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ORIGINAL CONTRIBUTION

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Activation and inhibition of lipoprotein lipase in mixed monolayers of medium or long chain-triglycerides and phospholipids

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Abstract We evaluated the activation and inhibition effects of phosphatidylcholine (PC) and sphingomyelin (SM) on lipoprotein lipase (LPL) for medium or long chain-triglycerides (TG) in monolayers at the air/water interface. Monolayers of medium chain-TG, being in an expanded state at a surface pressure of 15 mN/m, showed low susceptibility to LPL in the subphase. Adding 50 mole% of PC or SM into these monolayers reduced the partial molecular area of the TG and enhanced the LPL activity. Monolayers of long chain-TG, being in a condensed state, showed high susceptibility of LPL either with or without PC. SM added to the long chain-TG monolayers, however, inhibited the LPL action. We investigated the interaction between TG and phospholipid on the basis of the collapse pressure-measurements of mixed monolayers. For medium chain-TG monolayers, PC and SM gave similar collapse pressure-composition profiles. Contrary to this, SM gave a markedly higher collapse pressure of long chain-TG than PC: SM stabilized the monolayer state of long chain-TG. These results suggested that I) orientation of the acyl chains of TG molecule in a monolayer is crucial for the LPL activity, and that II) strong interaction between SM and long chain-TG retards the substrate-transfer from the mixed monolayer to the catalytic pocket of LPL.

Key words Lipoprotein lipase – inhibition – phosphatidylcholine – sphingomyelin – triglyceride – monolayer

Introduction

Lipoprotein lipase (LPL) hydrolyzes triglycerides in chylomicrons or very low density lipoproteins to mono-, diglycerides and fatty acids in animal plasma. The latter are taken up by cells and utilized as source of energy [1]. Primary sequence homology to pancreatic lipase and site-directed mutagenesis show that the catalytic domain of LPL consists of serine 132, aspartic acid 156 and histidine 241 [2, 3]. LPL contains a surface loop (lid) covering the catalytic pocket that modulates the selective access of long chain-triglycerides in lipoproteins or emulsions [4, 5]. The open lid-form has a bound lipid molecule in the catalytic pocket and the susceptible ester bond of the substrate is brought into close contact with the active site of the enzyme [6]. The selective access of a triglyceride molecule to the lid and the accommodation of the lipid into the pocket are essential processes in the lipolysis, and may be affected by lipid–lipase and lipid–lipid interactions at the protein/lipid interface.

Monolayers of triglycerides or triglyceride/phospholipid mixtures have been developed to understand several factors involved in interfacial lipolysis by LPL, e.g. surface pressure or packing density [7–9], apolipoproteins C [7–10], cholesterylesters [11] and sphingomyelin [12]. The lipolysis velocity and specific activity of the interface-bound LPL show bell-shaped curves as a function of lipid packing or surface pressure. Below and above an appropriate surface pressure range, LPL does not hydrolyze the monolayers. However, LPL is readily adsorbed by the lipid monolayers up to a surface pressure exceeding 40 mN/m (dyn/cm) [7, 8, 10]. Demel et al. [12] and Vainio et al. [7] have independently shown that the catalytic activity of LPL in various monolayers is dependent on the conformation or appropriate physical state of triglyceride at the interface.

Here, we measured the hydrolysis rates of saturated chain-triglycerides in mixed monolayers with phosphatidylcholine or sphingomyelin in an aqueous LPL solution. Triglycerides with medium (carbon numbers $8 \sim 12$) and long (14 and 16) saturated acyl chains were in different physical states at the interface and exhibited different LPL susceptibility. Furthermore, the effects of phospholipids on lipolysis for medium chain-triglycerides were distinct from those for long-chain triglycerides. These results are discussed in terms of triglyceride-phospholipid interactions at the interface.

