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Soybean Phosphatidylcholine-Induced Enhancement of Lymphatic Absorption of Triglyceride Depends on Chylomicron Formation in Rats

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We investigated whether chylomicron formation is involved in the dietary phosphatidylcholine (PC)-induced increase in triglyceride (TG) absorption using an inhibitor of chylomicron formation, pluronic L-81 (L-81). In rats, cannulas were implanted into the duodenum (exps. 1 and 2) and the mesenteric lymph duct (exp. 1), and an emulsified lipid solution containing the test lipids (soybean oil, SO or soybean oil plus phosphatidylcholine, LE) with or without L-81 was infused through a duodenal cannula at a rate 3 ml/h for 2 h, and followed by infusion of a glucose–NaCl solution for 2 h. Mesenteric lymph was collected for 4 h (exp. 1). In exp. 2, the mucosa and contents of the small intestine were collected at 20, 40, or 90 min after the start of duodenal infusion of the test lipid to evaluate accumulation of lipids incorporated into the mucosa in the rats without a lymph cannula. In exp. 1, lymphatic TG outputs rapidly increased with infusion of both test lipids without L-81, but L-81 abolished these increases. TG accumulated in the small intestinal mucosa with L-81 treatment in a time-dependent manner, but the levels of accumulation were similar between the SO and LE groups (exp. 2). There were no differences in the amounts of lipid remaining in the small intestinal lumen between the L-81-treated SO and LE groups. These results indicate that uptake of lipid into the mucosal cells was not increased by LE. We conclude that the formation of chylomicron is responsible for increases in the promotive effect of a high level of dietary PC on the lymphatic absorption of TG.

Key words: lymphatic transport; chylomicron; phosphatidylcholine

Lecithin (phospholipid, PL) is a widely distributed lipid in humans and animals, and a considerable amount of lecithin is ingested as a food component. Several studies[1–3] have suggested that endogenous phosphatidylcholine (PC) in the lumen enhances lymphatic transport of triglyceride (TG) in bile-diverted rats. In contrast, dietary PC has not clearly been shown to increase the lymphatic transport of TG[3–5] at low ratios of TG to PL (16:1–7:1). Our previous study, however, indicated that oral or intestinal administration of an emulsion containing PC at a higher ratio (TG:PC = 3:1) promoted lymphatic absorption of TG, and increased plasma TG concentrations in conscious rats fitted with a lymphatic cannula.[6]

Intestinal absorption of dietary TG into the body requires several steps, including the formation of lipid emulsion particles, digestion by lipase, solubilization into mixed bile salt micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the cells of the intestinal mucosa, and formation of chylomicron, but it is still not clear which is the rate-determining step for TG absorption from the intestine into the lymph.

We found that emulsion formation and lipid digestion were not involved in the excess dietary PC-induced increase in TG absorption in the above mentioned study.[6] This result suggests that dietary PC enhanced the absorption of TG at one of the steps after the formation of micelles, that is, during movement across the unstirred layer, uptake into the mucosal cells, or formation of chylomicron in the mucosal cells.

The aim of the present study was to determine which step after TG digestion is responsible for dietary PC-induced increase in TG absorption. We observed a time-dependent accumulation of lipid in intestinal mucosal cells using an inhibitor of chylomicron formation, pluronic L-81, which indicates the rate of mucosal uptake of lipids. Pluronic L-81 (L-81) is a hydrophobic detergent made up of polyoxyethylene and polyoxypropylene copolymers (average molecular weight, 2,750). Previous observations indicate that L-81 inhibits TG secretion into the lymph.[7–9]

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Abbreviations: TG, triglyceride; PL, phospholipid; PC, phosphatidylcholine; SO, soybean oil; LE, soybean oil + lecithin; SO+81, soybean oil + pluronic L-81; LE+81, soybean oil + lecithin + L-81; ANOVA, analysis of variance

Materials and Methods

The effect of pluronic L-81 on lymphatic TG absorption (exp. 1). Male Wistar/ST rats (Japan SLC, Hamamatsu, Japan), aged 9 weeks, were fed a semi-purified casein sucrose-based diet (AIN 76 formula) for 5 d. After a 24-h fast, a vinyl catheter (SV-35; 0.5-mm i.d., 0.8-mm o.d.; Natsume Seisakusyo, Tokyo, Japan) was implanted into the main mesenteric lymph duct,[10] and a silicone catheter (Silascon SH No. 00; 0.5-mm i.d., 1.0-mm o.d.; Kaneka Medix, Osaka, Japan) was implanted into the duodenum and another into the portal vein, under anesthesia (sodium pentobarbital, 40 mg/kg body weight).

