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Comparison of Lysophospholipid Levels in Rat Feces with Those in a Standard Chow

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ABSTRACT: Although lysophospholipids have attracted much attention due to their diverse physiological activities through their specific receptors, little is known about their metabolic fates in mammalian digestive systems after their ingestion as a minor food component. In this study, we analyzed five lysophospholipids in lipid extracts of a standard rat chow and feces of rats fed the chow by two-dimensional thin layer chromatography and liquid chromatography-tandem mass spectrometry. The most abundant lysophospholipid in the rat chow was lysophosphatidylcholine followed by lysophosphatidylethanolamine, lysophosphatidic acid (LPA), lysophosphatidylinositol and lysophosphatidylserine (LPS) in an increasing order, but their concentrations were very low in rat feces. Among the molecular species of LPS in the chow, only saturated species were detected in the feces in significant amounts. In addition, several molecular species of LPA remained in the feces in variable portions (saturated > monounsaturated > polyunsaturated). These results suggest that a portion of ingested LPA and LPS reach the rat large intestine, affecting physiological colon functions.

KEYWORDS: lysophospholipid, lysophosphatidic acid, lysophosphatidylserine, digestive absorption

INTRODUCTION

Dietary phospholipids are believed to have a diverse array of beneficial effects in the human body. For example, dietary phosphatidylserine (PS) has emerged as a brain-specific nutrient that ameliorates declining memory in human beings and model animals,[1] and supplementation of daily meals with PS-enriched ω3-fatty acids for 6 weeks resulted in improvement of memory recall in subjects with subjective memory complaints.[2] Dietary phosphatidylinositol (PI)[3] and phosphatidylcholine (PC)[4] were shown to prevent the development of nonalcoholic fatty liver disease in Zucker (*fa*/*fa*) rats and rats fed a high-fat diet, respectively. A randomized controlled trial examined the effect of oral administration of phosphatidylethanolamine (PE) as a possible precursor of oleoylethanolamine, a proposed inhibitor of food intake in model animals, on satiety and energy intake.[5] Phosphatidic acid (PA) is contained in soybean lecithin at about 7%.[6] The level of PA in cabbage homogenates in the human mouth was considerably increased by phospholipase D, which is released from cells of cabbage leaves during their mastication,[7] but its beneficial effects remain largely unknown.

Phospholipids in foods are known to be metabolized in the gastrointestinal lumen and absorbed into the body. Glycerol-based dietary phospholipids are hydrolyzed into free fatty acids and lysoglycerophospholipid by pancreatic phospholipase A_2 (PLA_2) for absorption into the bodies of humans and model animals, including the rat.[8−12] Although intestinal absorptions of triacylglycerol (TG) and PC have been extensively studied, there are few papers showing the effects of absorption of dietary phospholipids other than TG and PC on the absorption of major lipids including TG, PC, and cholesterol. Lymphatic transport of radioactivity derived from radiolabeled-PE fed to rats was found to be lower (8%) than the value for radiolabeled PC (17%),[9] and dietary PE caused a decrease in serum cholesterol and phospholipids.[13] PI was shown to promote cholesterol transport and excretion in rabbits[14] and to affect cholesterol metabolism and absorption of PI in rats.[15]

Lysophospholipids are minor components in foodstuffs as follows: pork muscle LPC, 4.9%; whole chicken egg LPC, 1.6%; whole milk LPE, 5.9%; wheat flour LPC, 31.1%; wheat flour LPE, 4.8%; pork liver LPC, 2.9%.[16] LPA in soybean lecithin, amounting to about 0.2%, was identified as a vasopressor substance on rats in 1978.[17] Since then, research on the physiological and pathophysiological roles of LPA has gradually advanced, attracting the interest of researchers in different fields. Lysophospholipids including LPA comprise an emerging lipid mediator family[18] and have now been recognized to exert diverse physiological activities through the binding to their specific G-protein-coupled receptors.[19,20] Attention has recently been also directed to the role of the lysophospholipid mediator family in the mammalian digestive system.[21,22] For instance, lysophosphatidic acid (LPA) was shown to inhibit cholera toxin-induced secretory diarrhea through CFTR-dependent protein interaction.[23] This is a practical concern when lysophospholipid-rich preparations from different foodstuffs are used in functional food supplements or rehydration solutions for antidiarrhea therapy. There are, however, few studies examining the absorption and stability of dietary lysophospholipids.[24,25] In this study, we attempted to obtain more information about the absorption of lysophospholipids by digestive cells by comparing the amounts of five lysophospholipids including LPA in a standard rat chow and feces of rats that had eaten the chow.

