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Cholesterol esterase accelerates intestinal cholesterol absorption

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Abstract

Mechanisms of acceleration of cholesterol absorption by cholesterol esterase were investigated in various experimental conditions. Lymphatic recovery of cholesterol intubated as a micellar solution containing phosphatidylcholine (PC) into the duodenum was enhanced by the co-administration of cholesterol esterase in rats drained of bile and pancreatic juice. However, no accelerated incorporation was observed when cholesterol was solubilized in PC-depleted micelles. Cholesterol esterase dose-dependently accelerated the incorporation of cholesterol into differentiated Caco-2 cells, only when cholesterol was solubilized in PC-containing micelles. The accelerated incorporation of cholesterol into Caco-2 cells by cholesterol esterase disappeared when the enzyme was preincubated with a suicide inhibitor of cholesterol esterase. Cholesterol esterase has an activity as phospholipase A_2. When 10% of PC in bile salt micelles was replaced by lysophosphatidylcholine (lysoPC), the incorporation of cholesterol into Caco-2 cells was significantly accelerated. Cholesterol esterase enhanced the incorporation of micellar cholesterol into brush border membranes prepared from the rat jejunum. The addition of cholesterol esterase to bile salt micelles accelerated the release of micellar cholesterol in a dose-dependent manner, only when the micelles contained PC. These observations strongly suggest that cholesterol esterase hydrolyzes PC in bile salt micelles and thereby, accelerating the release of cholesterol from bile salt micelles. This may be a major cause of the acceleration of cholesterol absorption by cholesterol esterase.

Keywords: Cholesterol esterase; Caco-2 cells; Cholesterol absorption; Phosphatidylcholine; Phospholipase A_2

1. Introduction

Pancreatic cholesterol esterase is an enzyme that hydrolyzes cholesterol ester to cholesterol and free fatty acid within the intestinal lumen [1]. This enzyme is also known as bile salt-stimulated lipase and carboxy ester lipase [1]. Because cholesterol ester is not directly absorbed in intestinal epithelial cells, the hydrolysis of cholesterol ester to cholesterol by cholesterol esterase in the intestinal lumen is an essential process for the incorporation of cholesterol into the cells.

It has been reported that cholesterol esterase is involved in the absorption of unesterified cholesterol [2]. Gallo et al. [3] observed that the addition of cholesterol esterase to a culture medium increased the esterification of cholesterol in isolated intestinal cells. They also showed that the depletion of cholesterol esterase in rat intestinal lumen markedly reduced cholesterol absorption in mesenteric lymph [4]. They proposed that cholesterol esterase might be essential for the absorption of unesterified cholesterol by involving cholesterol esterification in intestinal cells [4]. Bhat and Brockman [5] reported that cholesterol esterase increased the incorporation of cholesterol into rat intestinal sacs. They suggested that cholesterol esterase became anchored to the brush border membranes and acts as a cholesterol transporter. However, controversial observations have been reported. Although some investigations observed the accelerated absorption of cholesterol by cholesterol esterase [6,7], others did not

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demonstrate the acceleration of esterification and absorption of cholesterol in in vitro studies [8,9]. Howles et al. [10] showed that cholesterol esterase did not play a primary role in unesterified cholesterol absorption by using cholesterol esterase-knockout mice. It seems likely that unphysiological experimental conditions in some in vitro studies, as pointed out by Shamir et al. [9], make it difficult to clarify the precise mechanisms of the effect of cholesterol esterase.

In the present study, to reveal the causes of the discrepancy among various studies, we reinvestigated the effect of cholesterol esterase at various absorption stages of unesterified cholesterol. Our results suggest that although cholesterol esterase does not necessarily have an essential role on cholesterol absorption, it accelerates cholesterol absorption through hydrolysis of phosphatidylcholine (PC).

2. Materials and methods

2.1. Materials

Trypsin–chymotrypsin inhibitor, egg yolk PC, egg yolk lysophosphatidylcholine (lysoPC), 1-monooleoyl-rac-glycerol (monoolein), oleic acid and bovine pancreatic cholesterol esterase were purchased from Sigma (Sigma-Aldrich Japan, Tokyo, Japan). Human recombinant cholesterol esterase was kindly provided from Meiji Milk Products Co., Tokyo. Sodium taurocholate (purity >97%) was from Nacalai Tesque (Kyoto, Japan). [4-^{14}C]-Cholesterol was obtained from Amersham (Amersham Pharmacia Biotech, Tokyo). Cholesterol (>99%) and fatty acid-free bovine serum albumin (BSA) were from Daiichi Pure Chemical Co. (Tokyo) and Miles Scientific (ICN ImmunoBiologicals, Lisle, IL, USA), respectively. 3-Benzyl-6-chloropyrone (3-BCP), an suicide inhibitor of cholesterol esterase [11], was synthesized according to the precedents of Daniels et al. [12].

2.1.1. Preparation of micellar solutions

The composition and preparation of mixed micellar solutions were according to our previous studies [13,14]. A series of micellar solutions containing PC included 6.6 mM sodium taurocholate, 0.6 mM egg yolk PC, 1 mM oleic acid and 12.5 to 100 μM ^{14}C-cholesterol. PC-depleted micellar solutions contained 6.6 mM sodium taurocholate, 0.5 mM monoolein, 1 mM oleic acid and 50 or 100 μM ^{14}C-cholesterol. Micellar solutions were prepared in various culture media or buffers as described in the legends to Figures. The micelles prepared by sonication were passed through a 0.2-μm syringe filter (25 mm, GD/X, Whatman, Inc., Clifton, NJ, USA) and kept at 37 °C until use.

2.1.2. Cell culture

Cell culture was conducted according to the method of Ranheim et al. [15]. Caco-2 cells were grown on 60- or 100-mm plastic petri dishes at 37 °C in air and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL, Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS, GIBCO), 2 mM L-glutamate, 10 μg/ml insulin, 100 μg/ml streptomycin, 100 IU/ml penicillin, 1% non-essential-amino acids (ICN) and 3.7 g/l $NaHCO_3$. The culture medium was changed every other day. For the subculture, the medium was removed and the cells were detached from the culture dish with 0.25% trypsin in a Ca^{2+}-, Mg^{2+}-free phosphate-buffer saline (PBS) containing 0.2 g/l EDTA. Culture medium with FBS was added to stop trypsinization. Cells were suspended and seeded on a filter membrane or new plastic petri dishes.