Experimental

Materials

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Co. The purity (over 99.5%) was determined by thin-layer chromatography (TLC). Egg yolk sphingomyelin (SM) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1,2-Dimyristoylamide-1,2-deoxyphosphatidylcholine (DDPC) was purchased from Dojindo. Tricaprylin (TC) and tricaprin (TCa) were obtained from Funakoshi Co. and Wako Pure Chemicals, respectively. Trilaurin (TL), trimyristin (TM) and tripalmitin (TP) were purchased from Sigma Chemical Co. Each sample showed a single spot on TLC (solvent, chloroform/hexane (4:1, v/v)). Water was doubly distilled with a quartz still. Lipoprotein Lipase (EC 3.1.1.34) from bovine milk was purchased from Sigma Chemical Co. The specimen (Lot. No. 84H8065) showed a single band on SDS-polyacrylamide gel electrophoresis (molecular weight: 55 kDa). The molecular weight agreed with the reported value [17, 18]. LPL was stocked in a buffer consisting of 20 mM Tris-HCl and 3.8 M ammonium sulfate at 4 °C.

Monolayer assay of LPL activity

The hydrolytic activity of LPL for triglyceride monolayers was measured in a buffer (10 mM Tris-HCl/150 mM NaCl, pH 8.5) in a double-walled Teflon-coated trough ($10 \times 15.5 \times 0.8$ cm) at 30 °C. An aliquot of the stocked LPL was added to the subphase to give the final concentration of 5 μg/130 ml. Immediately after sweeping the air/water interface by the Teflon-coated barrier to remove impurities including adsorbed proteins, triglyceride or a triglyceride/phospholipid mixture was added by an Agla micrometer syringe using benzene as the spreading solvent. The movable barrier compressed monolayers up to a surface pressure of 15 mN/m at a rate of 2 cm/min. The lipolytic product, fatty acid, quickly left the interface, and the barrier automatically moved to keep the surface pressure constant (15 mN/m). Decreases in the surface area were recorded on a Film Balance Controller FSD-110 (U.S.I system, Fukuoka) equipped with a Whilhelmy's plate. The rate of lipid hydrolysis was evaluated from the initial slope of the decrease in surface area, since the lipid composition varied during hydrolysis. Results are represented as means $\pm$ S.D. of two or three independent experiments.

Surface pressure – area per molecule curve and collapse pressure

Triglycerides, phospholipids or their mixtures were dissolved in benzene. The solution was supplied from an Agla micrometer syringe at the surface of the buffer in the double-walled Teflon-coated trough. The monolayer spread was left for 1 min to evaporate the solvent. The area per molecule of insoluble lipid or the average area per molecule of mixed lipid in the monolayer, **A** (Å^2/molecule) was calculated as $\mathbf{A} = S/(n \cdot N_A)$, where S was the surface area of the subphase buffer solution between barriers, n was the moles of insoluble lipids spread on the surface, and N_A was the Avogadro number. **A** was changed by shifting the barrier position. The surface pressure and area were recorded on the Film Balance Controller equipped with a Whilhelmy's plate. The temperature was kept constant at 30 °C by circulating thermostated water through the outer compartment of the double-walled trough. The details of the monolayer techniques have been described elsewhere [14, 15]. Surface pressure-area per molecule curve of triglyceride or phospholipid became stationary at a defined surface pressure. The surface pressure of the monolayer transition point was identical with the spreading pressure of triglyceride or phospholipid [14, 15, 16] except TP, which is discussed later. The spreading pressure has been defined as the surface pressure of the monolayer

in equilibrium with the bulk phase (liquid, liquid crystal or solid). The stationary values of triglyceride or phospholipid monolayers and the inflections of mixed monolayer of triglyceride and phospholipid thus show the monolayer-collapse to the bulk phase of triglyceride [14]. The surface pressures of such transition points are represented as a function of monolayer composition.