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MATERIALS AND METHODS

Materials. 1-Oleoyl-2-lyso-*sn*-glycero-3-phosphate (18:1-LPA) was obtained from Sigma-Aldrich Co. (St. Louis, MO), and 1-heptadecanoyl-2-lyso-*sn*-glycero-3-phosphocholine (17:0-LPC) were obtained from Avanti Polar Lipids (Alabaster, AL). (The fatty acyl moieties of phospholipids are designated in terms of the number of carbon atoms and double bonds, for example, 16:0, palmitoyl group; 16:1, palmitoleoyl group; 18:0, stearoyl group; 18:1, oleoyl group; 18:2, linoenoyl group; 18:3, α-linolenoyl group.) 17:0-LPA was prepared from 17:0-LPC by *Streptomyces chromofuscus* phospholipase D as previously described.[26] All other reagents used were of analytical grade. 1-Heptadecanoyl-2-lyso-*sn*-glycero-3-phosphoethanolamine (17:0-LPE) was prepared by transphosphatidylation with phospholipase D from *Actinomadura* (Seikagaku Biobusiness, Tokyo, Japan) on 17:0-LPC in the presence of ethanolamine hydrochloride. LPS from bovine brain and LPI from bovine liver were purchased from Avanti Polar Lipids and Doosan Serdary Research Laboratories (Toronto, ON, Canada), respectively.

Chows and Feces. Male Wistar rats, 7−9 weeks old (Kyudo, Kumamoto, Japan), were kept in a 20−25 °C controlled environment with a light−dark cycle of 12 h each. The rats were housed separately in metabolic cages. All rats were handled in accordance with the principles and guidelines of the Experimental Animal Committee of Kyushu University of Health and Welfare. Rats were given free access to a standard chow (MF) purchased from Oriental Yeast Co. (Tokyo, Japan) consisting of powders of wheat, defatted soybean, alfalfa, defatted rice, defatted bovine milk, soybean oil, corn, white fish meal, and beer yeast according to the manufacturer and water. Feces were collected from four rats over a 24 h period. A portion of feces was dried to a constant weight to allow calculation of concentrations of lysophospholipids. Lipids were extracted from 0.2−0.3 g of another portion of feces in duplicate. We measured the total amounts of phospholipids extracted from the feces according to the modified method of Bligh and Dyer[28] using a malachite green method after heating treatment with perchloric acid,[27] as briefly described below.[22]

Extraction of Lysophospholipids in Chow and Feces. Lipids including LPC, LPE, LPS, and LPI were extracted from the dried chow (0.2 g) and feces (0.2−0.3 g) according to the modified method of Bligh and Dyer.[28] After additions of 5 nmol of 17:0-LPC and 1.2 nmol of 17:0-LPE, the pH of part of the aqueous layer was adjusted to 10−11 with 20% NH_4OH. The remaining aqueous layer was acidified with 1 N HCl to pH 2−2.5 and, after the addition of 0.1 nmol of 17:0-LPA, LPA, LPS, and LPI, were recovered in an organic layer from the acidified aqueous layer according to the method of Bligh and Dyer.[28] The second organic layer was dried under a stream of nitrogen gas, and the lipid extracts were dissolved in 0.1 mL of methanol/water (1:1, v/v) containing 5 mM ammonium formate for analysis of acidic lysophospholipids by liquid chromatography−tandem mass spectrometry (LC-MS-MS). For analysis of LPC and LPE, the first lipid extracted under basic conditions was dissolved in 1 mL of methanol/water (95:5) containing 5 mM ammonium formate.