2.1.3. Studies on incorporation of micellar cholesterol into Caco-2 cells

Cells were plated and grown to confluency on the apical side of presoaked membrane filters of 3.0-μm pore size and 23.1-mm diameter (Biocoat cell culture collagen type I, FALCON, Nippon Beckton Dickinson Co., Tokyo) in 2-ml culture medium supplemented with 10% FBS. To the lower well were added 2.5 ml of culture medium supplemented with 10% FBS. Cells at 14–16 days after reaching confluency were subjected to various studies on the incorporation of micellar cholesterol. The maturation of brush border membranes was confirmed by the increase in the activities of sucrase and alkaline phosphatase, which were marker enzymes of the membranes. The activities of both enzymes reached a maximum at 8 days after confluency. It has been reported that the differentiation state of Caco-2 cells may be achieved in 10–15 days after confluence [16,17]. The culture medium containing 10% FBS was replaced with the culture medium supplemented with 10% lipoprotein deficient serum (LPDS) 24 h prior to commencing the experiments. LPDS was obtained as follows: after FBS was adjusted to d=1.21 with KBr, it was ultracentrifuged at 100,000×g for 48 h. The LPDS fraction was then collected and dialyzed against PBS.

Mixed micellar solutions containing ^{14}C-cholesterol were prepared in culture medium. After filtration through a 200-nm filter, 2 ml of the micellar solution supplemented with 10% LPDS was added to the apical side of differentiated Caco-2 cells and 2.5-ml culture medium supplemented with 10% LPDS was added to the lower well (basolateral side). In the case where the effect of human cholesterol esterase was examined, it was added to the apical side. When the effect of 3-BCP on the acceleration of cholesterol absorption by cholesterol esterase was studied, human cholesterol esterase (318 μg) was preincubated with 300 μM 3-BCP for 5 min at 37 °C prior to the addition to the apical side. After 24 h of incubation, the cells were washed twice with PBS, scraped and suspended in saline. Aquasol II (Packard Japan, Tokyo) was added to an aliquot of the cell suspension solubilized in NCS II (Amersham). The radioactivities were counted with a liquid scintillation counter. Since a small portion of the cell-associated radioactive cholesterol was secreted to the basolateral side, the radioactivity in the basolateral medium was also measured. The amount of the

incorporation of micellar cholesterol was calculated as the sum of cholesterols associated with cells and secreted to the basolateral side. Lipids in cell suspension were extracted with chloroform/methanol=2:1 [18]. Esterified and unesterifed cholesterols were separated with thin-layer chromatography (hexane/diethylether/acetic acid=83:16:1).

2.1.4. Animals

Adult male SD rats (Seac Yoshitomi, Fukuoka, Japan) weighing 280–320 g were housed under a normal (12 h) light cycle and allowed free access to laboratory chow (NMF, Oriental Yeast Co., Tokyo) and water, ad libitum, prior to use. All animal studies were carried out under the guidelines for animal experiments of the Faculty of Agriculture, Graduate School, Kyushu University, and Law 105 and Notification 6 of the government of Japan.

2.1.5. Cannulation of thoracic and bile ducts

Bile and pancreatic juice was drained by an indwelling bile duct catheter inserted into the common bile duct near the junction to the duodenum [19]. The left thoracic lymphatic duct cephalad to the cisterna chyli was also cannulated. A third indwelling catheter was placed in the duodenum for administration of test micellar solutions. After surgery, the animals were placed in restraining cages, and intraduodenally given a continuous infusion of a mixed micellar solution containing 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC, 50 μM cholesterol, 62 mM NaCl and 139 mM glucose in 15 mM sodium phosphate buffer at pH 7.4 (PC-containing micelles), or 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.5 mM monoolein, 50 μM cholesterol, 62 mM NaCl and 139 mM glucose in 15 mM sodium phosphate buffer at pH 7.4 (PC-depleted micelles), at a rate of 3.4 ml/h until the end of the experiment. The next morning, animals with a constant lymph flow rate were administered 2 ml of one of the test micellar solutions containing ^{14}C-cholesterol with or without human cholesterol esterase (159 μg). The composition of the test micellar solutions was the same as the micellar solutions continuously infused overnight. Lymph was collected in ice-chilled tubes containing EDTA and the radioactivity was measured. Rats were not allowed drinking water to avoid dilution of the micellar solution during the course of lymph collection.

2.1.6. Preparation of brush border membranes

Intestinal brush border membranes were prepared from the proximal half of the small intestine as described by Kessler et al. [20]. Trypsin–chymotrypsin inhibitor was added in a homogenizing buffer to prevent proteolysis. The membranes were suspended in Hank's balanced salt solution containing 5 mM EGTA and 4% BSA (fatty acid-free) in 15 mM HEPES, pH 7.4, to give a final protein concentration of 2 mg/ml and kept at 0 °C until use. They were consistently enriched regarding the activities of sucrase and alkaline phosphatase 15-fold relative to the whole homogenate. Recovery of acyl CoA cholesterol acyltransferase [21], succinic dehydrogenase [22] and DNA [23], markers for microsomes, mitochondria and nuclei, respectively, in the brush border membranes was less than 0.5% of

