase of growth such that cell numbers at harvest approximated 40–50% saturation density (150–200 μg protein/dish). Ketoconazole, or [^{3}H]cholesterol or other agents were added in solution in absolute ethanol such that the final concentration of ethanol did not exceed 1% (v/v). Control cells received equivalent amounts of ethanol. Phosphocholine and ceramide were added in dimethyl sulfoxide (Me_2SO). Control dishes received equivalent amounts of Me_2SO. In experiments with sphingosine, equimolar concentrations of bovine serum albumin were added to prevent cell lysis; control dishes received equivalent amounts of bovine serum albumin. None of the treatments had any significant effect on the growth of the cells during the time course of these studies.

Human hepatoblastoma cells (HepG2) obtained from American Type Culture Collection (HB-8065) were maintained as monolayers in DMEM medium supplemented with 10% fetal calf serum (FCS) or calf serum (CS) (v/v) as described previously (9, 10). For experiments, cells were seeded at 5×10^4 cells into dishes (35 × 15 mm, 6-well plates) in DMEM with 10% CS or FCS (v/v). Prior to the addition of other agents or SMase, cells were incubated for 24–48 h with DMEM medium supplemented with lipoprotein-deficient serum protein (4 mg/ml). Human skin fibroblasts obtained from Human Mutant Genetic Repository (GM-0043) were maintained on DMEM medium supplemented with 10% FCS or CS (v/v). For experiments, cells were seeded at 1×10^5 cells into dishes (35 × 15 mm; 6-well plates) in DMEM medium supplemented with 10% CS or FCS (v/v). Prior to the addition of other agents or SMase, cells were incubated for 24–48 h with DMEM medium supplemented with lipoprotein-deficient serum protein (4 mg/ml).

Preparation of small unilamellar vesicles and efflux of [^{3}H]cholesterol

Small unilamellar vesicles (SUV) of dioleoyl phosphatidylcholine were prepared as described previously (11). IEC-6 cells were labeled with [^{3}H]cholesterol (0.5 μCi/dish) for 2 h on day 4. After labeling, the cells were washed with phosphate-buffered saline and indicated concentrations of SUV were added. Thirty minutes after the addition of SUV, SMase (10 mu/ml) was added. The cells and medium were harvested for determination of cholesterol efflux in the medium and cholesteryl ester content in the cell. For determination of cholesterol efflux, an aliquot of the medium was counted. The reductase activity was determined in cells in which [^{3}H]cholesterol was not added.

Measurement of HMG-CoA reductase activity

The activity of HMG-CoA reductase was determined in detergent extracts of monolayers as described previously (7–10). One unit of enzyme activity equals the formation of 1 pmol of mevalonate/min. The results are presented as the average of triplicate determinations (dishes) at each data point. The standard error of the mean did not exceed 5% unless otherwise mentioned.

Incorporation of [^{3}H]cholesterol into cellular plasma membrane and its esterification

To label the plasma membranes of IEC-6 cells with [^{3}H]cholesterol, cells were grown for 3 days as described above. On day 4, 0.5 μCi of [^{3}H]cholesterol/ml (48 Ci/mmol) was added from an ethanolic stock solution. After 2 h incubation, cells were washed twice with 2 ml

of phosphate-buffered saline (pH 7.4) and incubated further with or without SMase as described in the legend of the pertinent figure or table. After an additional 2 h of incubation, the medium was removed and cells were washed with ice-cold saline (2 × 2 ml). The cellular lipids were extracted with 5 ml of hexane–isopropanol 3:2 as described previously (7, 8). Cholesteryl[^{14}C]oleate (20,000 dpm; 25 μg/sample) was included to monitor the recovery. Cholesteryl ester was determined from the lipid extract after separation on TLC plates developed in petroleum ether–ethyl ether–acetic acid 80:20:1 (v/v).

Cholesterol esterification was also examined by following the incorporation of [^{3}H]oleic acid as described previously (7). On day 4, cells were fed LPDS medium with or without SMase (10 mu/ml). After 2 h of feeding, 0.1 mM [9, 10-^{3}H]oleate (100,000 dpm/nmol) bound to bovine serum albumin was added. Cells were harvested after an additional 2 h of incubation and processed as described above.

Measurement of the incorporation of ^{3}H-labeled precursors into nonsaponifiable lipids

IEC-6 cells were incubated with [^{3}H]acetate or [^{3}H]mevalonolactone or [^{3}H]squalene as described in the legend of the pertinent table or figure. At the end of incubation, the total lipids were extracted from the medium and cells as described previously (12, 13). The lipid extracts were combined and saponified, and nonsaponifiable lipids were extracted as described previously (13). Throughout the lipid extraction and fractionation procedures care was taken to minimize oxidation of sterols by including butylated hydroxytoluene (1 μg/ml) in all solutions. The hexane extract was evaporated under N_2 and the nonsaponifiable lipid residue was redissolved in methanol for separation by either TLC or HPLC. The HPLC was performed on a Zorbax ODS column (0.46 × 25 cms, Dupont Industries, Wilmington, DE) using 100% methanol as the mobile phase in an HPLC System 42 from Gilson (Gilson Electronics, Middleton, WI) equipped with a Gilson fraction collector (Model #201) and a V^{4} absorbance detector (Isco, Inc., Lincoln, NE). The chromatographic fractions were collected directly into scintillation vials followed by the addition of ScintiVerse E and counted in a Beckman Model 1801 liquid Scintillation counter. The retention times for authentic standards were: 25-hydroxycholesterol, 6.0 min; 25-hydroxylanosterol, 7.0 min; 24,25-epoxycholesterol, 7.9 min; 24,25-epoxylanosterol, 9.6 min; desmosterol, 19.3 min; lanosterol, 23.4 min; and cholesterol 24.5 min.