Lipid Class Analysis by Two-Dimensional TLC. Lipids extracted according to the modified method of Bligh and Dyer from rat feces and chow were applied to a silica gel 60 TLC plate (Merck, Darmstadt, Germany). The plate was developed with a solvent system of chroloform/methanol/28% ammonia (60:35:8, v/v) first and then with a solvent system of chloroform/methanol/acetone/acetic acid/water (50:10:20:13:5, v/v). Lipids were visualized by spraying with a 0.01% primulin solution in acetone/water (80:20). Then phospholipids and aminolipids on the TLC plate were detected by spraying with Dittmer−Lester and ninhydrin reagents, respectively.

Molecular Species Analysis of Lysophospholipids by LC-MS-MS. LC-MS-MS was performed using a quadrupole-linear ion trap hybrid MS, 4000 QTRAP (Applied Biosystems/AB Sciex, Concord, ON, Canada), with an Agilent 1100 LC system combined with an autosampler (Agilent Technologies, Wilmington, DE). Molecular species of LPC and LPE were separated on a Supelco Ascentis Express C18 column (100 mm × 2 mm) with methanol/water (95:5, v/v) containing 5 mM ammonium formate. Routinely, 5 μL aliquots of test solutions were applied to the mass spectrometer for analysis. The molecular species composition was analyzed by multiple reaction monitoring in positive ion mode. Q3 was set as $[\text{phosphocholine}]^+$ at m/z 184 for LPC and $[\text{MH} - \text{phosphoethanolamine (M} - 141)]^+$ at m/z for LPE in combination of the protonated molecular ion as Q1. The amounts of LPC and LPE molecular species were calculated from the ratios of their peak areas of positive ions to that of 17:0-LPC and 17:0-LPE, respectively, as internal standards.

Separation of molecular species of LPA, LPS, and LPI in the acidic polar lipid fraction by LC was performed using a Tosoh TSK-ODS-100Z column (150 mm × 2 μm; with 5 μm silica particles) developed with methanol/water (95:5, v/v) containing 5 mM ammonium formate at a flow rate of 0.22 mL/min in an isocratic elution mode. The molecular species of LPA, LPS, and LPI were analyzed by multiple reaction monitoring in a negative ion mode. In the negative ion mode, Q3 was set to $[\text{cyclic glycerol phosphate}]^-$ at m/z 153 for LPA, $[\text{cyclic glycerol phosphate}]^-$ at m/z 153 for LPS, and $[\text{inositolphosphate} - H_2O]^-$ at m/z 241 for LPI in combination with the deprotonated molecular ion as Q1. Amounts of molecular species of LPA were tentatively calculated from the ratios of their peak areas of negative ions to that of 17:0-LPA, an internal standard. Similarly, molecular species of LPS and LPI were quantified tentatively from the ratios of their ion peak areas to that of 17:0-LPA and correction factors of 1.54 and 2.04 for LPS and LPI, respectively. These correction factors were based on both the extraction efficiencies of bovine brain LPS and bovine liver LPI and their relative ion efficiencies against 17:0-LPA by multiple reaction monitoring under our conditions.

High-resolution mass spectrometry of phospholipids dissolved in methanol/water (95:5) containing 5 mM ammonium formate was performed on a time-of-flight mass spectrometer (Waters Micromass LCT-Premier).

Statistical Analysis. All data are expressed as the mean ± SE. The results were compared by using analysis of variance, followed by Student's unpaired *t* test for single comparisons.

RESULTS

Different Lipid Class Profiles of Chow and Feces of Rats. We calculated the amounts of total phospholipids (μmol/dry weight of samples) based on the values of lipid phosphorus in lipid extracts from the chow and feces as determined by using the malachite green method.[27] The mean values of total phospholipids in the chow and feces were 9.36 and 6.24 μmol/g, respectively. The same amounts of total phospholipids in lipid extracts from the chow and feces were spotted on the corner of a silica gel TLC plate, and lipids were separated by two-dimensional TLC. The major phospholipids in the chow were PC and LPC, whereas smaller amounts of PI, sphingomyelin (SPM), PA, and PE were detected (Figure 1A). A faint spot due to LPA was seen, as well as faint spots that seemed to be LPI, LPE, and LPS. On the other hand, the most predominant lipid spot from the feces was stained with Dittmer−Lester reagent, but not ninhydrin reagent (Figure 1B). The phospholipid spot was recovered from the silica gel according to the method of Bligh and Dyer, and analyzed by high-resolution mass spectrometry. The observed elemental composition of abundant negative ions seen at m/z 833, 835, 857, 831, and 859 was consistent with the