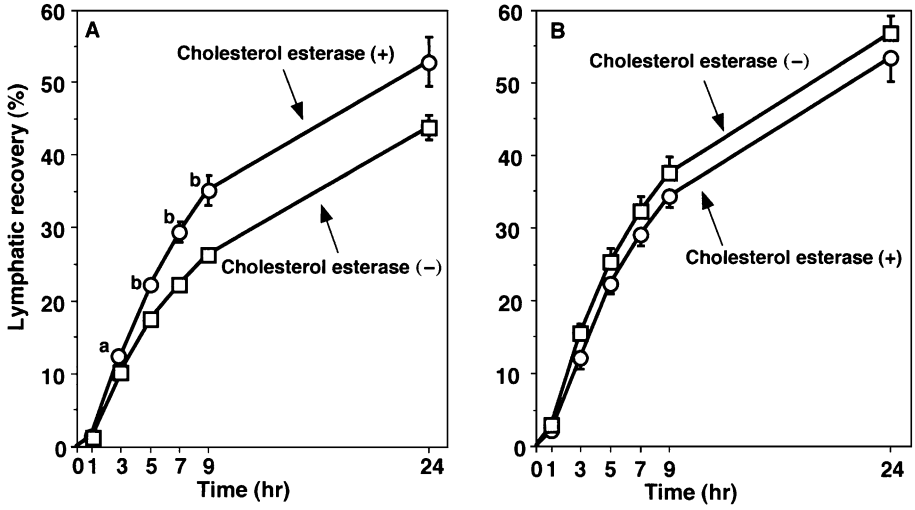

Fig. 1. Effect of human cholesterol esterase on lymphatic recovery of micellar cholesterol in thoracic duct-cannulated rats drained of pancreatic juice and bile. Bile- and pancreatic juice-diverted rats were given a micellar solution to the duodenum overnight. In (A), a micellar solution contained 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC, 50 μM cholesterol, 62 mM NaCl and 139 mM glucose in 15 mM sodium phosphate buffer at pH 7.4, and in (B), 0.5 mM monoolein was included instead of 0.6 mM PC. In the next morning, the rats were administered 2 ml of a test micellar solution containing ^{14}C-cholesterol with or without human cholesterol esterase (159 μg, 7.5 U). Lymph was collected for 24 h. One unit of human cholesterol esterase is equivalent to 1.0 μmol cholesterol oleate formed/min (21 μg protein). In (A), lymph flow was 37.5±2.9 and 31.9±1.0 ml/24 h in the Cholesterol esterase (−) and Cholesterol esterase (+) groups, respectively, and in (B), it was 39.1±1.3 and 39.1±3.6 ml/24 h, respectively. Data are means±S.E. of five or six rats. a,b: Significantly different from the Cholesterol esterase (−) group at $P<0.05$ and 0.01, respectively.

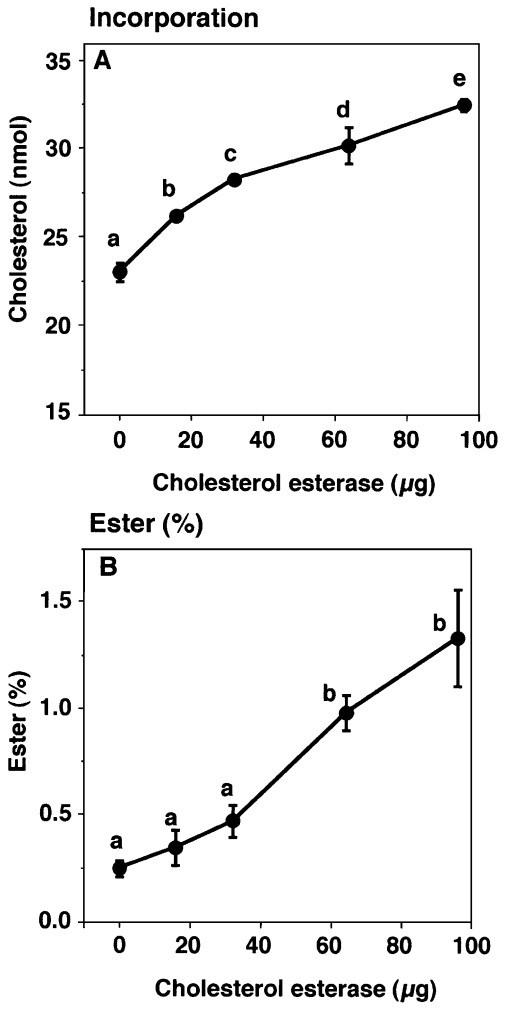

Fig. 2. Effect of human cholesterol esterase on the incorporation of micellar cholesterol and the esterification of incorporated cholesterol in Caco-2 cells. Two milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC and 50 μM cholesterol in culture medium supplemented with 10% LPDS was added with various amounts of human cholesterol esterase to the apical side of differentiated Caco-2 cells and incubated for 24 h. Culture medium (2.5 ml) supplemented with 10% LPDS was added in the lower well. The amount of the incorporation of micellar cholesterol (A) is the sum of cholesterols associated with cells and secreted to the basolateral side. See also the legend in Fig. 1. Data are means ± S.E. of four or five wells. a,b,c,d,e: Different letters show significant difference at $P < 0.05$.

the levels in the whole homogenate ob-served in our previous study [13].

2.1.7. Transfer of cholesterol from micellar solution to brush border membranes

The experimental conditions were according to our previous study [13,14]. Four milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 0.6 mM PC and 50 μM ^{14}C-cholesterol prepared in Hank's balanced salt solution containing 5 mM EGTA and 4% BSA (fatty acid-free) in 15 mM HEPES buffer, pH 7.4, were incubated with 1 ml of brush border membrane suspension (2 mg protein) at 37 °C. Bovine pancreatic cholesterol esterase was added before the addition of the brush border membrane suspension. To the control group was added the suspension buffer. At 0 and 30 min, 1 ml of the incubation solution was withdrawn and released into 5-ml iced 0.9% NaCl containing 7 mM sodium taurocholate. This was centrifuged at 27,000×g for 30 min at 5 °C. The resulting pellet was washed once with 5 ml of the same solution and then again collected by centrifugation. The brush border membrane pellet was suspended in 1.0-ml distilled water by sonication. An aliquot of the solution was subjected to sterol determination by radioactivity measurement and to protein determination.