Results

Effects of phospholipid on monolayer lipolysis of medium and long chain-triglycerides

We selected tricaprylin (trioctanoylglyceride, TC), tricaprin (tridecanoylglyceride, TCa), trilaurin (tridodecanoylglyceride, TL), trimyristin (tritetradecanoylglyceride, TM) and tripalmitin (trihexadecanoylglyceride, TP) as the interfacial lipolysis substrates. TM and TP monolayers were in the condensed state and TC, TCa, and TL monolayers were in the expanded state at 15 mN/m [16]. Dissolution of the hydrolyzed products, caprylic, capric, lauric and myristic acids from monolayers to the subphase was much faster than hydrolysis of the respective triglycerides by LPL (data not shown). The observed reduction in the monolayer area was directly related to the lipolysis velocity. On the other hand, the hydrolysis velocity of TP monolayers on the LPL solution was comparable to the dissolution velocity of palmitic acid monolayers, providing some qualitative information on the LPL activity.

Figure 1 shows changes in monolayer-area of TC, PC and a mixture of TC and PC (4/6 in mole ratio) as a function of the reaction time at 15 mN/m and 30 °C. The area of these monolayers did not appreciably change in the absence of LPL (data not shown). The PC monolayer was not hydrolyzed under our experimental conditions. Vainio et al. have shown the adsorption of LPL to PC monolayer in a similar condition to ours. The lipolysis activity for a medium chain triglyceride, TC, was very low in the monolayer, and the addition of PC (> 50 mole %) enhanced hydrolysis. On the other hand, the long chain-triglyceride, TM, was rapidly hydrolyzed by LPL in the absence of PC (data not shown). TC and TM monolayers had a molecular area of about 90 and 60 $Å^2$ respectively, at 15 mN/m in either the presence or the absence of LPL. When a TM monolayer was expanded to a molecular area of 90 $Å^2$ (surface pressure of 4 mN/m), the lipolysis activity steeply decreased to that of the TC monolayer at 15 mN/m. The high activity was recovered by recompressing the monolayer to 15 mN/m.

Figure 2 shows the lipolysis rates of a medium chain-triglyceride, TC in the mixed monolayers with either PC or

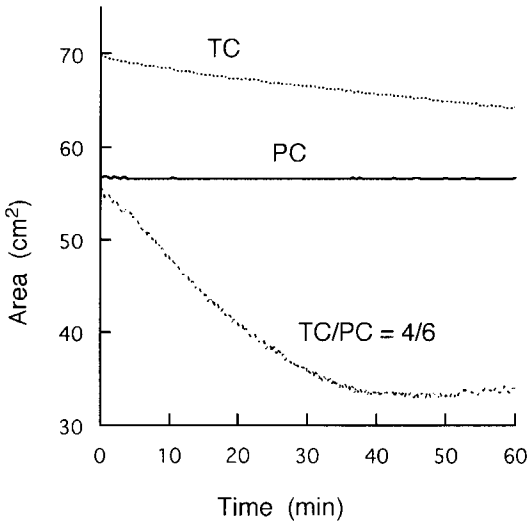

Fig. 1 Decrease in the monolayer area caused by LPL in the subphase. The surface pressure was kept constant (15 mN/m) during lipolysis. Temperature: 30 °C

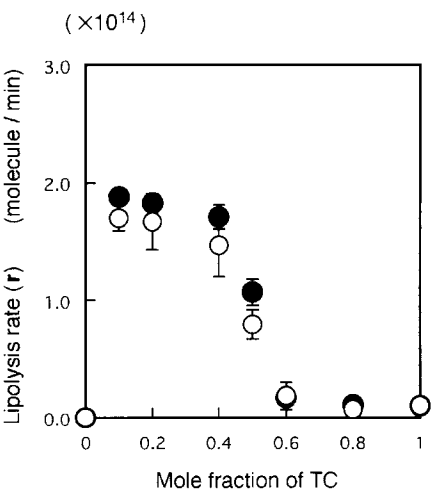

Fig. 2 Lipolysis rate (**r**) for mixed monolayers of the medium chain-triglyceride, TC, and a phospholipid (PC or SM) are represented as a function of the monolayer composition. ○: TC/PC mixtures; ●: TC/SM mixtures. see Eq. (1)