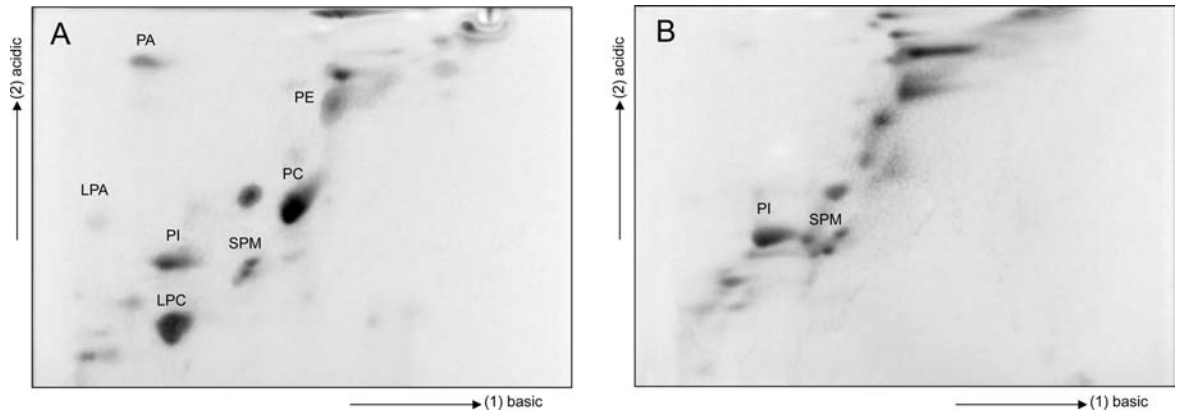

Figure 1. Two-dimensional TLC of lipid extracts from the chow fed to rats (A) and from their feces (B). The plate was developed first with an a basic solvent and then with an acidic solvent mixture. Lipid spots were visualized by primulin staining.

calculated values of PI with C34:2, C34:3, C36:4, C34:3, and C36:3, respectively. Characteristic double spots due to SPM were also detected in the feces. Bands due to PC, PE, PS, and PA were very faintly stained with primulin reagent. The less polar lipids detected in the feces were not stained with Dittmer—Lester reagent or ninhydrin reagent and, thus, were not characterized further. These results suggest that PC, PE, PS, and PA, but not PI, in the chow were effectively digested and metabolized in the rat digestive mucosa.

Comparison of Total Amounts and Molecular Species Composition of Lysophospholipids in Chow and Feces. Figure 2A compares the total amounts of five lysophospholipids in the chow with those in the feces of rats eating the chow. The most abundant lysophospholipid in the chow was LPC, followed by LPE, LPA, LPI, and LPS in that order. Interestingly, the major lysophospholipids in the feces were LPA, LPE and LPC. A lower amount of LPS was detected with a trace amount of LPI. Molecular species analysis of LPC (Figure 2B) revealed that the predominant molecular species in the chow were C16:0, C18:2, and C18:1 and that lower amounts of C20:5, C22:6, C18:0, C16:1, and C18:3 were also present. The amounts of all these molecular species in the feces were much lower than in the chow, indicating they were very effectively metabolized and subsequently absorbed in the rat digestive tract. Figure 2C shows the results for LPE, the molecular species composition of which was essentially similar to that of LPC in the chow. The abundant molecular species in the chow were C18:2, C16:0, and C18:1, but the percentages of C18:2 and C18:1 were lower than those of C16:0 and C18:0 in the feces, suggesting a moderate metabolic preference for unsaturated LPE over saturated LPE. In the case of LPI (Figure 2D), the results were similar to those obtained for LPE. The amounts of all molecular species of LPI in the feces were much lower than those in the chow, although the percentages of saturated molecular species were higher than those of other molecular species in the feces as compared with those in the chow: the level in the feces was about one-fourth that in the chow. Interestingly, the amounts of saturated (C16:0, C18:0, C20:0, and C22:0) and monounsaturated LPAs such as C16:1 and C18:1 in the feces were only moderately lower than those in the chow, whereas polyunsaturated species (C20:5, C22:5, and C22:6) in the feces were lower than those in the chow, suggesting that LPA was metabolized and absorbed to a much lesser extent in the digestive tract compared with LPC and LPE, although saturated species were more protected from metabolic absorption in the digestive tract than unsaturated molecular species. Results for LPS are shown in Figure 2F. The amounts of saturated species of LPS such as C18:0, C20:0, and C22:0 in the feces were similar to those in the chow, and the amount of C16:0 in the feces was about half that in the chow. However, amounts of both monounsaturated and polyunsaturated species in the feces were much lower than those in the chow, indicating quite selective protection of saturated LPS from metabolism and absorption by the rat digestive system. We calculated the ratios of amounts in the chow to those in the feces for three types of molecular species: saturated, monounsaturated, and polyunsaturated species. As shown in Figure 3, large differences in the ratios were observed for saturated species: LPS > LPA > LPE > LPI > LPC. In the case of monounsaturated species, the highest ratio was for LPA, followed by LPS. For polyunsaturated species, the ratios were very low except for LPA.