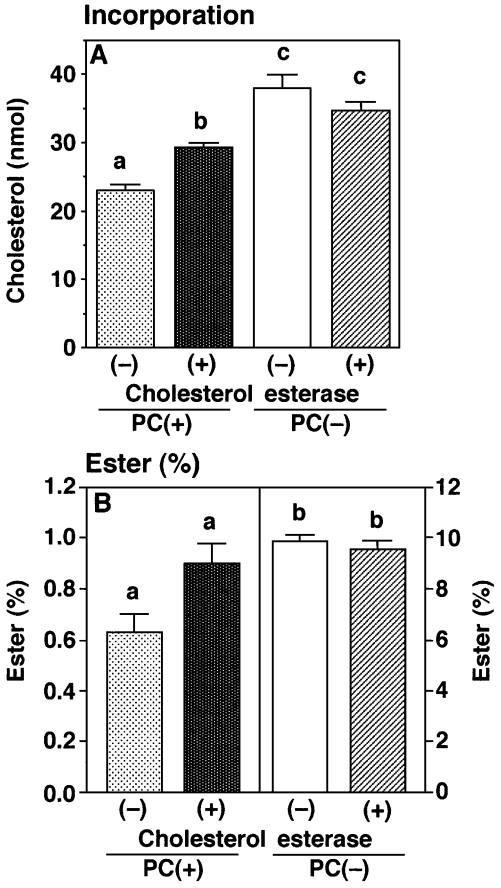

Fig. 3. Effect of human cholesterol esterase on the incorporation of cholesterol solubilized in PC-containing and PC-depleted micelles and the esterification of incorporated cholesterol in Caco-2 cells. Two milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC and 50 μM cholesterol (PC(+)), or 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.5 mM monoolein and 50 μM cholesterol (PC(−)) in culture medium supplemented with 10% LPDS was added with or without cholesterol esterase (32 μg, 1.5 U) to the apical side of differentiated Caco-2 cells and incubated for 24 h. Culture medium (2.5 ml) supplemented with 10% LPDS was added in the lower well. The amount of the incorporation of micellar cholesterol (A) is the sum of cholesterols associated with cells and secreted to the basolateral side. See also the legend in Fig. 1. Data are means ± S.E. of four wells. a,b,c: Different letters show significant difference at $P < 0.05$.

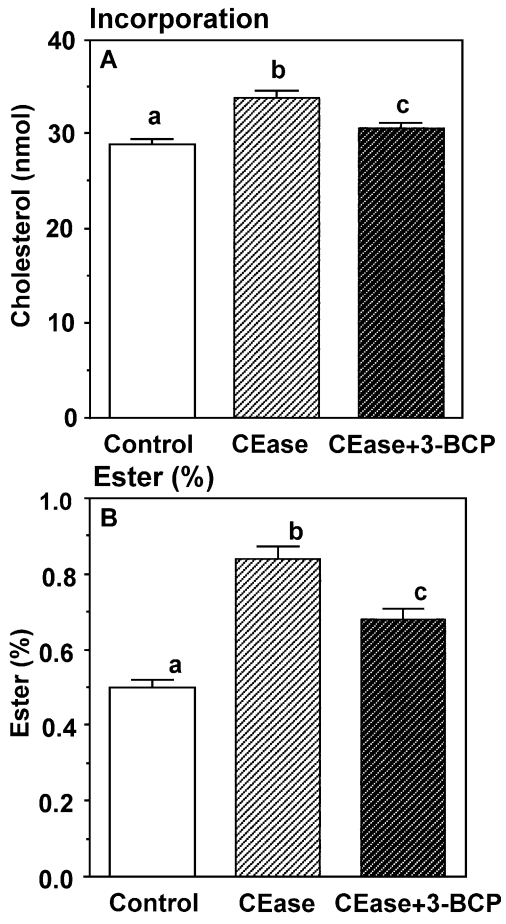

Fig. 4. Effect of human cholesterol esterase and 3-BCP, a suicide inhibitor of cholesterol esterase, on the incorporation of micellar cholesterol and the esterification of incorporated cholesterol in Caco-2 cells. Two milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC and 50 μM cholesterol in culture medium supplemented with 10% LPDS was added to the apical side of differentiated Caco-2 cells and incubated for 24 h. Cholesterol esterase (32 μg, 1.5 U) and cholesterol esterase (32 μg) preincubated with 3-BCP were also added to the apical side in the CEase or CEase +3-BCP groups, respectively. Culture medium (2.5 ml) supplemented with 10% LPDS was added in the lower well. The amount of the incorporation of micellar cholesterol (A) is the sum of cholesterols associated with cells and secreted to the basolateral side. See also the legend in Fig. 1. Data are means ± S.E. of four wells. a,b,c: Different letters show significant difference at $P < 0.05$.

2.1.8. Transfer of cholesterol from micellar solutions to triolein

The affinity of cholesterol for bile salt micelles was examined as described previously [14]. One and a half milliliters of a micellar solution, 0.5 ml of triolein and various amounts of cholesterol esterase were placed in a plastic tube, flushed with N_2 and sealed. Two micellar solutions were prepared. The composition of PC-containing micelles was 6.6 mM sodium taurocholate, 0.6 mM PC, 100 μM cholesterol and 132 mM NaCl in 15 mM sodium phosphate buffer (pH 7.4), and that of PC-depleted micelles was 6.6 mM sodium taurocholate, 0.5 mM monoolein, 100 μM cholesterol and 132 mM NaCl in 15 mM sodium phosphate buffer (pH 7.4). In PC-containing micelles, the tubes were incubated at 37 °C in an oscillating water bath for 6 h (190 oscillations/min), at which time the rate of cholesterol transfer from the micellar solution to triolein was linear. In PC-depleted micelles, incubation time was 2 h because the transfer rate was faster than in PC containing micelles and the transfer was almost completed for 6 h. At the end of the incubation, the contents of each tube were transferred to a Beckman Quick Seal tube, and