Separation of nonsaponifiable lipids by TLC

Nonsaponifiable lipids prepared as described above were separated by TLC on K-5 silica gel plates (20 × 20 cm) using petroleum ether–acetone 90:10 as the developing solvent. Typical R_f values were as follows: polar sterol fraction, 0.05–0.23; cholesterol (C_{27}) fraction, 0.25; 24(S), 25-expoxylanosterol, 0.31; lanosterol, 0.38; ubiquinone, 0.52; squalene 2,3:22,23-dioxide, 0.57; squalene 2,3-epoxide, 0.68; squalene, 0.75. The lipids were visualized by spraying the plates with *p*-anisaldehyde (13). The radioactivity of individual lipid fractions was determined in a Beckman LS-1801 liquid scintillation spectrometer. The results were corrected for the recovery of a [^{14}C]cholesterol internal standard.

Measurement of sphingomyelin hydrolysis in [methyl-^{3}H]choline-labeled cells

IEC-6 cells (2 × 10^{5}) were seeded in 5% FCS in 60-mm Petri dishes as described above. On day 2, cells were rinsed with phosphate-buffered saline and fed fresh DMEM medium containing lipoprotein-deficient serum protein (2 mg/ml) and 0.5 μCi/dish [methyl-^{3}H]choline and incubated for 48 h to allow incorporation of choline into sphingomyelin and phospholipids. After 48 h of incubation, cells were rinsed and fed fresh medium with or without ketoconazole (30 μM). Triplicate dishes were harvested to determine the radiolabel in the phospholipid fraction prior to any treatment. After 1 h of incubation, 10 milliunits (10 mu) of SMase were added. The medium and cells were harvested 2 h after the addition of SMase to determine the level of sphingomyelin and phosphatidylcholine in cellular lipids. Total lipids from the cells were extracted with chloroform–methanol 1:2 as described (13). The radioactivity in this extract, upper phase, and medium represented 100%. The phospholipids were present in the lower chloroform phase and these were separated using silica gel G-plates developed in chloroform–methanol–glacial acetic acid–water 75:45:12:6 (v/v) (14, 15). The radioactivity in the areas on the TLC plates comigrating with authentic phosphatidylcholine and sphingomyelin was collected and counted. The decrease of radioactivity in the sphingomyelin fraction was a measure of SMase activity.

RESULTS

Effect of exogenous SMase on reductase activity in cell cultures

Treatment of rat intestinal epithelial cells with increasing concentrations of SMase (2–50 mu/ml) caused an inhibition of reductase activity during an incubation period of 2–4 h which was concentration (**Fig. 1A**) and time-dependent (Fig. 1B). When SMase was heat-inactivated at 100°C for 10 min, the inhibitory effect on reductase activity was lost (data not shown). The addition of active enzyme to the in vitro cell digest had no effect on reductase activity (data not shown). Thus, the inhibition of reductase activity by SMase required intact cells.

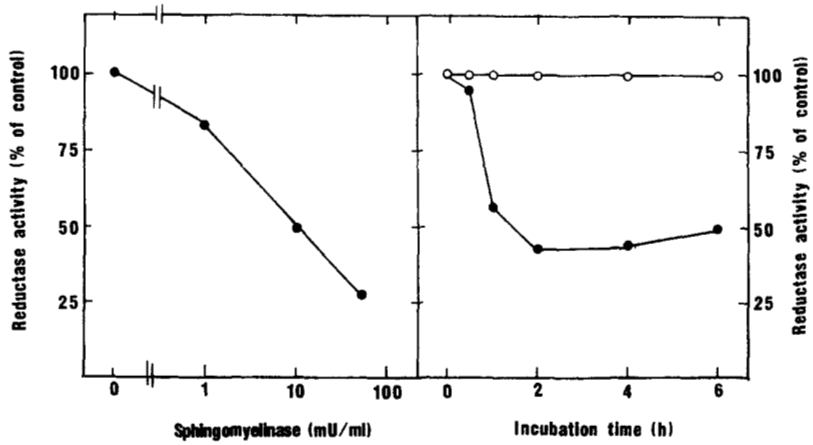

Fig. 1. Effect of varying concentrations of sphingomyelinase (A) and incubation time (B) on reductase activity in IEC-6 cells. IEC-6 cells were seeded (day 0) at 1 × 10^5 cells/well (35 mm, 6-well plates) in 2 ml DMEM medium supplemented with fetal calf serum (FCS medium). The cells were refed 2 ml DMEM medium containing lipoprotein-deficient serum (2 mg/ml, LPDS medium) on days 3 and 4. On day 4, neutral SMase was added to the medium at the indicated concentrations (A) or 10 mu/ml (B). After 4 h of incubation (A) or at the indicated times of incubation (B), cells were harvested for determination of reductase activity as described under Experimental Procedures. Values represent the mean of determinations from triplicate dishes; control (○); SMase (●).

SMase addition also caused inhibition of reductase activity in cell lines of other tissue origin (**Fig. 2**) e.g., human skin fibroblasts (GM-43) and human hepatoblastoma (HepG2) cells. Thus, the inhibition of reductase activity by exogenous SMase action appears to be a general phenomenon.

Effect of the products of sphingomyelin hydrolysis on reductase activity

The major products of sphingomyelin hydrolysis by SMase are phosphocholine and ceramide (16), with the latter being further converted to sphingosine and fatty acids (17) by ceramidase. To determine whether these products played a role in the inhibition of reductase activity, IEC-6 cells were incubated with either ceramide (1–10 μg/ml) or phosphocholine (1–10 μg/ml). No effects on reductase activity could be observed (**Table 1**). The addition of sphingosine, however, caused <50% stimulation of reductase activity. Since sphingosine addition resulted in the stimulation of reductase activity and SMase treatment caused inhibition of reductase activity, we investigated whether the addition of sphingosine could prevent the inhibition of reductase activity caused by SMase treatment. The results presented in **Fig. 3** show that although sphingosine increased reductase activity at all levels of SMase present in the culture medium, it had no effect on the percent inhibition of reductase activity by SMase addition. These results suggest that inhibition of reductase activity by SMase treatment, and stimulation of reductase activity by sphingosine addition, occur via different mechanisms.