DISCUSSION

Inhibition of luminal phospholipid digestion in rats resulted in decreased intestinal absorption of cholesterol, but targeted disruption of the mouse pancreatic PLA_2 gene caused no significant reduction of cholesterol absorption, indicating the presence of another enzyme that is capable of hydrolyzing phospholipid.[29] Phospholipase B in the distal intestinal brush border may replace group 1B PLA_2.[30,31] Thus, lysophospholipids derived from both foods and from metabolic degradation of diacyl phospholipids contribute to the regulation of intestinal absorption of other lipid components. An intraduodenal infusion of 1-oleoyl (18:1)-LPC was found to restore the intestinal absorption of retinol and α-tocopherol in rats fed a low-zinc diet.[32] A single intragastric injection of mixed micelles of luteolin with LPC enhanced the bioavailability of luteolin in rats.[33] In experiments with cultured intestinal cells, 1-palmitoyl (16:0)-lysophospholipids (LPC, LPS, LPE, and lysophosphatidylglycerol) were reported to enhance the uptake of β-carotene by differentiated human intestinal Caco-2 cells, irrespective of the polar headgroup in vitro.[34] The authors postulated that the lysophospholipids having an inverted conical shape contribute to both the assembly of nonbilayer phospholipid mixtures into micelles in an aqueous medium and induction of increased membrane permeability in Caco-2 cells. Similarly, 18:1 LPC was shown to increase apical absorption of cholesterol and oleic acid by differentiated human intestinal Caco-2 cells, and it doubled