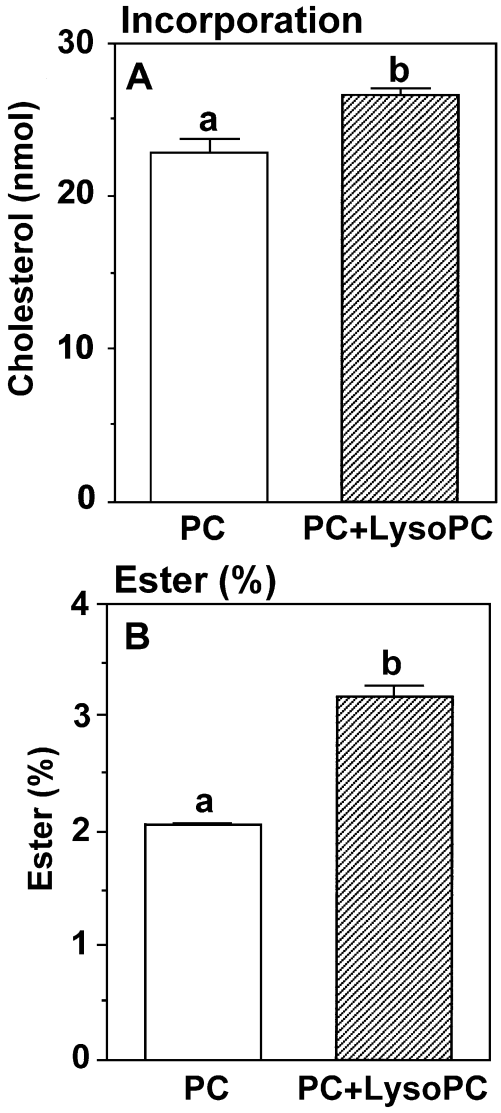

Fig. 5. Effect of lysoPC on the incorporation of micellar cholesterol and the esterification of incorporated cholesterol in Caco-2 cells. Two milliliters of a micellar solution supplemented with 10% LPDS was added to the apical side of differentiated Caco-2 cells and incubated for 24 h. Culture medium (2.5 ml) supplemented with 10% LPDS was added in the lower well. Two micellar solutions were prepared. One had the same composition as in Fig. 2 (PC group). Ten percent (0.06 mM) of PC was replaced by lysoPC in the other micellar solution (PC + LysoPC group). The amount of the incorporation of micellar cholesterol (A) is the sum of cholesterols associated with cells and secreted to the basolateral side. Data are means ± S.E. of five wells. a,b: Different letters show significant difference at $P < 0.01$.

the oil and aqueous phases were separated by ultracentrifugation (Optima TL Ultracentrifuge, Beckman, Pao Alto, CA) at 100,000×*g* for 1 h at 37 °C. The oil and aqueous phases were collected and analyzed for cholesterol concentration with GLC by using 5α-cholestane as the internal standard [14].

2.1.9. Protein and sterol analyses

Total protein was measured according to the method of Lowry et al. [24]. Sterols were estimated by liquid scintillation counting of radioactivity or by GLC determination of mass [19].

2.1.10. Statistical analysis

All data are expressed as means ± S.E. Student's *t*-test [25] or Duncan's new multiple range test [26] were used and *P* values of <0.05 were considered significant.

3. Results

3.1. Effect of human cholesterol esterase on lymphatic recovery of micellar cholesterol in lymph-cannulated rats

When a micellar solution contained PC, lymphatic recovery of cholesterol was increased by the addition of cholesterol esterase (Fig. 1A). In contrast, cholesterol esterase did not accelerate lymphatic recovery of cholesterol in the case of PC-depleted micelles (Fig. 1B). In the first study, cholesterol esterase did not influence the proportion of esterified cholesterol in lymph, i.e. 78% and 79% of cholesterol were esterified in the lymph of rats given a micellar solution with or without cholesterol esterase, respectively.

3.2. Effect of human and bovine cholesterol esterase on incorporation of micellar cholesterol and esterification of incorporated cholesterol in Caco-2 cells

The effect of human cholesterol esterase on micellar ^{14}C-cholesterol uptake was investigated in differentiated Caco-2 cells. The addition of cholesterol esterase to bile salt micelles containing PC accelerated the incorporation of micellar cholesterol into Caco-2 cells (Fig. 2A). The effect of cholesterol esterase was dose-dependent. The ratio of ^{14}C-cholesterol esterified in the cells was dose-dependently increased by the addition of cholesterol esterase (Fig. 2B). The same acceleration of micellar cholesterol uptake was observed when bovine cholesterol esterase was used in some studies (data not shown).

In the separate study, the effect of cholesterol esterase was compared between PC-depleted and PC-containing micelles. Although cholesterol esterase accelerated the incorporation of ^{14}C-cholesterol into Caco-2 cells in PC containing micelles, the addition of cholesterol esterase to PC-depleted micelles did not influence the incorporation of ^{14}C-cholesterol (Fig. 3A). The same tendency was observed in the percentage of esterified cholesterol (Fig. 3B).

3.3. Effect of cholesterol esterase preincubated with 3-BCP on incorporation of micellar cholesterol and esterification of incorporated cholesterol in Caco-2 cells

As in the case of Figs. 2 and 3, the addition of cholesterol esterase to a micellar solution containing PC accelerated the incorporation of cholesterol into Caco-2 cells (Fig. 4A). In contrast, cholesterol esterase preincubated with 3-BCP did not increase the incorporation of cholesterol into the cells.