After the operation, the rats were placed in individual restraining cages. During a 1-d recovery period, an isoosmotic solution containing 139 mM glucose and 85 mM NaCl was infused continuously until the end of lymph collection at a rate of 3 ml/h through the duodenal catheter, except during test lipid infusion. After collection of lymph for 30 min (initial lymph), the rats were divided into four groups, and the glucose-NaCl infusion was replaced with an emulsion containing 40 mg test lipids and 40 mg sodium taurocholate with or without 1.0 mg pluronic L-81 (L-81, BASF, Tokyo) in 3 ml of test solution. The test lipids were 40 mg soybean oil (SO) or 30 mg soybean oil plus 10 mg phosphatidylcholine (LE, 95% soybean phosphatidylcholine, Epikuron 200, Lucas Meyer, Hamburg, Germany). The test solution was infused at 3 ml/h for 2 h, and then the glucose-NaCl solution was infused at 3 ml/h for the next 2 h. The lymph was collected in a tube at 0.5-h intervals during the first 2 h and at 1-h intervals during the next 2 h. Fifty μl of blood was collected *via* the portal vein catheter at 30-min intervals for 4 h. The collected lymph was frozen immediately and kept at −40 °C until subsequent analysis. The portal blood was collected with a capillary tube containing heparin sodium. Plasma was separated from the portal blood by centrifugation (5,000 × *g*, 10 min, 4 °C) and stored at −40 °C.

Accumulation of lipid in the mucosa of the small intestine (exp. 2). Rats aged 9 weeks were fed a semi-purified casein sucrose-based diet (AIN 76 formula) for 5 d. After a 24-h fast, a silicone catheter (Silascon SH No. 00) was implanted in the duodenum under anesthesia (sodium pentobarbital, 40 mg/kg body weight), and then the rats were allowed a 3-d recovery period. After a second 24-h fast and saline infusion for 5 min, the rats were placed in individual restraining cages and infused with a solution containing an emulsion of the test lipids with L-81, as described for exp. 1, for 15, 35, or 85 min. Five min after the end of each infusion (20, 40, and 90 min after the start of infusion), the rats were anesthetized with diethyl ether and killed by exsanguination after the collection of portal blood (5 ml). The rats in the saline group were killed immediately after the saline infusion of 5 min. The portal blood was collected by syringe containing heparin sodium. The plasma separated from the portal blood by centrifugation (5,000 × *g*, 10 min, 4 °C) was stored at −40 °C. The small intestine was divided into the upper and lower halves, and they were washed out twice with 5 ml of 5 mM sodium taurocholate.[7] The wash-out solution was collected as the intestinal contents. Intestinal segments were frozen at −40 °C, and the mucosae of the segments were collected by pressure from outside the intestinal wall after thawing.[11] Total lipids in the upper and lower small intestinal mucosae and intestinal contents were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch *et al.*,[12] and the concentrations of triglyceride were measured. In rats infused the test lipids for 85 min (90 min after lipid infusion), a 2-cm segment of the duodenum from the pylorus was cut off, washed with saline, and fixed with 10% formalin in phosphate-buffered saline (for fat stain) to visualize the accumulation of lipid into the mucosa by histochemical analysis.

This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Chemical and histochemical analyses of intestinal tissues. Triglyceride (TG) and phospholipid (PL) concentrations in the lymph fluid, portal plasma, and the extracts of the mucosa and luminal contents were measured by enzymatic procedures (TG-EN and PL-EN, Kainos Laboratories, Tokyo). Free fatty acid and ketone body concentrations in the plasma were also measured by an enzymatic procedure (Cholesterol C-test Wako, Wako Pure Chemical Industries, Tokyo, and Ketone-test 'Sanwa,' Sanwa Kagaku Kenkyusyo, Nagoya, Japan, respectively).