Effect of SMase on cholesterol metabolism and fatty acid synthesis

Data presented in **Table 2** show that SMase treatment caused a two- to threefold increase in the incorporation of [^{3}H]cholesterol or [^{3}H]oleic acid into cholesteryl esters. The incorporation of [^{35}S]methionine into total protein was the same in presence (8.53 × 10^6 dpm/mg protein) and absence (7.93 × 10^6 dpm/mg protein) of SMase. Under the same conditions, incorporation of [^{3}H]acetate into total fatty acids was unaffected but the incorporation of [^{3}H]acetate into nonsaponifiable lipids was inhibited (35–45%) by SMase treatment which paralleled the inhibition of reductase activity (Fig. 2). However, further analysis of the nonsaponifiable lipid fraction from SMase treated cells by HPLC (**Fig. 4**) or TLC (**Table 3**) showed

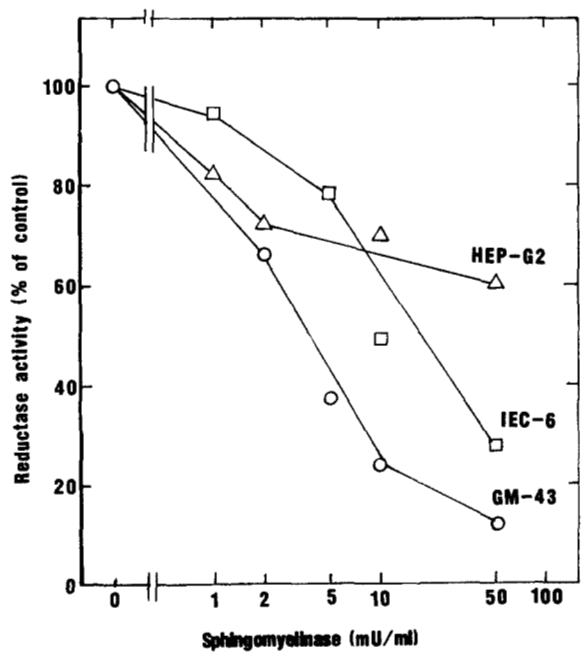

Fig. 2. The effect of sphingomyelinase on reductase activity in various cell lines. IEC-6 cells were grown as described in the legend to Fig. 1. Human skin fibroblasts (GM-43) and human hepatoblastoma cells (HepG2) were seeded on day 0 in a medium supplemented with 10% fetal bovine serum as described under experimental procedures. They were fed LPDS medium (4 mg protein/ml) on day 3. On day 4, cells were fed fresh LPDS medium containing indicated concentrations of SMase. After 4 h of incubation, cells were harvested for determination of reductase activity as described under Experimental Procedures.

TABLE 1. Effect of sphingomyelinase, sphingosine, ceramide, and phosphocoline on HMG-CoA reductase activity in IEC-6 cells

Addition	Concentration	Reductase Activity
		% of control
None	0	100
Phosphocholine	1 μg/ml	102
Phosphocholine	10 μg/ml	107
Ceramide	1 μg/ml	100
Ceramide	10 μg/ml	100
Sphingosine	1 μg/ml	106
Sphingosine	5 μg/ml	129
Sphingosine	10 μg/ml	148
Sphingomyelinase	5 mu/ml	78
Sphingomyelinase	50 mu/ml	27

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed LPDS medium. Indicated concentrations of phosphocholine, ceramide, sphingomyelinase, and sphingosine were added. The cells were harvested for determination of reductase activity after 4 h of the incubation as described in Experimental Procedures. The data presented are the means of triplicate determinations. Standard error of the mean did not vary >5%.

a significant increase over control in the radioactivity in the polar sterol zone (HPLC R_t 6–12 min and TLC R_f 0.05–0.23) expressed as total radioactivity/mg cell protein or as percentage of the total radioactivity in the nonsaponifiable lipid fraction. Several known potent inhibitors of reductase comigrate in this zone and thus it is likely that these fractions may contain several compounds. SMase treatment had no effect on percent distribution of radiolabel in other nonsaponifiable lipids, e.g., lanosterol, squalene epoxides, desmosterol, squalene, and ubiquinone. However, the absolute incorporation of [^{3}H]acetate (dpm/mg protein) into these fractions was inhibited to the same extent as the inhibition of nonsaponifiable lipids by SMase treatment.

Prevention of the SMase effect by inhibitors of oxysterol and cholesterol biosynthesis

We examined the question whether the increased level of polar sterols was involved in the regulation of reductase activity. One approach was to add inhibitors of the formation of oxysterols. Cells were pretreated with ketoconazole, a well-known inhibitor of lanosterol 14α-demethylase and cytochrome P-450-dependent reactions, (7, 18, 19) for 1 h followed by incubation with increasing concentration of SMase. As shown in **Fig. 5**, pretreatment with ketoconazole (30 μM) completely prevented the effect of SMase on reductase activity in IEC-6 cells (Fig. 5A) and HepG2 cells (Fig. 5B). Ketoconazole did not interfere with the action of SMase as shown by the effect of Ketoconazole on hydrolysis of sphingomyelin in cells prelabeled with [methyl-^{3}H]choline. Approximately 4.3% of the radiolabel was associated with cellular sphingomyelin (**Table 4**). After 2 h of SMase treatment, the radiolabel in the cellular sphingomyelin fraction decreased to 1.4%. In the presence of ketoconazole, SMase decreased the level of choline in sphingomyelin to the same extent. Ketoconazole also showed no effect on the activity of SMase when added to the in vitro assay (data not shown).

We considered the possibility that ketoconazole attenuated the SMase effect by inhibiting the formation of endogenous polar sterol in cell culture. The results shown in Table 3 show that pretreatment of cells with ketoconazole attenuated the effect of SMase on reductase activity and decreased the incorporation of [^{3}H]acetate into the polar sterol fraction. Other inhibitors of polar sterol formation or action, e.g., 3β-[2-(diethylamino)ethoxy]androst-5en-17-one (U18666A) (20) and progesterone (21, also attenuated the inhibitory effect of SMase on reductase activity (**Table 5**). Miconazole, a member of the same class of antifungal agents as ketoconazole, was unable to prevent the SMase effect. Sandoz compound 58-035, which inhibited cholesteryl esterification in IEC-6 cells (7, 18), had little effect on SMase action on reductase activity.