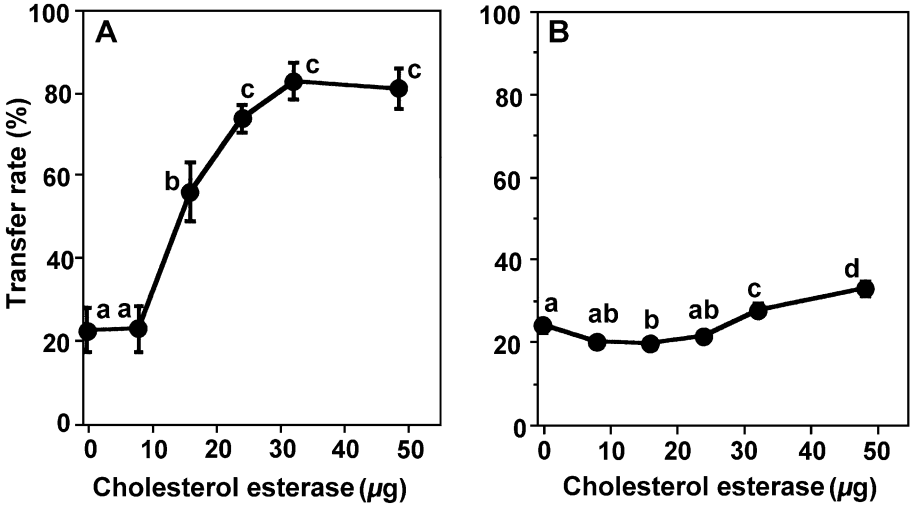

Fig. 6. Effect of bovine cholesterol esterase on transfer of micellar cholesterol to triolein. Triolein and a micellar solution containing (A) 6.6 mM sodium taurocholate, 0.6 mM PC, 100 μM cholesterol and 132 mM NaCl in 15 mM sodium phosphate buffer (pH 7.4), or (B) 6.6 mM sodium taurocholate, 0.5 mM monoolein, 100 μM cholesterol and 132 mM NaCl in 15 mM sodium phosphate buffer (pH 7.4), were incubated with various amounts of bovine cholesterol esterase for (A) 6 or (B) 2 h. Transfer of micellar cholesterol to triolein was measured. One unit of bovine cholesterol esterase is equivalent to 1.0 μmol cholesterol oleate formed/min (32 μg protein). Data are means ± S.E. of three determinations. a,b,c,d: Different letters show significant difference at $P < 0.05$.

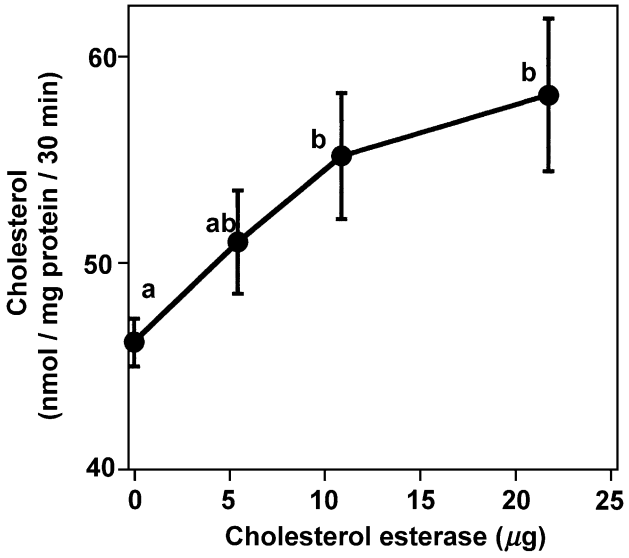

Fig. 7. Effect of bovine cholesterol esterase on the uptake of micellar cholesterol by rat intestinal brush border membranes. Four milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 0.6 mM PC, 50 μM ^{14}C-cholesterol prepared in Hank's balanced salt solution containing 5 mM EGTA and 4% BSA (fatty acid-free) in 15 mM HEPES buffer, pH 7.4 was incubated with 1 ml of brush border membrane suspension (2 mg protein) at 37 °C. Various amounts of bovine pancreatic cholesterol esterase was added to the incubation mixture. See also the legend in Fig. 6. Data are means±S.E. of five rats. a,b: Different letters show significant difference at $P < 0.05$.

The percentage of esterified cholesterol was significantly higher in the addition of cholesterol esterase compared with the control, whereas it was lower in the addition of cholesterol esterase preincubated with 3-BCP than in the addition of cholesterol esterase alone (Fig. 4B).

3.4. Effect of lysoPC on incorporation of micellar cholesterol and esterification of micellar cholesterol in Caco-2 cells

It has been reported that cholesterol esterase has the ability to hydrolyze PC to lysoPC [1]. About 10% of PC in the mixed micellar solution used in our Caco-2 cell studies was hydrolyzed to lysoPC by the addition of 32-μg human cholesterol esterase during 24-h incubation. Therefore, in a model study, 10% PC in a mixed micellar solution was replaced with lysoPC and added to the apical side of the differentiated Caco-2 cells and incubated for 24 h. The result showed that the incorporation of micellar cholesterol

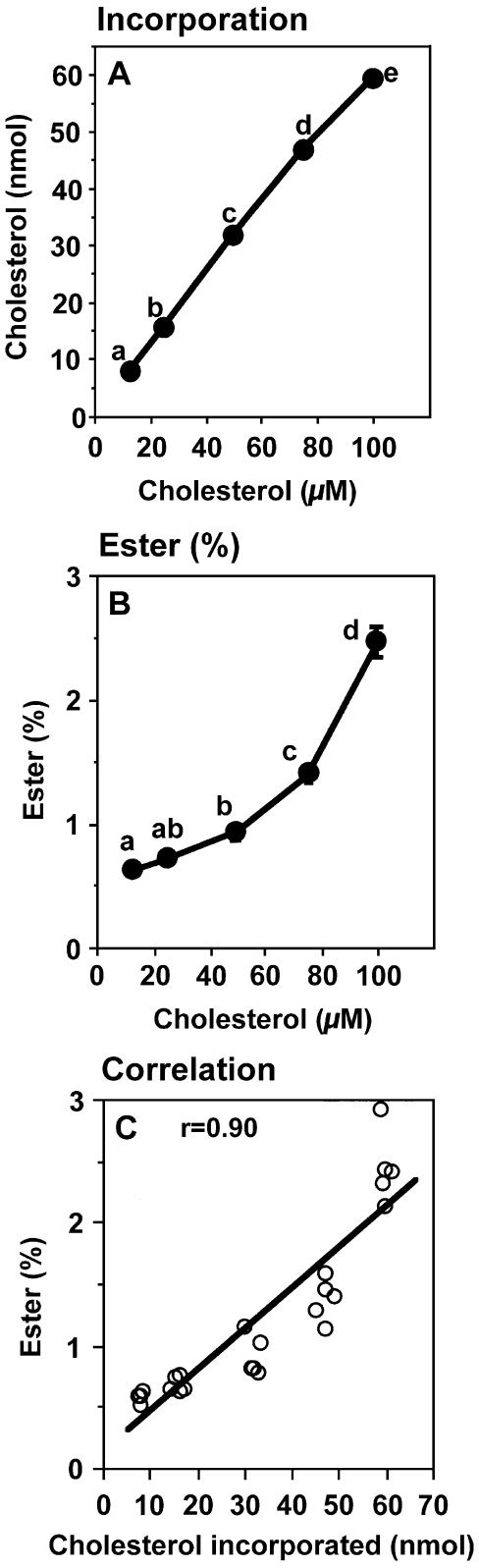

Fig. 8. Effect of cholesterol concentration in micelles on the incorporation of micellar cholesterol, the esterification of incorporated cholesterol and their correlation in Caco-2 cells. Two milliliters of a micellar solution containing 6.6 mM sodium taurocholate, 1 mM oleic acid, 0.6 mM PC and various amounts (12.5, 25, 50, 75 or 100 μM) of cholesterol in culture medium supplemented with 10% LPDS was added to the apical side of differentiated Caco-2 cells and incubated for 24 h. Culture medium (2.5 ml) supplemented with 10% LPDS was added in the lower well. Data are means±S.E. of four or five wells. a,b,c,d,e: Different letters show significant difference at $P < 0.05$.

and the esterification rate of the incorporated cholesterol were higher in a micelle containing lysoPC compared with a micelle containing PC alone (Fig. 5).