The fixed 2-cm duodenal segments were cut into 10-μm sections and fixed on a glass slide as cryostat sections. The slides were immersed in a 7% aqueous solution of Nile blue stain (Nile blue sulfate) for 10 min at 40 °C. The slides were washed with water to remove the excess stain, followed by a 10-min wash in 1% acetic acid. They were then rinsed in water, air-dried, and visualized under an optical microscope (×40 magnification).

Statistics. Data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA (lipid and time), and significant differences between groups were determined by Duncan's multiple range test. Values are shown as means ± SEM.

Results

The effect of pluronic L-81 on lymphatic absorption of lipids (exp. 1)

During infusion of the test lipid solution without

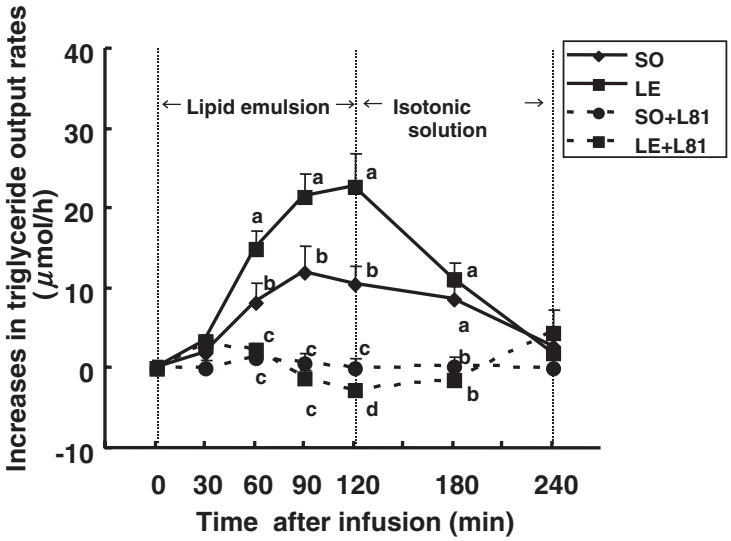

Fig. 1. Increases in Lymphatic Triglyceride Output Rates during and after a Duodenal Infusion of Test Lipid Emulsion.

The test emulsion was infused at 3 ml/h for 2 h, then a glucose-NaCl solution was infused at 3 ml/h for 2 h. Values are the means for 5–7 rats, with SEM shown as vertical bars. Values not sharing a common superscript are significantly different within the same time period ($P < 0.05$). The values are shown as increased rates from the initial value. The P values for lymphatic output increase in triglyceride estimated by two-way ANOVA were 0.021 for lipid (L), 0.388 for time (T), and 0.050 for L × T. SO, soybean oil; LE, soybean oil + phosphatidylcholine; SO+81, soybean oil + L-81; LE+81, soybean oil + phosphatidylcholine + L-81.

L-81, the increases in lymphatic output rates of TG in the rats given LE was much higher than that in the rats given SO for 2 h (Fig. 1). After the end of lipid infusion, the lymphatic output of TG in both groups decreased gradually and returned to the initial level. By addition of L-81 to the test lipids, the increase in the lymphatic output of TG was completely abolished for 4 h (Fig. 1). Increases in lymphatic PL output rates were much lower than those in lymphatic TG, but the manner of the changes were similar to those of the output rates of TG (data not shown). There were no differences in lymph flow rate between the groups. Total lymph volumes for 4 h were 4.2 ± 0.7, 5.5 ± 0.9, 5.1 ± 0.5 and 4.7 ± 1.0 ml in rats given SO, LE, SO+L-81 and LE+L-81, respectively ($n = 6$–7, $P = 0.679$).