SM. The rate (**r**) is defined as

$$\mathbf{r} = (\mathbf{v}/\mathbf{x}) = (\mathbf{1}/\mathbf{x})(\mathbf{1}/\mathbf{a}_1)(-\mathbf{dS}/\mathbf{dt}) . \quad (1)$$

Here, **v** is the observed velocity of the reducing area, **x** is the mole fraction of triglyceride, **S** is the total area of mixed monolayer, $\mathbf{a}_1$ is the partial molecular area of triglyceride and **t** is the reaction time (in min). The $\mathbf{a}_1$ values were separately obtained from the experimental relationship of **A** (average area per molecule) and **x** as described later. The susceptibility of TC to LPL was increased by adding 50 mole% of either PC or SM. The maximal rates

Table 1 Lipolysis rates (**r**) of triglyceride and triglyceride/phospholipid monolayers[a]

		Molecular Area of TG ($Å^2$)	$r \times 10^{-14}$ (molecule/min.)[b]		
			TG	TG/PC = 2/8	TG/SM = 2/8
Expanded monolayer	TC	90	0.101 ± 0.013	1.67 ± 0.24	1.89 ± 0.06
	TCa	91.5	0.135 ± 0.027	1.43 ± 0.06	1.81 ± 0.12
	TL	88	0.160 ± 0.035	1.47 ± 0.17	1.83 ± 0.08
Condensed monolayer	TM	62.5	1.35 ± 0.26	1.39 ± 0.17	N.D.[d]
	TP	60	(+)[c]	(+)[c]	N.D.[d]

[a] Measured at 15 mN/m.
[b] Values are represented as means ± S.D. of two or three independent experiments.
[c] Surface area decreased, however the hydrolysis velocity was comparable to the dissolving velocity of the hydrolysis products (palmitic acid).
[d] Not detectable.

of the TC/PC and TC/SM mixed monolayers were similar. The other medium chain triglycerides (TCa and TL) were in the expanded state at 15 mN/m and the lipolysis of these triglycerides was similarly enhanced by either PC or SM as that of TC (Table 1). Vainio et al. [10] have shown that the lipolysis velocity (**v**) for mixed monolayers of TC and dilauroylphosphatidylcholine gives a bell-shaped curve as a function of the TC mole fraction. The lipolysis rate (**r**) defined by Eq. (1) contains the factor (**1/x**) and shows biphasic curves for LPL activity in mixed monolayers of TC/PC and TC/SM.

Figure 3 shows the effects of PC and SM on the lipolysis of TM in the mixed monolayers. The long chain-triglyceride was equally hydrolyzed in the presence and absence of PC. The maximal rates of the TM/PC (Fig. 3) and TC/PC (Fig. 2) mixed monolayers were similar. In contrast, SM inhibited the hydrolysis of TM (Table 1). The other long chain-triglyceride, TP, was hydrolyzed in a mixed monolayer with PC (TP/PC = 2/8), but a mixed monolayer of TP and SM (2/8) failed to decrease the area by LPL in the subphase (Table 1). Thus, medium and long acyl chain triglyceride molecules affected the lipolysis activity of LPL in mixed monolayers differently.