Effect of endogenous SMase inhibitors on reductase activity

It has been reported that tricyclic antidepressants, such as chlorpromazine and desipramine, and calmodulin antagonists, such as W-7, caused an inhibition of endo-

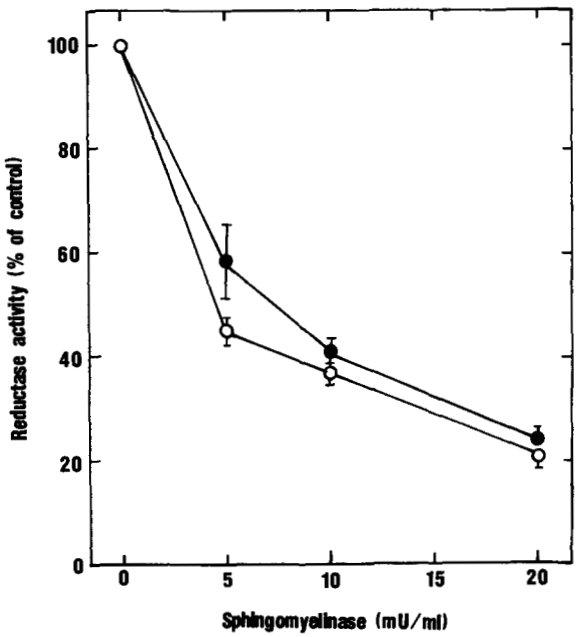

Fig. 3. Effect of sphingosine on the inhibition of reductase activity by sphingomyelinase in IEC-6 cells. Cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium containing BSA (100 μg/ml) with (●) or without (○) sphingosine (5 μg/ml). After 30 min incubation, SMase was added. Cells were harvested after 4 h incubation for the determination of reductase activity as described under Experimental Procedures. The 100% activity for control and sphingosine-treated cells was 304 ± 4 and 391 ± 35 pmol/min per mg protein, respectively.

TABLE 2. Effect of sphingomyelinase on cholesterol synthesis, cholesterol esterification, and fatty acid synthesis, in IEC-6 cells

Treatment	Cholesterol Esterification		[^{3}H]Acetate Incorporation		Nonsaponifiable Lipid Synthesis	
	Cholesterol	Oleic Acid	Free Fatty Acid	Nonsaponifiable Lipids	[^{3}H]Squalene	[^{3}H]Mevalonic Acid
	dpm × 10^2/*dish*					
None	694 ± 36	135 ± 47	1915 ± 157	1965 ± 78	196	67 ± 3
Sphingomyelinase	1585 ± 120	430 ± 40	2087 ± 120	1262 ± 36	172	76 ± 11

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. To determine the esterification of plasma membrane cholesterol, cells on day 4 were fed fresh LPDS medium containing [^{3}H]cholesterol (0.5 μCi/dish). After 2 h of incubation, triplicate dishes were harvested to measure the incorporation of radiolabel in cholesteryl ester before the addition of SMase. Other dishes were washed and fed medium with or without SMase (5 mu/ml). Cells were harvested for incorporation of cholesterol into cholesteryl esters. To determine the incorporation of [^{3}H]oleic acid into cholesteryl esters, [^{3}H]oleic acid–BSA complex (10 μCi/dish, 100 dpm per nmol) was added. Cells were harvested after an additional 2 h of incubation. To determine the [^{3}H]acetate incorporation into fatty acid and cholesterol, cells were fed LPDS medium on day 4 with or without SMase. After 3 h of incubation, [^{3}H]acetate (10 μCi/dish) was added. Cells were harvested after an additional 1 h incubation and were processed for fatty acid and cholesterol determination. To determine the incorporation of [^{3}H]mevalonic acid or [^{3}H]squalene into nonsaponifiable lipids, cells were fed medium containing either [^{3}H]mevalonic acid (20 μCi/dish) or [^{3}H]squalene (0.5 μCi/dish) on day 4. SMase was added immediately to treatment dishes. Cells were harvested after 4 h of incubation and processed as described under Experimental Procedures.

genous acid SMase activity (22, 23). Since endogenous SMase activity is involved in the turnover of cellular sphingomyelin, we tested whether addition of these agents would result in increased reductase activity due to an increase in sphingomyelin content (22, 24). Treatment of cells for 4 h with increasing concentrations of chlorpromazine, desipramine, and W-7 caused two- to threefold stimulation of reductase activity (**Fig. 6**). On the other hand, these agents were unable to prevent the inhibition of reductase activity of exogenous SMase (**Table 6**).

Effect of SUV on the inhibition of reductase activity

It has been observed that enhanced efflux of cholesterol from plasma membrane to an acceptor molecule, e.g., HDL, results in increased cholesterol synthesis and reductase activity (25). Since spingomyelinase treatment of cells results in the movement of cholesterol from the plasma membrane to an interior pool and subsequent inhibition of reductase activity, it was reasoned that if this movement of cholesterol was reversed, i.e., by the presence of an acceptor molecule in the medium, then the effect of SMase on reductase activity could be abolished. To examine this, we added to the medium SUV made of dioleoyl phosphatidylcholine; SUV have been shown to act as acceptors of cholesterol from cell membranes (11).

As shown in **Table 7**, SMase treatment caused an increase in the esterification of plasma membrane cholesterol and a 55% decrease in reductase activity, but there was no effect on the efflux of cholesterol. When cells were treated with SUV we observed a large increase in cholesterol efflux that was unaffected by SMase treatment. SUV addition, however, resulted in the inhibition of the increase in cholesterol esterification caused by SMase. HMG-CoA reductase activity, on the other hand, was stimulated by SUV to the same extent regardless of the presence of SMase (**Fig. 7**). SUV addition did not affect the hydrolysis of sphingomyelin by SMase (Table 7). In order to determine whether the absence of cholesterol in the SUV acceptor influenced the results, we substituted HDL_3 as an example of an acceptor that does contain cholesterol and, as shown by Rothblatt and Phillips (26), results in a lesser efflux of cholesterol compared to phosphatidylcholine vesicles. As shown in **Table 8**, when HDL_3 was the acceptor molecule in the medium there was an increase in reductase activity and efflux of cholesterol. As expected, the apparent extent of cholesterol efflux was considerably less than that observed with SUV, but SMase treatment was unable to affect the increases in reductase activity and of cholesterol efflux.