There were no differences in portal concentrations of free fatty acid or TG between the groups and no changes over the 4-h infusion period. The average concentrations of TG and free fatty acid were 0.437 mM ($P = 0.838$ for lipid, $P = 0.074$ for time, and $P = 0.595$ for lipid × time) and 0.93 meq/l ($P = 0.923$ for lipid, $P = 0.116$ for time, and $P = 0.296$ for lipid × time) respectively.

Accumulation of lipid in the mucosa of the small intestine and changes in portal lipids (exp. 2)

Accumulation of lipid in the mucosa of the proximal intestine was expressed as the increase in TG, which was calculated by subtraction of the mean value of the saline group from the values for individual rats in the test lipid groups (Fig. 2). Triglyceride did not increase in the mucosa in the groups without L-81 at 90 min after the start of test lipid infusion (SO, 0.09 ± 0.70; LE, 0.15 ± 0.76 μmol/proximal intestinal mucosa). Owing to the L-81-treatment, the accumulation of TG in the mucosa increased in a time-dependent manner after the start of infusion, and there were no differences between the SO and LE groups. Increases in TG accumulation into the mucosa of the distal intestine were much lower than those of the proximal segment, and there were no differences among groups.

The TG remaining in the small intestinal lumen was expressed as the ratio of the lipid remaining in the small intestine to the total administered lipid, after removing the mean value of lipid in the small intestine contents of the saline group from those for individual rats in the test lipid groups (Fig. 3). The TG of the small intestinal lumen in saline group was 3.46 ± 0.26 μmol. The amount of TG remaining in the small intestinal lumen was less than 5% of the amount of administration, and was not different between the SO and LE groups with L-81. The values in the groups without L-81 were very similar to those with L-81 at 90 min (SO, 2.17 ± 0.80%; LE, 5.61 ± 1.47%).

The lipid concentrations in the portal plasma 90 min after the start of test lipid infusion are shown in Table 1. In rats without L-81 treatment, the concentration of TG was higher in the LE group than in the SO group. In rats treated with L-81, the concentrations in both the lipid groups were very similar to those in the saline group (0.53 ± 0.06 mmol/l). There were no differences in PL concentrations among the groups (data not shown). The concentrations of free fatty acid were lower in rats with L-81, and the concentrations of ketone bodies were higher in rats with L-81. There were no differences between the SO and LE groups regardless of treatment with L-81.

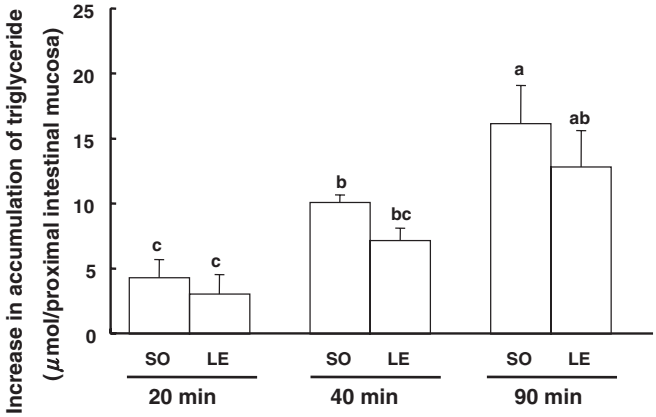

Fig. 2. Increases in Accumulation of Lipid in the Proximal Intestinal Mucosa 20, 40, and 90 min after the Start of Duodenal Infusion of Lipid Emulsion with Pluronic L-81.

Values are the means for 6–7 rats, with SEM shown as vertical bars. Values not sharing a common superscript are significantly different between the different sampling times within the same lipid group ($P < 0.05$). P values estimated by two-way ANOVA were 0.102 for lipid (L), < 0.001 for time (T), and 0.837 for L × T. SO, soybean oil; LE, soybean oil + phosphatidylcholine.