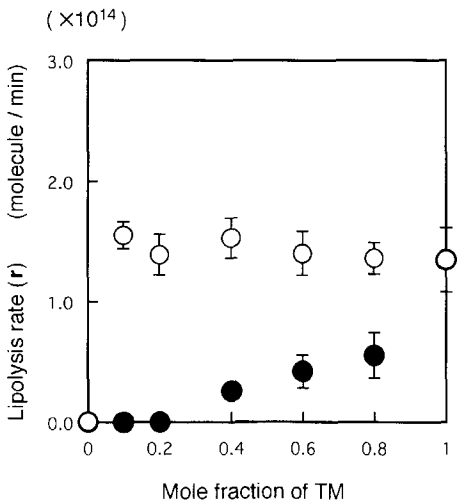

Fig. 3 Lipolysis rates (**r**) for mixed monolayers of a long chain-triglyceride, TM, and a phospholipid (PC or SM) are represented as a function of the monolayer composition. ○: TM/PC mixtures; ●: TM/SM mixtures

Partial molecular area and collapse pressure of triglyceride in mixed monolayers with phospholipid

Partial molecular areas of TC and TM in mixed monolayers with phospholipid were evaluated from the intercept of the tangent to the average molecular area-mole fraction curve (data not shown). Figure 4 represents the lipolysis rate of TC and TM as a function of the partial molecular area, $\mathbf{a}_1$, in mixed monolayers. LPL activity was very low when the $\mathbf{a}_1$ value was larger than 85 $Å^2$. The rate steeply increased around $\mathbf{a}_1 = 80$ $Å^2$ in mixed monolayers except the TM/SM mixtures. As described before, the LPL activities for the TM monolayer at the molecular area (or the partial molecular area) of 60 $Å^2$ (condensed state) and 90 $Å^2$ (expanded state) were reversibly changed by expansion and compression.

The decrease in the partial molecular area of a medium chain-triglyceride, TC, caused by SM enhanced the activity of LPL (Figs. 2 and 4). In contrast, SM had the intense inhibitory effects on the long chain triglycerides, TM and TP in mixed monolayers (Figs. 3 and 4). SM (TM/SM = 2/8) contracted the TM molecule to a partial molecular area of less than 53 $Å^2$ and eliminated the susceptibility of the triglyceride to LPL. These results indicated that long and medium chain-triglycerides interacted quite differently with SM in the mixed monolayers.

Lipid–lipid interactions in a monolayer have been evaluated by means of the collapse pressure measurements [13–15]. The collapse pressure is the surface pressure of a monolayer in equilibrium with the bulk phase of the

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triglyceride segregated (collapsed) from the monolayer. When the mole fraction of triglyceride in a mixed monolayer decreases (that is, the increase in the phospholipid fraction), the activity of the triglyceride decreases. In the equilibrium of a triglyceride between a mixed monolayer and a bulk phase, a higher surface pressure is required to compensate the decrease in the activity [15]. The collapse pressure of mixed monolayer of TC increased with the mole fraction of PC or SM in a similar manner (Fig. 5a). PC and SM also had comparable thermodynamic effects on the monolayer collapse pressure of the other medium chain-triglycerides (TCa and TL) (data not shown).

Figure 5b shows the collapse pressures of the mixed monolayers of TM with either PC or SM. The collapse pressure of the TM/SM mixed monolayer was much higher than that of the TM/PC mixed monolayer, indicating that the TM molecules were stabilized in the mixed monolayer with SM. The segregation of the TM molecules out of the mixed monolayer was retarded. In addition, the diamidophospholipid, 1,2-dimyristoylamide-1,2-deoxyphosphatidylcholine (DDPC) and PC similarly affected the LPL activity and monolayer collapse (data not shown).

The collapse pressure of the other long chain-triglyceride, TP (30 mN/m) was much higher than the spreading pressure (~ 0 mN/m). Because a reversible transition between monolayer and bulk states could provide the identical values for the surface pressures of a single component system [13, 15], the discrepancy was presumed to arise from the polymorphism of TP [19]. Anyway, collapse pressure of the TP/SM mixed monolayer was much higher than that of the TP/PC mixed monolayer (data not shown), suggesting that SM more effectively stabilized the TP molecules in the mixed monolayer more than PC, and that the separation of the TP molecules from the mixed monolayer was also retarded by SM.