DISCUSSION

An important element underlying consideration of the results of these experiments is the ratio of cholesterol to phospholipid in the plasma membrane, especially the ratio of sphingomyelin to cholesterol and the role it plays in maintaining cellular cholesterol homeostasis. Thus, depletion of the sphingomyelin level of plasma membrane results in movement of cholesterol to the cell interior where it may be esterified via ACAT. Cholesterol may also be shunted to a special pool where regulatory events occur which influence cholesterol synthesis/metabolism (6, 27, 28).

Our experiments on the effects of the addition of SMase in IEC-6 cells essentially confirm the observations of Slotte and Bierman (6) with respect to activation of ACAT, the movement of cholesterol from the plasma membrane to the endoplasmic reticulum, and inhibition of the incorporation of [^{3}H]acetate into cholesterol.

With respect to the reduction in cholesterol synthesis, our experiments show that the primary effect of SMase

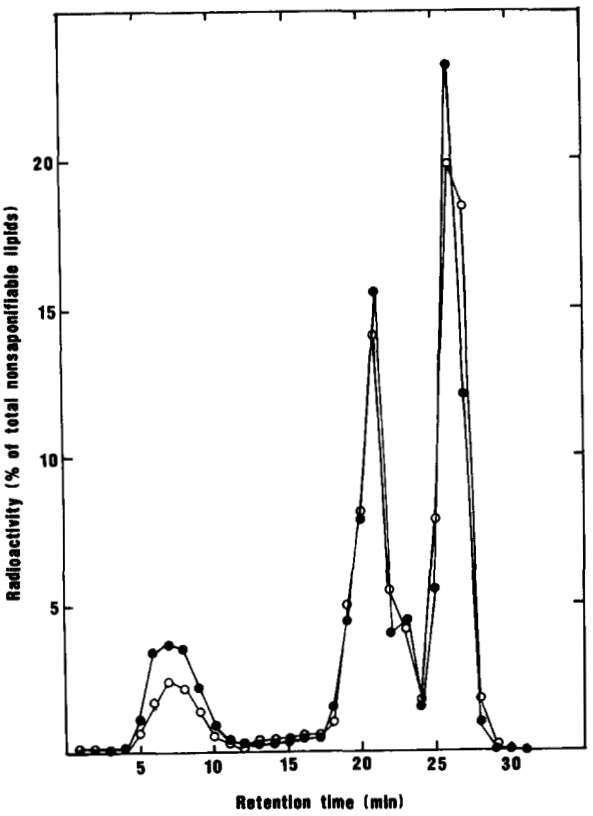

Fig. 4. Reverse phase (C18) HPLC chromatographic separation of cellular sterol extract from control (○) and sphingomyelinase-(●) treated cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were either fed LPDS medium or LPDS medium containing sphingomyelinase (10 mu/ml). Immediately after feeding medium, [^{3}H]acetate (10 μCi/dish) was added. Cells and medium were harvested after 4 h for the extraction of sterols as described under Experimental Procedures. The sterols were separated on a Zorbax ODS column with methanol as the mobile phase at a flow rate of 1 ml/min. The radioactivity in each fraction was counted. The radioactivity in total nonsaponifiable lipids fraction (dpm/mg cell protein) of control and SMase treated cells was 6.36 × 10^5 and 5.49 × 10^5, respectively. The amount of radiolabel (dpm/mg cell protein) in 6–12 min zone was 61,117 ± 12, 209 and 83,515 ± 830 for control and SMase-treated cells, respectively. The percentage of total nonsaponifiable lipid radiolabel incorporated into the 6–12 min zone was 9.61 and 15.1; in the 18–21 min zone, 37.07 and 37.32; and in the 25–28 min zone 48.05 and 43.35 for control and SMase treatment, respectively.

treatment is inhibition of HMG-CoA reductase. There was little change in the overall incorporation of mevalonic acid or squalene into the nonsaponifiable lipid fraction or percent incorporation into post-squalene intermediates of cholesterol biosynthesis. However, we did observe a rise in the level of a polar sterol fraction of the nonsaponifiable lipids after SMase treatment, whose chromatographic characteristics resemble those of known oxysterol inhibitors of the HMG-CoA reductase. Since this zone (Fig. 4) was relatively broad, the presence of more than a single compound might be expected.

We have previously shown that the formation of these endogenously generated polar sterols is inhibited by ketoconazole (7) and that HMG-CoA reductase activity was increased in the presence of this drug. In this work we show that ketoconzóle also prevented the effects of SMase action on both reductase activity and increased formation of the polar sterol fraction. Ketoconazole itself did not prevent exogenous SMase activity. In addition, we observed that ketoconazole had no effect on the efflux of cellular cholesterol to LPDS medium with or without HDL_3 as an acceptor molecule (data not shown).

The precise identification and determination of the exact amount of polar sterol generated by SMase treatment has remained elusive because of the small amounts generated. However, the following rough calculation allows the assumption that the amounts generated are sufficient to affect reductase activity. Since there is a 4–6% increase in polar sterol over the basal level and if we assume that IEC-6 cells contain 20 μg cholesterol/mg cell protein (7), the amount of polar sterol can be roughly calculated as 800–1200 ng/mg cell protein. In our experiments the amount of cell protein varied from 150 to 200 μg/dish. Thus, the concentration of polar sterol could be in the range 120–240 ng/dish. Such concentrations of hydroxysterol or epoxysterol have been shown to inhibit reductase activity in IEC-6 cells (7, 8). Thus our overall results provide support for the suggestion that the putative regulatory molecule generated from a regulatory pool of cholesterol might be a polar sterol.