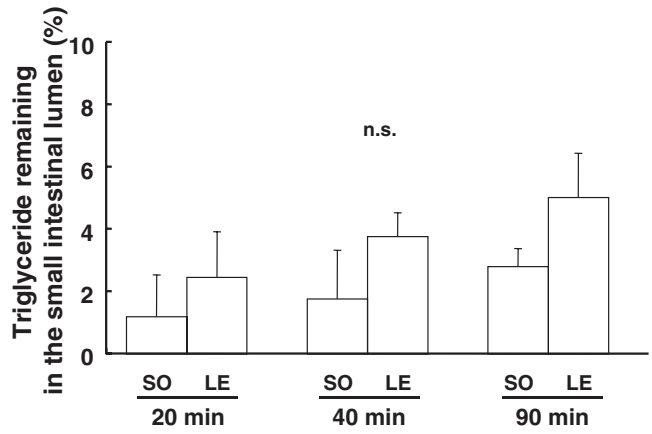

Fig. 3. Percentages of Triglyceride Remaining in the Small Intestinal Lumen after the Start of Duodenal Infusion of Lipid Emulsion with and without Pluronic L-81.

Values are the means for 6–7 rats, with SEM shown as vertical bars. Values not sharing a common superscript are significantly different ($P < 0.05$). P values estimated by two-way ANOVA were 0.075 for lipid (L), 0.232 for time (T), and 0.916 for L × T. SO, soybean oil; LE, soybean oil + phosphatidylcholine.

Table 1. Effects of Lipid and Pluronic L-81 on Lipids in Portal Plasma at 90 min after Lipid Infusion

	n		Triglyceride (mmol/l)	Free fatty acid (mmol/l)	Total ketone bodies (mmol/l)
With L-81	6	SO	0.61 ± 0.03 c	0.51 ± 0.02 b	4.21 ± 0.26 a
	6	LE	0.55 ± 0.07 c	0.52 ± 0.02 b	4.31 ± 0.28 a
Without L-81	6	SO	0.85 ± 0.10 b	0.64 ± 0.06 a	3.70 ± 0.12 b
	6	LE	1.14 ± 0.13 a	0.58 ± 0.01 a	3.21 ± 0.43 b
P values					
Lipid			0.148	0.757	0.526
L-81			<0.001	0.016	0.019
Lipid × L-81			0.068	0.465	0.363

Values are the means for 6–7 rats with SEM. P values were estimated by two-way ANOVA. Values not sharing a common superscript are significantly different ($P < 0.05$). SO, soybean oil; LE, soybean oil + phosphatidylcholine.

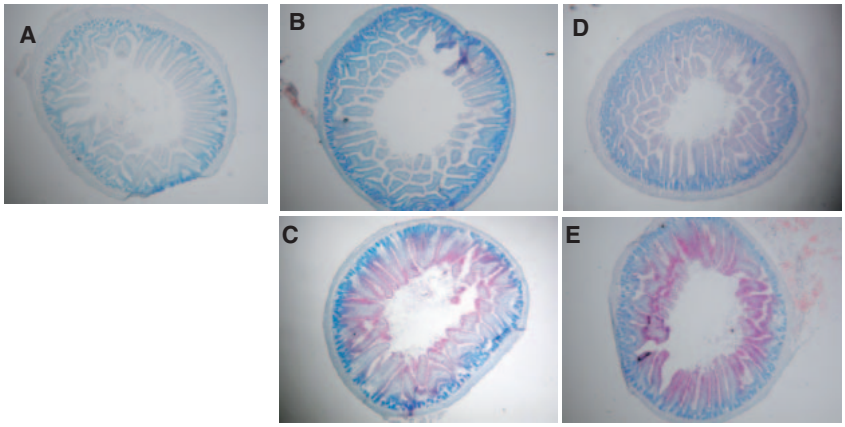

Fig. 4. Optical Micrographs of Duodenal Segments Stained with Nile Blue 90 min after the Start of Duodenal Infusion of Lipid Emulsion. A, Saline; B, soybean oil; C, soybean oil + L-81; D, soybean oil + phosphatidylcholine; E, soybean oil + phosphatidylcholine + L-81.

The sections of the duodenum of the rats treated with L-81 were stained red, which indicates accumulation of TG in the mucosa (Fig. 4C and E). No differences were observed between the SO and LE groups treated with L-81. In the L-81-untreated groups, the red portion of the section was negligible (Fig. 4B and D).