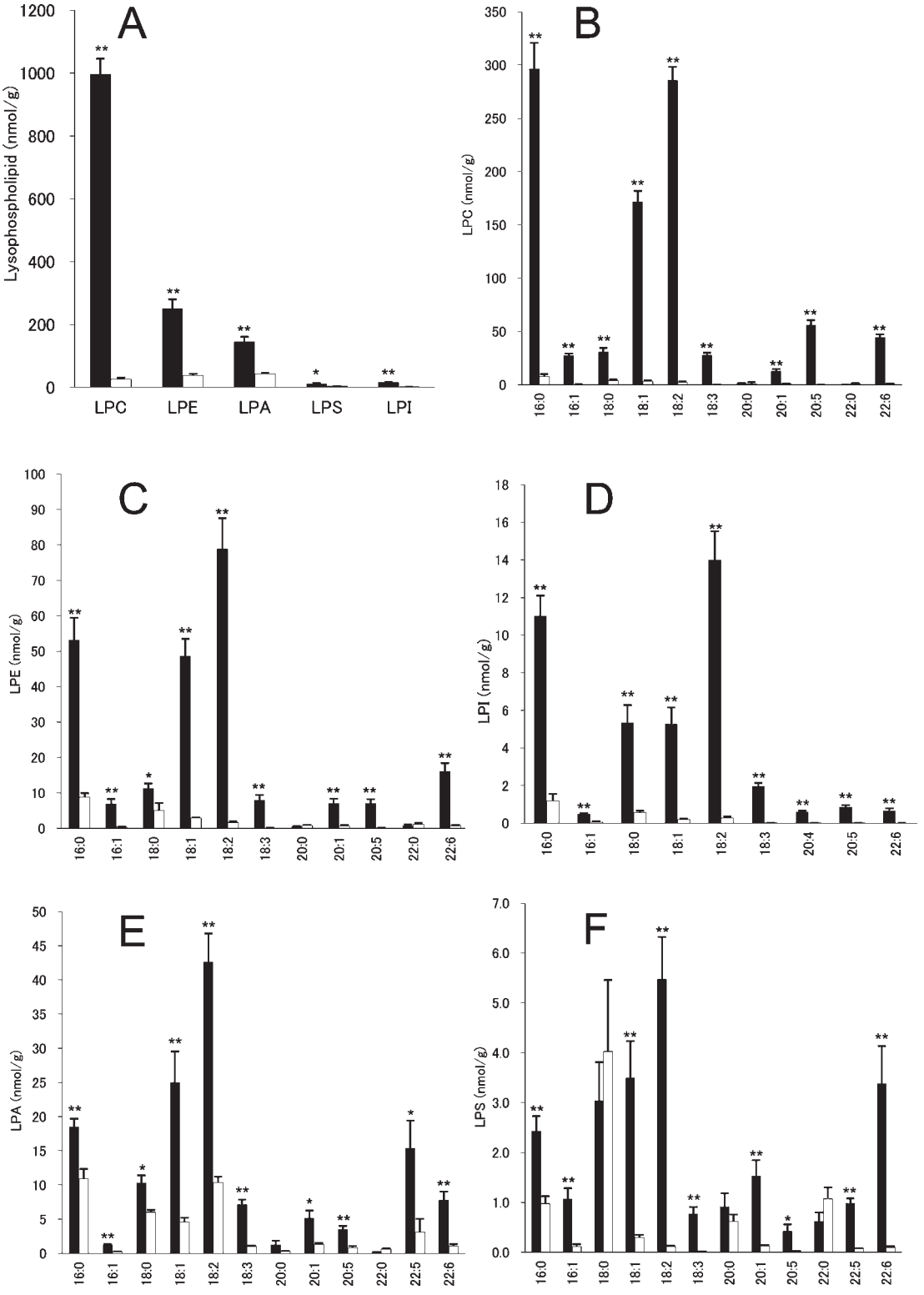

Figure 2. Comparison of total amounts (A) and molecular species compositions (B−E) of five lysophospholipids in lipid extracts from the chow and feces: (B) LPC; (C) LPE; (D) LPI; (E) LPA; (F) LPS. Black and white columns with bars show the mean value ± SE of five and four separate experiments with lipids extracted from the chow and feces, respectively. ∗ and ∗∗ represent $P < 0.05$ and $P < 0.01$, respectively.

basolateral secretion of triacylglycerol-rich lipoproteins from the intestinal cell line.[35] However, until now there has been no systematic study of the absorption of lysophospholipids into intestinal cells in vitro or the intestinal epithelium in vivo. To the best of our knowledge, the present study is the first to evaluate the relative susceptibility of molecular species of different lysophospholipids to metabolic intake through the digestive system of an animal model.

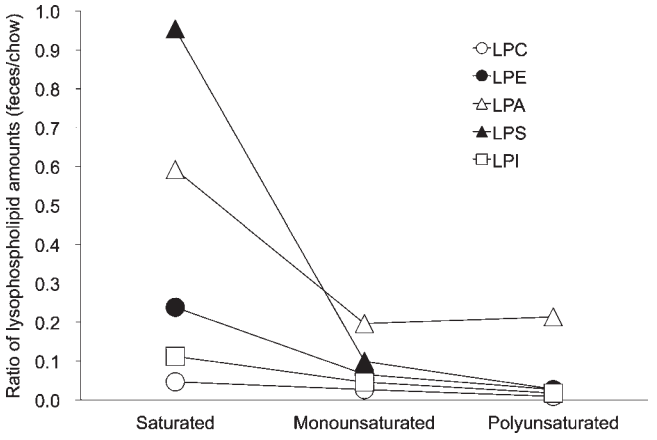

Figure 3. Ratios of amounts of lysophospholipids having a saturated, monounsaturated, or polyunsaturated fatty acyl group in the feces to those in the chow. Results are the mean ± SE of five and four samples for chow and feces, respectively. Open and solid circles show values for LPC and LPE, respectively. Open and solid triangles show values for LPS and LPA, respectively. Open squares show values for LPI.