We considered the possibility that ketoconazole might act by restricting the bulk movement of unesterified cholesterol to a regulatory site, either directly from the plasma membrane or from lysosomes. Liscum (29) and Liscum and Faust (30) have presented evidence in

TABLE 3. Effect of ketoconazole on polar sterol formation and reductase inhibition caused by sphingomyelinase treatment in IEC-6 cells

Treatment	Polar Sterol		Reductase Activity
	HPLC	TLC	
	% of total nonsaponifiable lipids		*pmol/min/mg protein*
None	7.30 ± 1.26	7.60 ± 0.33	160 ± 4
SMase	11.40 ± 0.29	12.78 ± 0.56	58 ± 3
KC	3.54 ± 0.39	3.45 ± 0.32	252 ± 6
KC + SMase	3.29 ± 0.09	3.31 ± 0.34	222 ± 8

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium with or without ketoconazole (30 μM). After 1 h treatment with the drug, SMase (10 mu/ml) was added to a group of dishes. All dishes received [^{3}H]acetate (10 μCi/dish). After 4 h of incubation, cells from triplicate dishes were harvested and processed for separation of nonsaponifiable lipid fraction either by HPLC or TLC as described under Experimental Procedures. The retention time of the polar sterol fraction by HPLC was 6–9 min. The R_f value of the polar sterol fraction by TLC was 0.05–0.23. The radiolabel in total nonsaponifiable lipid (dpm/mg cell protein) was 1.64 × 10^5 for control, 1.32 × 10^5 for SMase, 2.69 × 10^5 for ketoconazole, and 2.51 × 10^5 for ketoconazole and SMase. The percentage of radiolabel in the polar sterol dpm/mg protein was determined as outlined in the legend to Fig. 4.

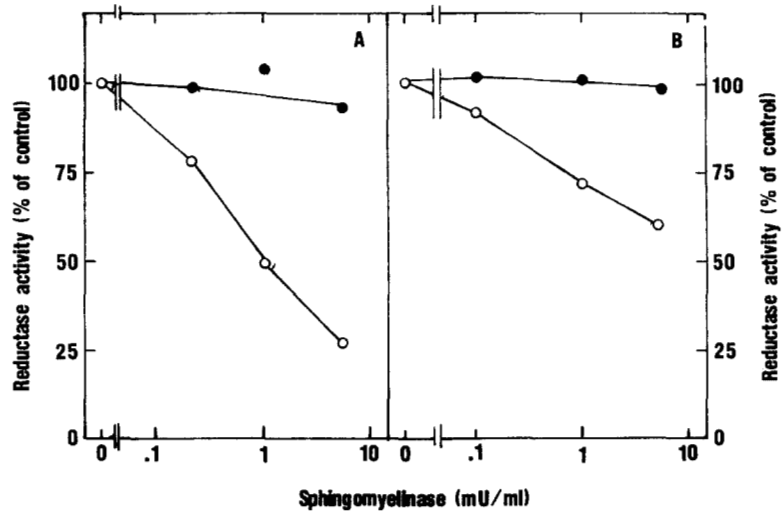

Fig. 5. Ketoconazole effect on inhibition of reductase activity by sphingomyelinase in IEC-6 cells (A) and HepG2 cells (B). IEC-6 cells and HepG2 cells were grown for 3 days as described in the legends to Fig. 1 and Fig. 2. On day 4, they were fed LPDS medium with (●) or without (○) ketoconazole (30 μM). After 1 h of incubation, SMase (10 mu/ml) was added. Cells were harvested after an additional 4 h incubation for the determination of reductase activity as described in Experimental Procedures.

Chinese hamster ovary cells that ketoconazole and U18666A sequester LDL cholesterol in lysosomes, which may partially account for the ability of these drugs to prevent the down-regulation of HMG-CoA reductase by LDL. In IEC-6 cells we were unable to demonstrate that ketoconazole sequestered LDL cholesterol (7). There are two reports which, combined with our data, suggest that the cholesterol released from plasma membrane may be transported to the regulatory site via a route that does not involve lysosomal interaction. Rodriquez-Lafrasse et al. (31) have shown that imipramine-treated fibroblast cultures displayed impaired down-regulation of cholesterol synthesis and mobilization of LDL cholesterol from lysosomes. If this was a factor in our studies, then one would expect that desipramine, which has an almost identical structure to imipramine and with similar effects on LDL metabolism, would prevent the effect of SMase. As we show in this study, this is not the case. The second study was by Slotte, Hedstrom, and Bierman (32) in Nieman-Pick C cells which display impaired transport of LDL cholesterol and decreased esterification of cholesterol and down-regulation of cholesterol synthesis by LDL. Treatment of these cells with SMase caused increased cholesterol esterification and down-regulation of cholesterol biosynthesis. These observations, in conjunction with our data, suggest that free cholesterol released from plasma membrane as a result of SMase action travels to a regulatory site independent of lysosomal involvement. We are unable to rule out, however, the possibility that ketaconozole may have directly inhibited this putative pathway and further studies will be needed to clarify these points.

TABLE 4. Effect of ketoconazole on sphingomyelin hydrolysis in IEC-6 cells

	% of [^{3}H-Methyl]Choline in Phospholipids	
Treatment	Sphingomyelin	Phosphatidylcholine
Oh	4.30 ± 0.07	64.84 ± 0.25
None	4.00 ± 0.18	57.38 ± 1.16
SMase	1.40 ± 0.06	54.09 ± 1.05
KC	3.32 ± 0.09	56.26 ± 0.63
KC + SMase	1.38 ± 0.05	56.67 ± 0.51

IEC-6 cells were grown as described in the legend to Fig. 1. On day 2, cells were fed LPDS medium containing 0.5 μCi/dish [^{3}H-methyl]choline. After 48 h of incubation, three dishes were harvested. The rest of the dishes were washed and refed fresh medium with or without ketoconazole (30 μM). SMase (10 mu/ml) was added to a group of dishes. After an additional 2 h of incubation, the media and cells from triplicate dishes was collected. To determine the radiolabel in sphingomyelin and phosphatidylcholine, the cells were processed as described in Experimental Procedures. Results are expressed as the percentage of radiolabeled choline in the total lipid extract appearing in sphingomyelin and phosphatidylcholine. A measure of SMase activity is the decreased level of choline radioactivity in the residual sphingomyelin, fraction.