Discussion

We have found that PC increases TG output into the lymph by a bolus injection of test lipids into the duodenum. In this study, we pursued our research under the condition that the TG:PC ratio was 3:1, because we found in the previous study that the TG and PC emulsion mixed in this ratio clearly enhanced the lymphatic output of TG.[6] First we examined the reproducibility of the promotion of lymphatic TG output with LE by continuous infusion to determine the effect of pluronic L-81 as an inhibitor of chylomicron formation, because L-81 was not effective in inhibiting TG output into the lymph by bolus administration (data not shown). All previous studies we know used L-81 with continuous infusion.[7,8] Lymphatic absorption of TG was clearly higher after continuous infusion of a test lipid containing PC (LE) than after infusion of the lipid without PC (SO), indicating that the effect of a high level of PC on lymphatic TG absorption was reproduced under continuous lipid infusion. We also demonstrated that L-81 infusion completely inhibited TG output into the lymph. Pluronic L-81 is a non-ionic, hydrophobic surfactant that selectively and reversibly blocks chylomicron transport. It has been reported that L-81 induces accumulation of absorbed lipids in the intestinal mucosa by blocking lipid transport into the lymph from the intestine, while L-81 does not affect intestinal lipid digestion or uptake of lipids into mucosal cells.[13,14] It has been shown that inhibition of apolipoprotein incorporation into chylomicron by L-81 results in inhibition of chylomicron formation.[8]

There were no differences in the accumulation level of the lipid in the mucosa between the L-81-treated SO and LE groups at any time after the infusion of test lipids (20, 40 or 90 min). Triglyceride in the mucosa of the small intestine time-dependently increased with treatment of L-81. The remaining lipids in the intestinal lumen were very low in amount in both the SO and LE groups, with a tendency to be higher in the LE group ($P < 0.075$). These results indicate that PC does not increase lipid uptake into the intestinal mucosal cells, and that the stages after lipid uptake into the mucosal cells were responsible for the PC-induced increases in lymphatic output of TG. In our previous studies, we also showed that the TG hydrolytic rate in emulsions containing PC was lower than in the emulsions without PC in *in vitro* digestion using rat bile-pancreatic juice.[6] The most likely mechanism for the promotion of TG output into the lymph is an increase in the formation of chylomicron by PC. The formation and secretion of chylomicron require apolipoproteins. It has been demonstrated that bile diversion significantly reduces synthesis and release of apo B-48 by the intestinal mucosa, which suggests that endogenous biliary PC plays a role in the intestinal synthesis of apoB-48.[15,16] However, the addition of a low dose of dietary PC does not affect lymphatic TG transport, as described above. This suggests that endogenous PC is sufficient for apolipoprotein synthesis, and suggests that apoprotein synthesis is not associated with our findings at relatively high doses of PC. Some mechanism other than apoprotein synthesis might be involved in the promotion of lymphatic TG output with excess dietary PC. Recently, it has been proposed that microsomal triglyceride transfer protein (MTP) also plays a role in the association of intestinal lipoprotein formation.[17,18] We intend to clarify the effect of PC on this stage in the future.

The concentration of plasma TG was higher in the LE than in the SO group in L-81-untreated rats, but not in L-81-treated rats, and the plasma TG concentration did not increase during the infusion of test lipids without L-81

in lymph-cannulated rats. These results indicate that plasma TG levels reflect lymphatic absorption, and suggest that TG was not absorbed by anything other than the lymphatic pathway, for example *via* portal blood. Free fatty acid levels in the portal blood were lower and keton body levels were higher in rats with L-81 than in those without it. The reason for these changes is not known, but possibly L-81 changes lipid metabolism.

In our previous[6] and present studies, we found that excess dietary PC caused an increase in TG absorption in normal rats without the removal of biliary PC. This finding may indicate that excess dietary PC is beneficial in that it enables patients with impaired digestive function to metabolize energy and lipids efficiently. Moreover, we found that dietary PC increased the lymphatic output of lycopene as well as TG.[19] Our findings might help to understand the mechanism that promotes lycopene absorption by PC, which is very low. We conclude that a high level dietary PC causes an increase in the lymph output of TG dependent on the formation of chylomicron.

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