In brief, our experimental approach was comparison of the concentrations of five lysophospholipids in the chow and feces to understand their relative susceptibilities to metabolism in the lumen of the digestive tract and digestive absorption. However, our results must be interpreted carefully with consideration of several points. First, lysophospholipids in the feces, if present, may also involve undigested ones derived not only from the chow but also from corresponding diacylphospholipids in food[36,37] and/or secreted by digestive epithelial cells.[38] Indeed, PC was shown to be secreted into in the ileal lumen of rats,[39] and the protective role of PC in mammalian gastrointestinal mucus has recently been well-documented.[40] Second, a large proportion of the PC in the intestinal lumen would be derived from bile, although PC was both present in the chow and secreted in the intestinal mucosa. Third, *Helicobacter pylori* in the stomach and commensal bacteria in the colon may affect the phospholipid profiles in the lower digestive tracts, by secreting phospholipids and phospholipase or activating their release from the luminal surface of the digestive system.[41,42] Unfortunately, the amounts of various phospholipids secreted into the digestive tract by bacteria and from the apical lumen of the digestive tract are impossible to quantify, although one can estimate such parameters by measuring the amounts of phospholipids in foods and feces. Despite these limitations, we thought that our investigation would be useful if quite definite differences were seen among the five classes of lysophospholipids or among the molecular species of a single class of lysophospholipid. Our results showing much lower amounts of LPCs and LPEs in rat feces compared with the chow suggest their quite efficient absorption into the digestive tract. PC and LPC excretions into feces, however, are increased in children with cystic fibrosis, which is associated with plasma homocysteine, *S*-adenosylhomocysteine, and *S*-adenosylmethionine.[43] Unlike major glycerophospholipids such as PC and PE, <25% of administered SPM was shown to escape absorption and thus be excreted in rat feces (10% SPM, 80−90% ceramide, 3−6% sphingosine).[44] In rats, the digestion of SPM is most efficient in the middle of the small intestine.[45] The delayed and incomplete absorption of SPM and its metabolites was shown to be relevant to inhibition of the serum LDL cholesterol concentration and colon carcinogenesis.[46] Our TLC analysis showed that PI was the most abundant phospholipid in the feces in contrast to PC in the chow, indicating the relative resistance of PI to metabolic absorbance through the digestive wall. This may be due to low phospholipase A_1 and A_2 activities in the digestive mucosa toward PI, but not other major phospholipids (PC, PE, PS, and PA). Because the LPI concentration in the feces was very low compared with that in the chow, LPI is apparently absorbed quite efficiently in the digestive system, like LPC and LPE. Thus, it is likely that phospholipids could be absorbed into the body if PI in the chow was degraded to LPI. In rats, orally administered ^{3}H-glycerol-labeled PS was mainly converted to TG and PE in the intestinal mucosa, possibly due to its absorption after conversion to LPS in the intestinal lumen.[24] The result suggests that the extensive hydrolysis occurred with decarboxylation of the serine moiety. However, a significant portion of radioactivity was recovered as PS, suggesting its effective intestinal absorption, possibly via its conversion to LPS. Our results on LPS support previous findings and add an interesting suggestion that only LPS, having a saturated fatty acyl residue, is resistant to luminal metabolism and absorption through the digestive mucosa. Less molecular species-selective resistance (saturated species > monounsaturated species and polyunsaturated species) to digestive absorption was observed for LPA in this study with rats. This finding suggests that the rat colon is exposed to a portion of the LPA derived from foods. It should be mentioned that intrarectal administration of LPA not only had beneficial effects including prevention of cholera toxin-induced diarrhea in the rat colon,[23] but also ameliorated harmful effects such as induction of tumorigenesis in the colon and aggravation of colonic cancer in rats.[21] Further studies are needed to clarify the molecular mechanisms of the molecular species-selective evasion of LPA and LPS from digestive absorption in rats and to understand their physiological and pathological significances.

■ ABBREVIATIONS USED

LC-MS-MS, liquid chromatography−tandem mass spectrometry; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PS, phosphatidylserine; SPM, sphingomyelin; TG, triacylglycerol.

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