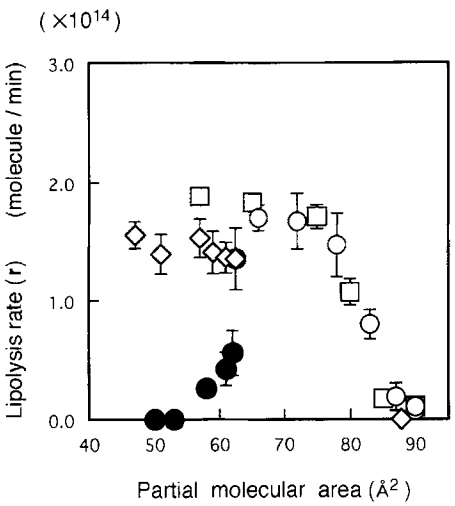

Fig. 4 Activation and inhibition of triglyceride lipolysis by PC or SM. The lipolysis rates (**r**) are represented as a function of the partial molecular area of triglyceride in mixed monolayers with PC or SM. ○: TC/PC mixtures; □: TC/SM mixtures; ◇: TM/PC mixtures; ●: TM/SM mixtures

Discussion

Physical state of triglyceride molecules and LPL activity

The results in Figs. 2, 3 and 4 show that triglycerides were susceptible to LPL at a partial molecular area ($\mathbf{a}_1$) of less than 80 Å^2, but LPL activity was very low at the larger $\mathbf{a}_1$ value. The high activity was recovered when the expanded monolayer was compressed to the lower $\mathbf{a}_1$ value. Therefore, the low lipolysis activity was not due to the interfacial denaturation of LPL at the low surface density of lipid at 90 Å^2 per molecule. The TC monolayer was not compressed beyond the molecular area of 80 Å^2 because of the collapse of monolayer, but the addition of PC led to smaller values of $\mathbf{a}_1$ even at a surface pressure of 15 mN/m.

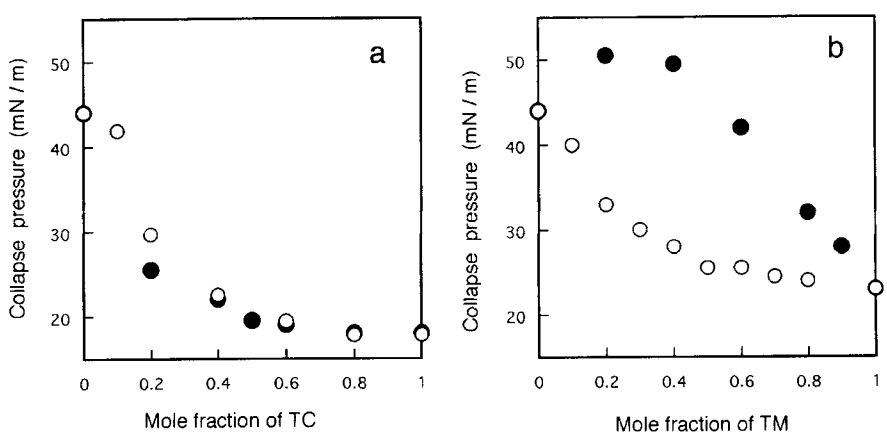

Fig. 5 Collapse pressures of triglyceride/phospholipid mixed monolayers are shown as a function of the monolayer composition. a: TC/PC (○) and TC/SM (●) mixed monolayers. b: TM/PC (○) and TM/SM (●) mixed monolayers