TABLE 5. Effect of various ACAT and cholesterol biosynthesis inhibitors on sphingomyelinase action on reductase activity in IEC-6 cells

		HMG-CoA Reductase Activity	
Drug	Concentration	None	SMase (10 mu/ml)
		pmol/min/mg protein	
Control		254 ± 16	94 ± 6 (37)[a]
Ketoconazole	30 μM	366 ± 22	354 ± 19 (97)
U18666A	5 μg/ml	173 ± 9	157 ± 21 (91)
Progesterone	10 μg/ml	219 ± 12	278 ± 28 (127)
Miconazole	10 μM	433 ± 13	259 ± 18 (58)
Compound 58-035	5 μg/ml	365 ± 10	211 ± 29 (58)

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium with or without indicated concentrations of the drugs. After 1 h pretreatment, SMase (10 mu/ml) was added. Cells were harvested after 4 h of the additional incubation for the determination of reductase activity as described in Experimental Procedures.

[a]Values in parentheses are % activity of values in absence of SMase.

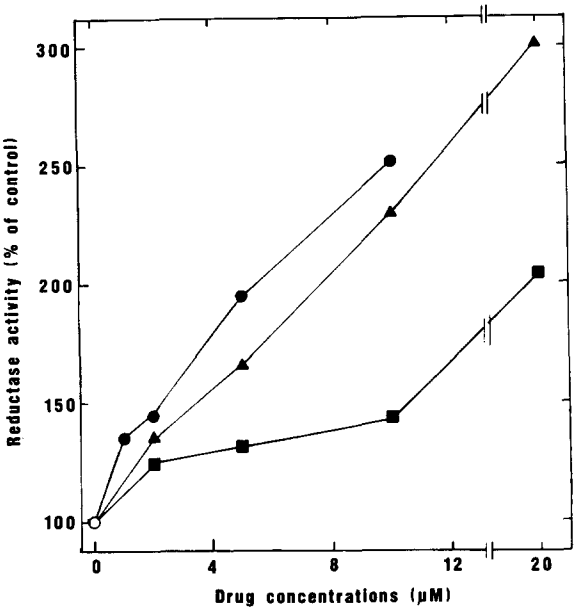

Fig. 6. Effect of the inhibitors of lysosomal sphingomyelinase on reductase activity in IEC-6 cells. Cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium either containing indicated concentrations of chlorpromazine (●), or desipramine (▲) or W-7 (■) in ethanol. Cells were harvested after 4 h of the incubation for determination of reductase activity as described under Experimental Procedures. The control reductase activity was 206 ± 5 pmol/min per mg protein.

Since ketoconazole has been shown to inhibit cholesterol esterification, the possibility exists that SMase action on reductase activity may be linked to the observed stimulation of cholesterol esterification by ACAT. We incubated cells with compound Sandoz-58-035, a potent ACAT inhibitor (7, 18), and observed no effect on the inhibition of reductase by SMase (Table 5). This finding suggests that inhibition of ACAT activity is not related to the prevention of reductase inhibition by ketoconazole.

TABLE 6. Effect of chlorpromazine, desipramine, and W-7 on the inhibition of reductase activity by sphingomyelinase in IEC-6 cells

Drug	Concentration	Reductase Activity	
		SMase (1 mu/ml)	SMase (5 mu/ml)
	μM	% of control	
Control	0	70	61
Chlorpromazine	10	71	60
Desipramine	20	74	46
W-7	20	44	39

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium containing either chlorpromazine (10 μM), desipramine (20 μM), W-7 (20 μM), or ethanol. After 1 h of pretreatment, SMase was added. The cells were harvested from triplicate dishes after 4 h of additional incubation for determination of reductase activity as described in Experimental Procedures. The 100% activity values for control, chlorpromazine-, desipramine-, and W-7-treated cells were 222 ± 32, 419 ± 39, 713 ± 10, and 258 ± 14, respectively.

It is well documented that efflux of cholesterol from the plasma membrane to an acceptor molecule in the medium results in the stimulation of reductase activity and cholesterol biosynthesis (25). If sphingomyelinase treatment resulted in the movement of cholesterol from the plasma membrane to an interior regulatory pool and inhibition of reductase activity, then reversal of transport of cholesterol from the plasma membrane to an internal pool should result in the prevention of SMase effect on reductase activity. We found that treatment of cells with SUV of dioleoyl phosphatidylcholine abolished the effect of SMase on both inhibition of reductase activity and increase in the esterification of plasma membrane cholesterol. Under similar conditions SUV caused an increase in the efflux of cholesterol in presence and absence of SMase. The hydrolysis of [methyl-^{3}H]choline-labeled sphingomyelin was not affected by SUV (Table 7). HDL_3,

TABLE 7. Effect of small unilamellar vesicles on sphingomyelinase action on cholesterol movement, sphingomyelin hydrolysis, and reductase activity in IEC-6 cells

Analysis	Treatment Conditions			
	− SUV		+ SUV	
	None	SMase	None	SMase
Cholesteryl esters (dpm × 10^2)	56	130	24	25
[^{3}H]Cholesterol in medium (dpm × 10^3)	30	33	122	120
Residual sphingomyelin (dpm × 10^3)	16	3	13	4
HMG-CoA reductase (pmol/min/mg protein)	202	105	395	399

IEC-6 cells were grown as described in the legend to Fig. 1. For determination of cholesterol movement, on day 4 the cells were fed LPDS medium containing [^{3}H]cholesterol (0.5 μCi/dish). After 2 h of incubation, medium was removed and cells were washed with phosphate-buffered saline. Fresh medium containing either SUV (500 μM) or equivalent amount of buffer was fed. Sphingomyelinase (10 mu/ml) was added 30 min after the addition of SUV. Medium and cells were harvested after 4 h of incubation and analyzed for cholesterol efflux and cholesteryl esters as described in Experimental Procedures. For determination of sphingomyelin hydrolysis, a similar protocol was followed as described in Table 4, except that SUV was used in place of ketoconazole.