3.5. Transfer of micellar cholesterol to triolein

Effect of bovine cholesterol esterase on transfer of micellar cholesterol to triolein was measured. We previously used the method as a model of measuring the affinity of cholesterol against bile salt micellar solutions [14]. The addition of cholesterol esterase to a micellar solution containing PC significantly increased the transfer of micellar cholesterol to triolein in a dose-dependent manner (Fig. 6A). In contrast, only a small acceleration was observed in PC-depleted micelles (Fig. 6B).

3.6. Effect of bovine cholesterol esterase on uptake of micellar cholesterol by rat intestinal brush border membranes

Brush border membranes and bile salt micelles containing ^{14}C-cholesterol were incubated with or without bovine cholesterol esterase (Fig. 7). Cholesterol esterase dose-dependently increased the incorporation of cholesterol into brush border membranes.

3.7. Effect of different concentration of micellar cholesterol on incorporation of cholesterol and esterification of incorporated cholesterol in Caco-2 cells

The incorporation of micellar cholesterol dose-dependently increased with the increase of micellar cholesterol concentration (Fig. 8A). The percentage of esterified cholesterol was also dose-dependently increased (Fig. 8B). A highly positive correlation was observed between the percentage of esterification and cholesterol incorporated into the cells ($r = 0.90$) (Fig. 8C).

4. Discussion

The acceleration of cholesterol absorption by cholesterol esterase was confirmed by our in vivo study. Cholesterol esterase accelerated lymphatic absorption of cholesterol in pancreatic juice-drained rats, only when PC-containing micelles were infused into intestinal lumen (Fig. 1). Cholesterol esterase dose-dependently accelerated the incorporation of micellar cholesterol into Caco-2 cells (Fig. 2) and the acceleration occurred only when bile salt micelles contained PC (Fig. 3). We also showed that when cholesterol esterase was inhibited by the preincubation with 3-BCP, a suicide inhibitor of cholesterol esterase, the acceleration of cholesterol incorporation disappeared (Fig. 4). These observations strongly suggest that the acceleration of the absorption of unesterified cholesterol by cholesterol esterase occurs only when bile salt micelles contain PC, and the enzyme activity rather than the protein may be responsible for the acceleration. Gallo et al. [4] observed that depletion of cholesterol esterase and pancreatic juice in the intestinal lumen dramatically reduced lymphatic cholesterol absorption and cholesterol esterase activity in intestinal mucosal cells. They concluded that cholesterol esterase played an essential role in the regulation of cholesterol absorption. We do not think that cholesterol esterase plays a primary role on cholesterol absorption, because a significant amount of cholesterol was incorporated into Caco-2 cells even when no cholesterol esterase was added (Fig. 2) and it was absorbed in lymph of rats drained of pancreatic juice (Fig. 1).

It has been known that cholesterol esterase hydrolyzes phospholipids [1]. In our experimental conditions, the addition of 1.5 U (32 μg protein) of cholesterol esterase to micellar solutions containing PC hydrolyzed 10% of PC to lysoPC during 24-h incubation at 37 °C. When 10% of PC in the micelles were replaced by lysoPC, the incorporation of cholesterol into Caco-2 cells was accelerated (Fig. 5). This observation suggests that the acceleration of cholesterol absorption by cholesterol esterase is mediated by the hydrolysis of PC. There have been several in vivo and in vitro studies in which PC suppressed cholesterol absorption [27–34]. Recently, Homan and Hamelehle [35] showed that lysoPC formed by the action of phospholipase A_2 relieved PC-dependent reduction in cholesterol absorption. Richmond et al. [36] also reported that compensatory phospholipid digestion was required for cholesterol absorption in pancreatic phospholipase A_2-deficient mice. Therefore, it is reasonable to assume that cholesterol esterase accelerates cholesterol absorption via the hydrolysis of PC.

Our results also showed that the dose-dependent acceleration of the incorporation of cholesterol occurred at the surface of intestinal brush border membranes (Fig. 7). We previously reported that the difference in the affinity of various sterols for mixed bile salt micelles was one of the major determinants for their absorbability [14]. Since sitosterol has higher affinity for bile salt micelles than cholesterol and therefore, it is thought that the former sterol is less absorbable than the latter. The affinity of sterols for the micelles was assessed with measuring sterol movement from micellar to oil phases (triolein) [14]. We used the same procedure to assess the effect of cholesterol esterase on the affinity of cholesterol for bile salt micelles. The addition of cholesterol esterase in bile salt micelles containing PC accelerated the transfer of cholesterol from micellar to oil phases in a dose-dependent manner (Fig. 6). However, in PC-depleted micelles, the effect of cholesterol esterase on the transfer of cholesterol to triolein was marginal. We think that PC hydrolysis by cholesterol esterase facilitates the release of cholesterol as a monomer from bile salt micelles by weakening the affinity of cholesterol for bile salt micelles containing PC, and hence, the incorporation of micellar cholesterol into intestinal absorptive cells is accelerated.

Triolein is a substrate of cholesterol esterase. In a preliminary study measuring sterol movement from micellar

to oil phases, 32 μg of cholesterol esterase hydrolyzed 5% and 9% of triolein in PC-containing micelles during 6-h incubation and in PC-depleted micelles during 2-h incubation, respectively. We cannot deny a possibility that the hydrolysis products of triolein influenced the transfer rate of micellar cholesterol to triolein. However, as seen in Fig. 6, although the rate of hydrolysis of triolein was higher in PC-depleted micelles than in PC-containing micelles, the transfer of cholesterol in the former micelles was not increased by the addition of cholesterol esterase. Therefore, we conclude that hydrolysis products of triolein do not substantially influence the increased transfer of cholesterol in PC-containing micelles.