Vainio et al. have shown that the interfacial LPL concentration is not significantly affected by the monolayer composition of phosphatidylcholine and triglyceride [10]. They have also shown that although the adsorption of LPL to the mixed monolayers changes slightly, the specific activity of the bound enzyme shows a bell-shaped curve as a function of surface pressure or packing density [7]. In this study, the moles of LPL per lipid molecule might change by 25% at the most with the monolayer composition, while the LPL activity changed more than 10 times for medium chain-triglycerides (Fig. 2). The **r** value calculated by Eq. (1) showed biphasic, not bell-shaped profiles (Figs. 2 and 4). Furthermore, the high susceptibility of long chain-triglycerides to LPL was independent of the PC content (Fig. 3). The results for the TC/PC, TM/PC and TC/SM mixtures in Fig. 4 suggested that the interfacial orientation of triglyceride was crucial in the LPL lipolysis. Rogalska et al. have demonstrated that the stereoselectivity of LPL towards racemic diglycerides in monolayers depends on the surface pressure, namely the interfacial orientation of the lipid-substrate [20].

LPL consists of a large NH_2-terminal domain containing catalytic residues and a small COOH-terminal domain promoting interactions with a surface of lipid emulsion or lipoprotein particles [5, 21]. The NH_2-terminal domain contains a surface loop covering the catalytic pocket that may modulate the access of the substrate to the pocket. Secondary structure analysis of this loop reveals a helix-turn-helix motif with two short amphipathic helices [4]. The interaction between the lipoprotein surface and the helices may in part determine the LPL-substrate specificity. The lipolytic ratio (**r**) to the partial molecular area ($\mathbf{a}_1$) correlation in Fig. 4 suggests that the vertical orientation of the acyl chains in monolayer is favorable for the specific interaction with the surface loop (lid) of LPL.

Inhibition of LPL activity by SM

Amide type inhibitors hydrogen bonded directly to the imidazole ring of His in the catalytic domain of phospholipase A_2 [22, 23]. The catalytic site of LPL consists of a Ser-Asp-His triad [4] and the SM molecule contains an amid bond. Figures 2, 3 and Table 1 show that the lipolysis of medium chain-triglycerides was enhanced, but that of long chain triglycerides was inhibited by adding SM to the monolayers, indicating that direct interaction between SM and LPL did not play a major role in regulating the enzyme activity. In fact, an amid-type PC analog, DDPC, did not inhibit the enzyme activity, but had similar effects on the triglyceride lipolysis to PC. Figures 2 and 3 show a marked difference in LPL activity between TC/SM and TM/SM monolayers at a high mole fraction of SM, where the adsorption amounts of LPL to these mixed monolayers may not so greatly differ as the lipolysis activity. We presumed that the observed distinction in LPL activity reflects different interactions of LPL between the medium and long-chain triglyceride in the SM monolayer.

The collapse pressure against mole fraction curves in Fig. 5a show that medium chain-triglycerides segregate as easily from the mixed monolayers of SM as they do from those of PC. On the other hand, the separation of long chain triglycerides, TM and TP, was depressed from the mixed monolayers of SM. The collapse pressure is the surface pressure at which chemical potentials of triglyceride in the monolayer and bulk phase become equal. When the mole fraction of triglyceride decreases, the increase in surface pressure (collapse pressure) compensates the decreasing activity. The activity was defined as the product of the mole fraction and the activity coefficient. Figure 5b indicates the remarkable decrease in the activity coefficient (stabilization) of the long chain-triglyceride, TM, caused by adding SM. The strong interaction of the SM and the long saturated chain-triglyceride molecules is assumed to retard the substrate-transfer from the interface to the catalytic pocket of LPL.

In conclusion, we showed that i) triglyceride molecules in the condensed monolayer were highly susceptible to LPL, ii) the addition of phosphatidylcholine or sphingomyelin to an expanded monolayer of medium chain-triglyceride decreased the partial molecular area of the triglyceride and increased the susceptibility to LPL, and that iii) strong interactions between sphingomyelin and long chain-triglyceride retarded the substrate-transfer from the mixed monolayer to the catalytic pocket of LPL. Further studies on the interactions between SM and long saturated chain-triglycerides are in progress.

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