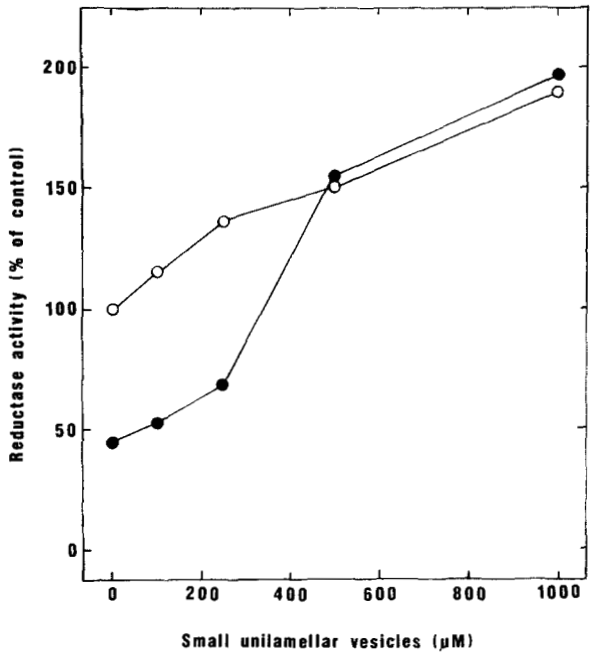

Fig. 7. Effect of small unilamellar vesicles of dioleoyl phosphatidylcholine on the inhibition of reductase activity by sphingomyelinase in IEC-6 cells. Cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium or LPDS medium with indicated concentrations of SUV. After 30 min of incubation, SMase (10 mu/ml) was added to each dish. Cells were harvested for the determination of reductase activity after 2 h of incubation as described under Experimental Procedures. The control reductase activity was 203 ± 4 pmol/min per mg protein; (○) LPDS medium minus SUV; (●) LPDS medium plus SUV.

which elicits a lesser efflux of cholesterol than SUV consisting of phosphatidylcholine (26), also abolished this effect of SMase on reductase activity (Table 8). Thus, these observations with different cholesterol acceptors in the medium support the concept that inhibition of reductase activity by SMase treatment was due to movement of cholesterol from the plasma membrane to a regulatory site where conversion of cholesterol to a regulatory molecule might take place, and that this movement was reversed in the presence of cholesterol acceptors SUV and HDL_3. However, the possibility that these receptor molecules in the medium acted by removal of the putative regulatory polar sterol cannot be ruled out at this time.

We investigated whether products of SMase action, e.g., ceramide and phosphocholine, were involved in the inhibition of reductase activity. Ceramide or phosphocholine showed no effect on reductase activity when they were added to the culture medium. The possibility was considered that in situ products of further degradation of ceramide such as sphingosine might be involved in the inhibition of reductase activity. Hannun and Bell (33) have reviewed the evidence that sphingosine and lysosphingolipids are biologically active molecules that may function as endogenous modulators of cell function. We observed that addition of sphingosine to the culture medium of IEC-6 cells resulted in an increase of reductase activity, suggesting that the possible generation of sphingosine from products of SMase treatment may not be involved in the inhibition of reductase activity.

It has been suggested that lysosomal enzymes are involved in the transport and degradation of membrane sphingolipids (34). Thus it might be expected that inhibition of endogenous (lysosomal) SMase activity would result in enhanced cholesterol synthesis and increased HMG-CoA reductase activity, presumably due to accumulation of sphingomyelin (22, 23) in the plasma membrane. It has been observed that certain drugs, e.g., chlorpromazine, desipramine, and W-7, are inhibitors of lysosomal SMase activity (22, 23). Our experiments show that addition of these substances to the medium increased the activity of HMG-CoA reductase (Fig. 6) but they failed to prevent the inhibitory effect of exogenous SMase on reductase activity (Table 6). This observation is consistent with previous findings where it was shown that these drugs had no effect on SMase activity when added to the in vitro assay. In view of the pleiotropic effects of these substances on other systems, e.g., Ca^{2+}/calmodulin-linked enzyme systems (35–38), further work is necessary to sort out these relationships. From this latter point of view the effect of sphingosine in stimulating HMG-CoA reductase is interesting since it has also been shown to modulate Ca^{2+}/calmodulin-linked enzyme systems (39).

SMase treatment of cultured cells results in several effects that are analogous to the down-regulation of reductase activity by LDL (7). In the case of SMase treatment, plasma membrane cholesterol moved to the interior of the cell and stimulated esterification of cholesterol (this report and ref. 6). Similarly, in the case of LDL addition, there is a release of free cholesterol by lysosomal hydrolysis of cholesteryl ester carried on LDL and subsequent stimulation of cholesterol esterification (7, 40). In both cases, there was an inhibition of reductase activity. Pretreatment

TABLE 8. Effect of HDL_3 on efflux of cholesterol and reductase activity in the presence and absence of SMase

Treatment	Reductase Activity	[^{3}H]Cholesterol Efflux
	pmol/min/mg protein	*dpm × 10³*
None	166 ± 5	27 ± 1.4
HDL_3	226 ± 12	38 ± 1.3
SMase	95 ± 12	31 ± 0.5
HDL_3 + SMase	250 ± 5	38 ± 0.7

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium containing [^{3}H]cholesterol (0.5 μCi/dish) for cholesterol efflux or LPDS medium for reductase activity determination. After 2 h of incubation, the medium was removed, cells were washed with PBS, and fresh medium with or without HDL_3 (250 μg/ml) was fed. SMase (10 mu/ml) was added after 30 min of incubation. Medium and cells were harvested after an additional 4 h of incubation for the determination of [^{3}H]cholesterol efflux into medium and reductase activity as described under Experimental Procedures.

with ketoconazole abolished the action of both SMase (this report) and low density lipoprotein inhibition of reductase activity (7). Other agents, e.g., progesterone (21) and U18666A (20), that abolished the inhibitory action of LDL on reductase activity also prevented the inhibition of reductase by SMase (Table 5). Of further interest is the fact that miconazole (10), which was unable to prevent LDL suppression of reductase activity, was also unable to attenuate the inhibitory effect of SMase on reductase activity.

As we have pointed out previously in all of the foregoing situations the evidence is in agreement with the possibility of movement of free cholesterol to a putative regulatory site (pool) and that the molecule generated at that site is an oxysterol. However, we are unable to rule out the possible involvement of the well-known capability of cholesterol to perturb membrane structure with subsequent effects on enzyme function (41, 42).

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