Gallo et al. observed that the preincubation of isolated intestinal cells with micellar cholesterol and pancreatic cholesterol esterase increased the esterification of cholesterol in the cells [3], and that cholesterol esterase penetrated from the intestinal lumen into intestinal villous cells [37]. Based on these observations, they propose that cholesterol esterase incorporated into mucosal cells accelerates esterification of cholesterol and hence enhances cholesterol absorption. Lopez-Candales et al. [6] also reported that esterification of cholesterol increased with the addition of cholesterol esterase in Caco-2 cells. Although several studies showed that cholesterol esterase was immunologically detected in the cytosol of mucosal cells, Field [38] concluded that the cholesterol esterase is a contamination of pancreatic origin. Even if cholesterol esterase is incorporated into the cells, it is not clear whether or not cholesterol esterase esterifies cholesterol, because this enzyme requires bile salt for activation [1].

In Caco-2 cells, we observed that the esterification rate of labeled cholesterol incorporated into Caco-2 cells increased with the addition of cholesterol esterase in the apical micellar solution (Fig. 2). However, when the amount of cholesterol incorporated into the cells was increased by the stepwise increase in the concentration of micellar cholesterol (Fig. 8), the rate of esterification of the incorporated cholesterol was also enhanced. A highly positive correlation was observed between the amount of the incorporation and the esterification rate of cholesterol. These observations strongly suggest that the enhanced incorporation of cholesterol induces an alternative esterification enzyme, acyl CoA cholesterol acyltransferase (ACAT), in Caco-2 cells. In this context, it is widely accepted that ACAT is more likely to be responsible for the esterification of cholesterol in intestinal mucosal cells [38]. Ellsworth and Starr [39] also showed by using inhibitors of ACAT that the esterification of cholesterol incorporated into Caco-2 cells was not due to cholesterol esterase added to culture medium, but was completely dependent on ACAT activity. Although cholesterol esterase accelerated the lymphatic absorption of cholesterol (Fig. 1), the esterification of cholesterol in lymph was not influenced by the treatment of cholesterol esterase. Therefore, we conclude that the increased esterification of cholesterol in Caco-2 cells by cholesterol esterase cannot be ascribed to the action of cholesterol esterase, but rather to the induced activation of ACAT in the case of Caco-2 cells in which esterification of cholesterol is extremely low.

Our conclusion is that cholesterol esterase hydrolyzes PC to lysoPC through the phospholipase A_2 activity, and hence, it releases the PC-dependent suppression of cholesterol absorption as shown in Fig. 9. Therefore, pancreatic phospholipase A_2 may be more responsible for cholesterol absorption compared with cholesterol esterase [40]. However, we contend that cholesterol esterase has an important role to play with regard to cholesterol absorption, because an inhibitor of pancreatic cholesterol esterase reduced cholesterol transport in rat lymph, while also reducing the plasma cholesterol concentration [41].

In contrast to our observation, no accelerated effect of cholesterol esterase on cholesterol absorption was reported in Caco-2 cells [8,9]. Watt and Simmonds [42] did not observe any change in lymphatic cholesterol absorption in pancreatic juice-diverted rats. A group of Hui showed that in cholesterol esterase knockout mice, cholesterol absorption was similar to that seen in normal mice [10]. We believe that these inconsistent observations are caused by differences in the extent of phospholipid hydrolysis by cholesterol esterase ascribed to the different experimental conditions and also by differences in the involvement of pancreatic phospholipase

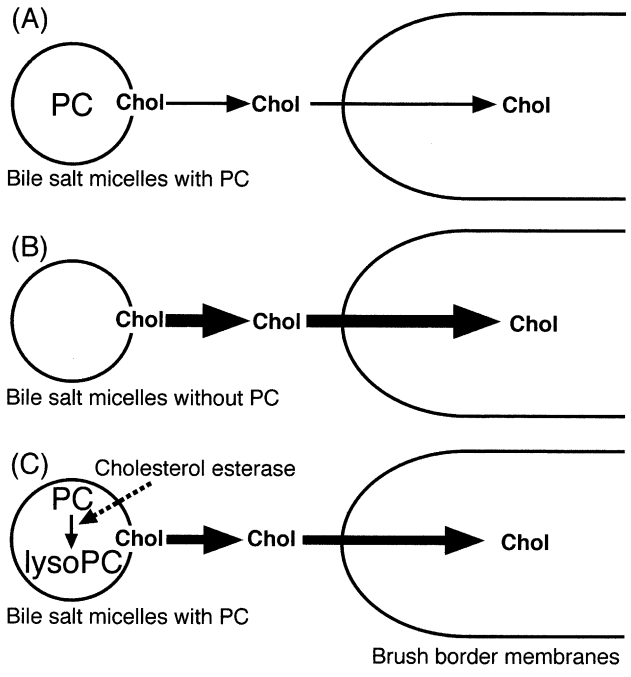

Fig. 9. A scheme of the proposed mechanism for the role of cholesterol esterase on cholesterol absorption. (A) Since cholesterol (Chol) has a higher affinity for bile salt micelles containing PC, it is less released from the micelles as a monomer and less absorbed to brush border membranes. (B) When PC is not contained in bile salt micelles, cholesterol is more releasable and absorbable than in the case of (A). (C) When PC in bile salt micelles is hydrolyzed to lysoPC by cholesterol esterase, the affinity of cholesterol for the micelles is weakened and the release of cholesterol from the micelles as a monomer is facilitated. Therefore, the incorporation of cholesterol into brush border membranes is increased.

A_2 in in vivo studies. A recent work by a group of Hui reported that phospholipid digestion was required for efficient cholesterol absorption in pancreatic phospholipase A_2-deficient mice and suggested a possibility that cholesterol esterase enhanced cholesterol absorption through phospholipid hydrolysis in intestinal lumen [36].

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